Pokeweed antiviral protein regulates the stability of its own mRNA by a mechanism that requires depurination, but can be separated from depurination of the α−sarcin/ricin loop of rRNA

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Running title: PAP regulates the stability of its own mRNA
SUMMARY

Pokeweed antiviral protein (PAP), a single chain ribosome inactivating protein (RIP) isolated from pokeweed plants (*Phytolacca americana*), removes specific adenine and guanine residues from the highly conserved, α–sarcin/ricin (S/R) loop in the large rRNA, resulting in inhibition of protein synthesis. We recently demonstrated that PAP could also inhibit translation of mRNAs and viral RNAs that are capped by binding to the cap structure and depurinating the RNAs downstream of the cap. Cell growth is inhibited when PAP cDNA is expressed in the yeast, *Saccharomyces cerevisiae* under the control of the galactose inducible *GAL1* promoter. Here, we show that overexpression of wild type PAP in yeast leads to a decrease in PAP mRNA abundance. The decrease in mRNA levels is not observed with an active site mutant, indicating that it is due to the N-glycosidase activity of the protein. PAP expression had no effect on steady state levels of mRNA from four different endogenous yeast genes examined, indicating specificity. We demonstrate that PAP can depurinate the rRNA in *trans* in a translation-independent manner. When rRNA is depurinated and translation is inhibited, the steady state levels of PAP mRNA increase dramatically relative to the U3 snoRNA. Using a PAP variant which depurinates rRNA, inhibits translation, but does not destabilize its mRNA, we demonstrate that PAP mRNA is destabilized after its levels are up-regulated by a mechanism that occurs independently of rRNA depurination and translation. We quantify the extent of rRNA depurination in vivo using a novel primer extension assay and show that the temporal pattern of rRNA depurination is similar to the pattern of PAP mRNA destabilization, suggesting that they may occur by a common mechanism. These results provide the first in vivo evidence that a single chain RIP targets not only the large rRNA, but also its own mRNA. These findings have implications for understanding the biological function of RIPS.
INTRODUCTION

Pokeweed antiviral protein (PAP), a single chain ribosome inactivating protein (RIP) isolated from the leaves of pokeweed plants (*Phytolacca americana*), removes specific adenine and guanine residues from the highly conserved, α–sarcin/ricin (S/R) loop in the large rRNA (1-3). The enzymatic removal of specific purines from the S/R loop has been reported to interfere with the binding of elongation factor 2 (eEF-2) and inhibit protein synthesis at the translocation step (4,5). RIPs are protein toxins produced by organisms ranging from bacteria to plants. Because of their selective toxicity, they have been used as biological weapons, to protect plants against pathogens and as therapies against cancer. Their biological function in the organisms that produce them is unknown. PAP is thought to be a defense protein because it is a potent inhibitor of animal and plant viral pathogens, including HIV, poliovirus, herpes simplex virus, influenza, potato virus X and brome mosaic virus (6-10). Due to its cytotoxicity to dividing cells, PAP is currently under clinical trials as a potent anti-cancer agent (11). The mechanism by which PAP inhibits cell growth or viral infection is not well understood. Translation inhibition by PAP and the resulting host cell death have been hypothesized to be responsible for the antiviral activity of PAP. However, a nontoxic C-terminal deletion mutant of PAP inhibited viral infection without depurinating host ribosomes, indicating that antiviral activity could be separated from rRNA depurination (12). Furthermore, expression of nontoxic forms of PAP in transgenic plants induced a stress-associated signal transduction pathway and provided resistance to viral and fungal infection (13,14). PAP is very active against both animal and plant ribosomes. It accesses the S/R loop by binding to ribosomal protein L3 (*RPL3*), a highly conserved protein associated with the peptidyltransferase center of ribosomes (15). Our recent results indicate that in a cell-free system PAP can inhibit translation of mRNAs and viral RNAs that are capped by recognizing the cap structure and depurinating the capped RNAs (3). Incubation of BMV RNAs or capped luciferase RNA with PAP resulted in depurination of either RNA. In contrast, uncapped luciferase RNA was not depurinated after incubation with identical concentrations of PAP (3). Analysis of the interaction between the cap structure and PAP indicated that PAP binds to the m’GpppG cap structure, but does not remove the cap (16). PAP depurinates the RNA downstream of the cap at specific sites (16). The relative affinity of PAP for capped RNA is similar to its affinity for the S/R loop of rRNA, suggesting that rRNA might not be the only target of PAP (16).

In our previous studies with transgenic plants, we observed that mRNA corresponding to wild type PAP was not detected by Northern blot analysis (13), even though PAP protein was detected (12). In contrast, PAP
mRNA was detected in transgenic lines expressing the inactive form, PAPx (or PAP<sub>E176V</sub>) that contains the point mutation, E176V, at its active site (13). In the present study, we examined the effect of PAP on the stability of its own mRNA and cellular mRNAs in the yeast, <i>Saccharomyces cerevisiae</i> where PAP expression can be tightly controlled. We have previously shown that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17). Our results demonstrate that PAP expression in yeast duplicates the effects of PAP in plant cells (12, 17).

**EXPERIMENTAL PROCEDURES**

**Media and Growth Conditions.** <i>Saccharomyces cerevisiae</i> strain <i>W303</i> (MATa ade2-1 trp1-1 ura3-1 leu2-3, 112 his3-11, 15 can1-100) was used for all of the assays. Yeast cells were grown at 30°C in YPD rich medium (1% yeast extract, 2% peptone, and 2% glucose) or synthetic dropout (SD) medium (0.67% Bacto-yeast nitrogen base) supplemented with appropriate amino acids (17,18). To induce expression of PAP and mutant forms of PAP, transformed yeast were initially grown at 30°C in 150 ml selective media containing 2% raffinose to an initial OD<sub>600</sub> of 0.6. At zero time the medium was replaced with 300 ml selective media (SD-leu) containing 2% galactose to a starting OD<sub>600</sub> of 0.3. Subsequently, 5 ml of culture was taken for protein isolation, 25 ml for RNA isolation, and 1 ml for a growth reading (OD<sub>600</sub>) at various times post-induction. The media was periodically diluted to maintain the cells in the logarithmic phase (OD<sub>600</sub> between 0.3 and 0.6). Doubling times were calculated based on exponential growth between 4 and 10 hours post-induction. For measuring the effect of translation and transcription on rRNA depurination, yeast were grown at 30°C in 150 ml SD-leu, 2% raffinose to an initial OD<sub>600</sub> of 0.6. Cells were then pelleted, washed once, and resuspended in 13 ml of SD-leu, 2% galactose to induce PAP expression. At one hour post-induction, 2% glucose with or without cycloheximide to a final concentration of 100 µg/ml was added to the medium and 2ml pellets were collected for RNA and protein isolation at the indicated times.
Plasmids. PAP expression plasmids used in this study were described previously (3,17). Expression of PAP in NT188 and the nontoxic PAP variants, PAP_E176V and PAP_L71R in NT224 and NT538, respectively is under control of the galactose-inducible GAL1 promoter in the YEp351-based high-copy plasmid. The NT616 contained the firefly luciferase cDNA from pLUC0 (18) downstream of the GAL1 promoter in YEp351.

RNase protection assays. Total RNA from cells expressing PAP, PAP_E176V, and PAP_L71R was analyzed with the RNase protection assay according to Tumer et al. (1998) (18). RNase protection assay was performed using several gene-specific antisense RNA probes to measure the steady state levels of mRNA. A 281-nt CYH2 specific probe was generated from a SP6 RNA polymerase runoff transcript of HincII digested p3433 (18). A 90-nt U3 specific probe was generated from a T3 RNA polymerase runoff transcript of SspI digested pJD161 (19). The U3 small nucleolar RNA (snoRNA) is constitutively expressed from an RNA polymerase III promoter and was used as loading control. A 252-nt RNA probe that hybridizes to the 3’-end of PAP mRNA was generated from a SP6 RNA polymerase transcript of XhoI-digested pMON8588. A 200-nt XRN1 specific probe was generated from a T3 RNA polymerase runoff transcript of DdeI-digested pNT404. The pNT404 has a 2-kb BglII/HincII fragment of the XRN1 gene from pXRN1 cloned into the BamHI/HincII sites of pBluescript KS+ (Stratagene). A 260-nt LEU2-specific probe was generated from a T7 RNA polymerase transcript of EcoRI-digested pNT403. pNT403 was constructed by inserting a 745-bp HincII/ClaI fragment of the LEU2 gene from YEp351 into pGEM3Zf(+) digested with the same restriction enzymes. A 250-nt RPL3-specific probe was generated from a T7 RNA polymerase transcript of XbaI-digested pRPL3, which carries the ribosomal protein gene RPL3. A 180-nt PGK1-specific probe was generated from a T7 RNA polymerase runoff transcript of Sall-digested pRS314-PGK1. Protected fragments were separated on a 7M urea 5% acrylamide denaturing gel, visualized with radiographic film (Kodak) and quantified on a PhosphorImager (Molecular Dynamics).

Analysis of Protein Expression. Protein from frozen yeast cells expressing PAP, PAP_E176V, and PAP_L71R harvested during the time course of induction, was extracted as described by Hudak et al. (1999) (15). Total protein (7.5 µg) from each time point was separated on 15% SDS-PAGE, transferred to nitrocellulose, and probed with affinity purified anti-PAP polyclonal antibody (1:5000). PAP was visualized by chemiluminescence using the Renaissance kit (NEN Life Science Products). The blots were then stripped for 30 min with 8M guanidine hydrochloride and reprobed with antibody to glucose 6-phosphate dehydrogenase (G6PD) (1:5000) as an internal loading control.
**rRNA depurination assay.** Ribosomal RNA depurination was assayed by primer extension analysis as described previously (20). Briefly, 2 µg of total yeast RNA from cells expressing PAP was hybridized with 10⁶ CPM of reverse primer [5'-AGCGGATGGTGCTTCGCGGCAATG -3’]. This depurination primer was end-labeled by T4 kinase (Invitrogen) in the presence of [γ-³²P]ATP and it hybridized 73-nt 3’ of the depurination site. The presence or absence of depurination was noted by synthesis of a 73-nt extension product that terminated at the depurination site. Superscript II-reverse transcriptase (Invitrogen) was used in the primer extension assay following the protocol described in Hudak et al. (2000) (3). Extension products were separated on a 7M urea 5% polyacrylamide denaturing gel, and visualized and quantified on a PhosphorImager (Molecular Dynamics). Further studies requiring more accurate quantification of depurination employed the use of a second primer serving as an internal control. For these analyses, either 1.25 µg of total yeast RNA isolated from yeast expressing PAP, PAP_{E176V}, or PAP_{L71R} as described above or 1.0 µg rRNA isolated from ribosomes was hybridized to two different reverse primers. The second primer hybridized upstream of the depurination site close to the 5’ end of the 25S rRNA. For in vitro depurination assays, yeast ribosomes were isolated as previously described (20). Ribosomes were then either incubated in buffer alone or depurinated with 250 ng purified PAP to completion as described previously (20). To quantify the extent of depurination, the target RNA was initially hybridized in the presence of excess amounts (700pmol) of the two [γ-³²P]ATP end-labeled negative strand primers. The depurination primer described above annealed 73-nt 3’ of the depurination site (A₃₁₃₇) on the 25S rRNA. The 25S control primer [5’-TTCACTCGCCGTTACTAAGG -3’] annealed 100-nt 3’ of the 25S rRNA 5’ end. To allow for accurate quantification, the labeled 25S control primer was diluted 1:4 with unlabeled 25S control primer. Superscript II-reverse transcriptase was used in the primer extension assay as above. Extension products for the control and depurination fragments (100-nt and 73-nt, respectively) were separated on a 7M urea 5% polyacrylamide denaturing gel, and visualized and quantified on a PhosphorImager. The amount of total yeast RNA and rRNA used was previously determined to be in the linear range of detection.

**In vivo [³⁵S]methionine incorporation.** Yeast cells were grown to an OD₆₀₀ of 0.6 in SD-leu, -met, 2% raffinose. Cells were then resuspended at an OD₆₀₀ of 0.3 in 2% galactose for 4 to 10 hours in order to induce either wild type PAP or mutant PAP expression. At time zero, [³⁵S]methionine was added to cells growing on galactose. At the times indicated, 800 µl of yeast cells were removed for growth measurements, and additional aliquots of 800 µl were assayed for methionine incorporation in duplicate as described by Carr-Schmid et al with minor modifications.
Briefly, the yeast were added to 200 µl of 100% TCA, incubated for 10 minutes on ice, followed by 20 minutes at 70°C. The precipitate was then filtered through 24mm glass microfiber filters (VWR), washed with ice cold 5% TCA followed by ice cold 95% ethanol. Filters were dried for several hours and incorporation was quantified in a scintillation counter. The CPM was normalized to the OD₆₀₀ reading. Rates of translation were determined from these results and tabulated as CPM per OD₆₀₀ per minute. To obtain translation inhibition in PAPₑ₁₇₆ᵥ cells that was comparable to wild type PAP inhibition at four hours post-induction, approximately 5 µg/ml final concentration of anisomycin was added at two hours post-induction to the media. This amount was previously titrated to achieve 75% translation inhibition (data not shown).

RESULTS

PAP inhibits the growth of *S. cerevisiae*. To determine if PAP affects the stability of its own mRNA in vivo, cDNAs encoding the wild type PAP or nontoxic variants were placed under the regulation of the *GAL1* promoter and expressed in the yeast, *Saccharomyces cerevisiae*. The variants used in this study are shown in Figure 1. They include the nontoxic PAPₑ₁₇₆ᵥ, which contains a point mutation (E176V) at its active site and produces an inactive protein, and PAP₇₁ᵥ, which contains a point mutation (L71R) at the putative RNA binding domain and has reduced toxicity compared to wild type PAP. We have previously shown that yeast cells transformed with plasmids carrying PAPₑ₁₇₆ᵥ or the vector alone (YEp351), were able to grow on SD-leu plates containing galactose (17). However, yeast cells harboring the wild type PAP plasmid (NT188) failed to grow on plates containing galactose (17). As shown in Figure 2, growth of cells expressing the wild type PAP, but not PAPₑ₁₇₆ᵥ was inhibited in liquid SD-leu media containing galactose, compared to cells harboring the same vector (YEp351) with luciferase cDNA (VC). The doubling time of cells expressing PAPₑ₁₇₆ᵥ was similar to the vector control, 3.9 +/- 0.1 h, while the doubling time of cells expressing wild type PAP was 11.9 +/- 1.7 h. Growth of cells expressing PAP₇₁ᵥ was also inhibited in liquid media containing galactose, but not to the same extent as wild type PAP. This was evident from its doubling time of 8.5 +/- 0.8 h and ability to grow on plates containing galactose (data not shown). Addition of anisomycin to cells expressing PAPₑ₁₇₆ᵥ resulted in complete inhibition of growth (doubling time: 24.9 +/- 2.0 h). These results suggested that the inhibition of growth observed in cells expressing wild type PAP might be due to inhibition of translation.
Correlation between inhibition of growth and inhibition of translation. To determine if reduction of growth correlated with inhibition of translation, we examined total translation in cells expressing PAP, PAP_{E176V} and PAP_{L71R}, compared to control cells harboring the same vector with luciferase cDNA (VC). Total translation was examined by $^{35}$S methionine incorporation at 4 and 10 hours post-induction (21). As shown in Figure 3A, cells grown in galactose for 4 hours to induce expression of PAP_{E176V} were not significantly inhibited in translation as compared to vector control cells. The rate of translation in cells expressing PAP_{E176V}, judged from the slope of the curve in Figure 3A, was $88.7 \pm 2.9\%$ of the rate of translation in vector control cells (Table I). The rate of translation in yeast expressing active PAP was $27.4 \pm 3.0\%$ of the vector control as determined by averaging results of five independent experiments (Table I). These results indicate that total translation is significantly inhibited in cells expressing PAP, but not PAP_{E176V}.

The growth inhibition observed in the presence of anisomycin in Figure 2 suggested that inhibition of growth might be due to inhibition of translation. Under these conditions, we would expect cells expressing PAP_{L71R} to be inhibited in translation. As shown in Figure 3A, total translation was significantly inhibited in cells expressing PAP_{L71R} at 4 hours post-induction. The rate of translation in cells expressing PAP_{L71R} was $33.8 \pm 0.7\%$ of the vector control (Table I). Analysis of total translation at 10 hours post-induction indicated that translation remained inhibited in cells expressing wild type PAP and PAP_{L71R}, but not in PAP_{E176V} (Figure 3B). These results provide evidence that the reduction in growth observed in cells expressing wild type PAP and PAP_{L71R} correlates with the inhibition of translation observed in these cells.

PAP has a specific effect on the stability of its own mRNA in yeast. Since our previous results indicated that PAP can inhibit translation by depurinating capped RNAs (3), to determine if translation inhibition correlated with the activity of PAP on mRNAs in vivo, we examined the abundance of PAP mRNA and cellular mRNAs in yeast expressing the wild type and the mutant forms of PAP. Cells were harvested at various times after induction on galactose, and the level of PAP mRNA was measured by RNase protection assay (Figure 4A). A 252-nt $^{32}$P-labeled antisense RNA probe corresponding to the 3’ end of PAP mRNA was transcribed and hybridized with total RNA extracted from cells harboring PAP, PAP_{E176V} and PAP_{L71R} plasmids. A 90-nt $^{32}$P-labeled antisense probe specific for U3 snoRNA, which is constitutively expressed from an RNA polymerase III promoter, was used as a loading control (19). Samples were electrophoretically separated and the intensities of the protected bands were quantified using a PhosphorImager. The ratios for signals of the PAP, PAP_{E176V} or PAP_{L71R} mRNAs to the U3
snoRNA were used as relative measures of the steady state abundance of the PAP, PAP_{E176V} or PAP_{L71R} mRNAs. The cells harboring the vector alone (YEp351) grown for 8 hours on raffinose or galactose did not show any detectable background (data not shown). Similarly, RNase protection analysis using tRNA did not show any protected fragments corresponding to either PAP or U3 (Figure 4A). As expression of wild type PAP was induced, the level of PAP mRNA decreased dramatically relative to the U3 snoRNA (Figure 4A). By 10 hours post-induction, PAP mRNA levels decreased to about 10% of the levels observed at 4-hours post-induction (Figure 4B). In contrast, PAP_{E176V} mRNA levels increased up to 10 hours post-induction and reached steady state levels after 10 hours. Though the overall increase in PAP_{E176V} mRNA was highly reproducible, the extent of the increase was subject to some variation. The amount of mRNA present at 10 hours in cells expressing PAP_{E176V} was between 2.5 and 8 times greater than that at 4 hours. The error bars in Figure 4B represent averaging of three independent quantifications, and the RNase protection analysis has been repeated at least three times with similar results. These results indicated that PAP mRNA is destabilized in cells expressing wild type PAP. This regulation is impaired when the active site mutant, PAP_{E176V}, is expressed in yeast, indicating that mRNA destabilization is due to the N-glycosidase activity of PAP. RNase protection analysis beyond 10 hours post-induction indicated that PAP mRNA is not detectable after 12 hours, while PAP_{E176V} mRNA remains at steady state levels (data not shown).

RNase protection analysis of yeast expressing PAP_{L71R} indicated that as observed with wild type PAP, mRNA levels increased up to 4 hours post-induction. However, PAP_{L71R} mRNA remained at elevated steady state levels and was not destabilized after 4 hours of induction (Figure 4). These results indicated that PAP_{L71R} behaved similar to wild type PAP during the early stages of induction. Although both growth and translation were inhibited in cells expressing PAP_{L71R}, mRNA was not destabilized, indicating that the L71R mutation did not affect the ability of PAP to inhibit translation, but did affect its function in mRNA destabilization.

**PAP does not affect the stability of the cellular mRNAs examined.** Since PAP is cytotoxic to yeast (17,18), its expression could lead to a general reduction in the steady state levels of all mRNAs in the cell. To test this hypothesis we analyzed the steady state levels of four yeast genes that are constitutively expressed: XRN1, LEU2, PGK1 and RPL3 (22-27). These genes encode the major 5'-to-3' exoribonuclease (XRN1); isopropylmalate dehydrogenase (LEU2) involved in leucine biosynthesis; 3-phosphoglycerate kinase (PGK1) involved in glucose metabolism; and ribosomal protein L3 (RPL3). As shown in Figure 5, the levels of mRNA corresponding to these four genes were unaffected in yeast expressing either PAP or PAP_{E176V}. In both PAP and PAP_{E176V} expressing cells,
the levels of *XRN1*, *LEU2*, and *RPL3* transcripts remained relatively unchanged; however, the level of *PGK1* mRNA decreased. Since *PGK1* mRNA levels decreased in the presence of both PAP and PAP<sub>E176V</sub>, this effect is possibly not due to the activity of PAP (Figure 5). These results indicate that PAP has a specific effect on the accumulation of its own mRNA.

**PAP protein accumulates during galactose induction.** To verify that the effects of PAP on growth and mRNA abundance were due to PAP expression, immunoblot analysis was performed on aliquots harvested from the same cells grown on galactose medium in Figure 4. As shown in Figure 6, protein levels corresponding to mature PAP increased during the 10 hour timecourse of induction in cells expressing the wild type PAP despite the translation inhibition and the reduction in PAP mRNA abundance. PAP is synthesized as a precursor and processed at both N-terminal and C-terminal ends to form the mature protein (7). The higher molecular weight form was previously observed and co-migrates with the precursor form of PAP, which is incompletely processed in yeast (15). Both forms accumulated in cells expressing PAP<sub>E176V</sub>, since translation was not inhibited in these cells (Figure 6). In cells expressing PAP<sub>L71R</sub>, both forms accumulated by four to six hours post-induction and remained at constant levels after six hours, possibly due to inhibition of translation (Figure 6). The immunoblots were stripped and reprobed with antibodies against glucose 6-phosphate dehydrogenase (G6PD) to show that equal amounts of protein were loaded on the gels.

**Correlation between inhibition of translation and mRNA accumulation.** RNase protection analysis in Figure 4 indicated that PAP mRNA levels peak at 4 hours post-induction in cells expressing wild type PAP and PAP<sub>L71R</sub>. This increase is not observed in cells expressing PAP<sub>E176V</sub>, even though the expression of all three genes is driven by the same promoter. PAP<sub>E176V</sub> mRNA levels increase gradually up to 10 hours, as reported with other genes driven by the *GAL1* promoter, such as β-galactosidase which can require 6 hours to reach maximal levels (28). These results suggested that the increase in PAP mRNA levels at 4 hours post-induction could be due to inhibition of translation, since translation is inhibited in cells expressing both the wild type PAP and PAP<sub>L71R</sub>, but not PAP<sub>E176V</sub> at 4 hours post-induction (Figure 3).

To determine if inhibition of translation is responsible for the increase in mRNA levels, we added the translational inhibitor anisomycin to PAP<sub>E176V</sub> cells at 2 hours post-induction. Titration experiments indicated that addition of 15 OD<sub>280</sub> units (approximately 5µg) of anisomycin per ml to PAP<sub>E176V</sub> expressing cells resulted in a similar level of translation inhibition as observed in PAP expressing cells (Table I). As shown in Figure 7A, RNase
protection analysis indicated that PAP<sub>E176V</sub> mRNA levels increased to higher levels after the addition of anisomycin compared to mRNA levels in the absence of anisomycin. PAP<sub>E176V</sub> mRNA levels at 4 hours after induction were 8-fold higher in the presence of anisomycin than the levels at 4 hours in the absence of anisomycin (Figure 7B). The PAP<sub>E176V</sub> mRNA remained at high levels after 4 hours in the presence of anisomycin. These results strongly suggest that the inhibition of translation elongation is responsible for the initial increase observed in mRNA levels in cells expressing PAP or PAP<sub>L71R</sub>.

Immunoblot analysis indicated that PAP<sub>E176V</sub> protein accumulated during the induction in the absence of anisomycin (Figure 8). After addition of anisomycin to PAP<sub>E176V</sub> expressing cells at 2 hours post-induction, a slight increase in protein levels was observed between 2 and 4 hours (Figure 8). This could be due to the slow uptake of anisomycin into the yeast cells. After 4 hours however, both forms of PAP<sub>E176V</sub> protein remained at constant levels and did not increase to the levels observed without anisomycin, confirming that translation was inhibited. The blot was stripped and reprobed with G6PD to demonstrate equal loading of protein on the gels.

**Ribosome depurination in yeast expressing PAP.** Previous results showed that inhibition of translation did not correlate with the decrease in mRNA accumulation, suggesting that the translation inhibition is not due to the reduction in mRNA levels. Since PAP has been reported to inhibit translation by depurinating the S/R loop of the rRNA (4,5), we examined rRNA depurination in cells expressing PAP. Ribosomes isolated from yeast expressing PAP, but not PAP<sub>E176V</sub>, were previously shown to be depurinated (20). PAP may depurinate ribosomes in cis only during its translation. Alternatively, PAP may depurinate ribosomes in trans after it is synthesized in a manner that is independent of translation. To determine if PAP depurinates yeast ribosomes in trans, we induced PAP expression by growing yeast cells for one hour in galactose containing medium, followed by inhibition of PAP gene transcription by shifting cells to medium containing 2% glucose. As shown in Figure 9A, PAP was not detected after one hour on galactose, but accumulated when transcription, but not translation was repressed with glucose. To examine depurination of the rRNA, we used the primer extension assay described previously (3). Ribosomes were not depurinated after one hour on galactose (Figure 9B). However, depurination was observed one hour after repression of transcription (Figure 9B). To determine if translation is required for depurination of rRNA, following a one-hour induction on galactose, cells were resuspended in media containing glucose and cycloheximide to inhibit transcription and translation, respectively. PAP did not accumulate when transcription and translation were both inhibited (Figure 9C). As shown in Figure 9D, ribosomes were depurinated one hour after repression of
transcription and translation equally as well as when only transcription was repressed (Figure 9B). These results demonstrate that rRNA depurination does not require ongoing translation and can occur in trans in a way that is independent of translation.

**Correlation between mRNA destabilization and rRNA depurination.** To examine the relationship between rRNA depurination and mRNA destabilization, a novel dual-oligo primer extension assay was developed to quantify the extent of rRNA depurination at different times after induction of PAP expression. Equimolar amounts of the two oligonucleotides were end-labeled and hybridized to total RNA. One primer hybridized downstream of the depurination site and was used to examine the extent of depurination, while the other primer hybridized upstream of the depurination site close to the 5’ end of the 25S rRNA and was used to quantify the total amount of rRNA (Figure 10A). The ratio of the depurination fragment compared to the control fragment allowed for accurate quantification of the extent of depurination. This ratio was compared to the level of depurination obtained when ribosomes were treated with purified PAP in vitro (PAP in Figure 10B). The primer extension products of total RNA from the same cells expressing PAP and PAP<sub>L71R</sub> shown in Figure 4, were then resolved on a denaturing polyacrylamide gel. As shown in Figure 10B, primer extension analysis indicated that rRNA depurination was detected two hours after induction in cells expressing wild type PAP. Maximal depurination of rRNA occurred at 4 hours after induction and then rapidly decreased (Figure 10C). Depurination of rRNA was also detected in cells expressing PAP<sub>L71R</sub> at 2 hours post-induction. Depurination increased up to 4 hours post-induction and in contrast to cells expressing wild type PAP, it remained at constant levels after 4 hours (Figure 10C). These studies indicated that not all the ribosomes are depurinated in yeast expressing wild type PAP or PAP<sub>L71R</sub>. Maximal depurination in vivo corresponded to 47% of the depurination observed in vitro (Figure 10C). Furthermore, as shown in Figure 10D, the temporal pattern of rRNA depurination was similar to the temporal pattern of mRNA accumulation in cells expressing wild type PAP and PAP<sub>L71R</sub>, indicating that there is a direct relationship between rRNA depurination and mRNA abundance. These results suggest that PAP may depurinate the rRNA and destabilize its own mRNA by a common mechanism.

**DISCUSSION**

The results presented here demonstrate that in addition to depurinating the rRNA, PAP regulates its own expression by reducing the abundance of its own mRNA. This effect is dependent on the N-glycosidase activity of
PAP, since an active site mutant fails to alter its mRNA levels. We present evidence that inhibition of growth observed in PAP expressing cells is due to inhibition of translation. Depurination of the rRNA and inhibition of translation by PAP leads to up-regulation of the steady state levels of PAP mRNA, which occurs prior to mRNA destabilization. Using a PAP variant which depurinates rRNA, inhibits translation, but does not destabilize its own mRNA, we show that destabilization of PAP mRNA can be separated from rRNA depurination and translation inhibition. We examine the relationship between the rRNA depurination and destabilization of PAP mRNA and present evidence that they may be mechanistically related.

Total translation was reduced by 65-75% in cells expressing wild type PAP and PAP_{L71R} at 4 hours post-induction and remained at that level throughout the timecourse. In contrast, total translation was not significantly inhibited in cells expressing PAP_{E176V}. These results indicated that protein synthesis is inhibited, but not completely abolished in cells expressing PAP. Many RIPs have been shown to depurinate DNA, RNA and poly (A) RNA (29). We have previously shown that PAP can inhibit translation in a cell-free system by depurinating capped RNAs. To determine if PAP targets capped RNAs in vivo, we examined the stability of PAP mRNA and cellular mRNAs in yeast and showed that induction of PAP expression led to a dramatic decrease in PAP mRNA abundance. Steady state levels of four different cellular mRNAs were not affected by PAP, demonstrating that PAP mRNA is not destabilized simply as a consequence of host cell death. These results indicated that PAP expression does not destabilize every RNA and therefore exhibits specificity. DNA microarray analysis of yeast expressing PAP confirmed these results and indicated that PAP expression affects the abundance of specific mRNAs in yeast (data not shown). We observed no effect on the U3 snoRNA, which is capped with a trimethyl guanosine (TMG). These results are consistent with our prior observations, which indicate that PAP destabilizes mRNAs containing a 7-methyl guanosine cap and suggest that PAP may not recognize the trimethyl guanosine cap present on the U3 RNA.

As summarized in Table II, analysis of PAP mRNA accumulation in two different PAP mutants with reduced toxicity indicated that destabilization of PAP mRNA required an intact active site, since the active site mutant, PAP_{E176V} did not decrease the abundance of its mRNA. The rRNA was depurinated and translation and growth were inhibited in cells expressing PAP_{L71R} just like wild type PAP. However, mRNA was not destabilized, indicating that destabilization of PAP mRNA can be dissociated from depurination of the S/R loop and inhibition of translation (Table II). Unlike previously characterized pathways of mRNA degradation, which are interconnected with translation (30), PAP-mediated degradation of PAP mRNA occurs when translation is inhibited.
Inhibition of translation correlated well with the up-regulation of PAP mRNA levels in cells expressing wild type PAP and PAP_{L71R} at 4 hours post-induction. This increase was not observed in cells expressing PAP_{E176V}, which did not inhibit translation. To determine if inhibition of translation elongation would lead to stabilization of PAP mRNA levels, we added the elongation inhibitor anisomycin to cells expressing PAP_{E176V}. RNase protection analysis indicated that PAP_{E176V} mRNA levels increased dramatically after addition of anisomycin. These results suggest that the increase observed in PAP mRNA levels at 4 hours post-induction in cells expressing wild type PAP and PAP_{L71R} is due to inhibition of translation elongation. They are consistent with previous reports where elongation inhibitors have been shown to stabilize mRNAs in yeast (30).

We show that very small amounts of PAP synthesized during one hour of induction on galactose depurinated ribosomes in trans, demonstrating that ribosome depurination occurs in a way that is independent of translation. Similar results are reported with ricin, where one molecule of ricin has been shown to inactivate 300 ribosomes in trans (31). A novel dual-oligo primer extension assay was devised to examine the relationship between rRNA depurination and mRNA decay. In cells expressing wild type PAP, depurination of rRNA was detected prior to destabilization of PAP mRNA, indicating that rRNA can be depurinated under conditions when PAP mRNA is not degraded. Since rRNA depurination can be separated from mRNA destabilization, PAP may destabilize its own mRNA in trans when it is on the ribosome, but depurinate the ribosomes whether or not they translate its mRNA. Our previous observations indicate that PAP mRNA degradation most likely occurs on the ribosomes. We demonstrated that PAP is associated with and binds to ribosomes in yeast through its ability to physically interact with the ribosomal protein L3 (RPL3) (15). A chromosomal mutant of yeast, harboring the mak8-1 allele of RPL3, is resistant to PAP because PAP cannot interact with the mutant L3 in vivo. Although PAP protein accumulated in mak8-1 cells, PAP was not associated with ribosomes and ribosomes were not depurinated (15). RNase protection analysis showed that PAP transcripts were not destabilized in mak8-1 cells expressing PAP or PAP_{E176V} (15), suggesting that PAP mRNA destabilization occurs on the ribosome.

We present evidence that the temporal patterns of rRNA depurination in cells expressing wild type PAP and PAP_{L71R} are very similar to the patterns of mRNA accumulation (Figure 10D), suggesting that they are mechanistically related. This is further supported by the observation that destabilization of PAP mRNA occurs independently of translation, suggesting that like rRNA, PAP destabilizes its own mRNA in trans. Analysis of PAP
mRNA turnover in the presence of cycloheximide or anisomycin demonstrated that PAP mRNA is destabilized in trans in a manner that is independent of translation (data not shown).

Our previous results indicate that PAP depurinates capped, but not uncapped RNAs in a cell-free system (3). We have recently characterized this activity further and showed that PAP binds to the cap structure (16). PAP does not remove the cap structure or depurinate the cap, but depurinates the mRNA at specific adenine and guanines downstream of the cap (16). If a single site of PAP binds to both cap and purines, it implies that a single molecule of PAP cannot do so simultaneously. Incubation of ribosomes with PAP and increasing concentrations of the cap analog m7GpppG resulted in a decrease in the level of rRNA depurination, indicating that the cap structure competes with the rRNA for binding to PAP (16). We have shown that the affinity of PAP for capped message is only 4-fold lower than its affinity for rRNA (16), indicating that at increased levels, such as those seen as a result of translation inhibition, capped RNA may become a substrate for PAP.

A model that takes these observations into account is one that separates early events (pre-destabilization) from late events (destabilization). Galactose induction causes PAP mRNA and protein to accumulate. Only a small amount of protein is required for S/R loop depurination to occur and this depurination can proceed in trans. By 4 hours post-induction, rRNA depurination reaches maximal levels, translation is inhibited and there is a dramatic increase in PAP mRNA abundance, which is not observed in cells expressing PAP_E176V. Since translation is inhibited very early in cells expressing PAP, this inhibition most likely causes the accumulation. PAP mRNA may be sequestered on depurinated ribosomes by the interaction between PAP and the cap structure of the mRNA. Translation remains inhibited late in the timecourse, but PAP protein accumulates, indicating that its translation is somehow insensitive to PAP mediated translation inhibition. Previous studies also showed that unlike ricin, PAP does not inhibit translation of its own mRNA in vitro in rabbit reticulocyte lysate (17). As the timecourse continues, enough PAP is translated to create a high concentration of protein on the ribosome. Although translation remains inhibited late in the timecourse, there is now enough active PAP present to depurate the mRNA on the ribosomes. Under these conditions, the depurination of the PAP mRNA would occur independently of depurination of the rRNA and inhibition of translation. The depurinated RNA may be degraded as a result of cleavage by cellular lyases as observed for rRNA in wheat germ (32). RNase protection analysis indicates that PAP mRNA levels decrease and PAP mRNA is not detectable after 12 hours. In some experiments, we have observed shorter fragments of PAP mRNA, indicating that it is not simply sequestered; it is being degraded (data not shown).
Our model implies that the destabilization observed may not be specific for only PAP mRNA, but may affect other mRNAs translated by depurinated ribosomes. We present evidence that PAP does not affect every capped mRNA in the cell. In vitro results also indicate that capped viral RNAs differ in their sensitivity to PAP indicating that the presence of the cap structure is not the only determinant for depurination by PAP. This activity may be modulated in vivo by association with cellular proteins or through recognition of particular secondary structures in addition to cap, which would enable PAP to target particular transcripts.

Mature PAP is an extracellular protein stored in the apoplast of pokeweed plants where it can accumulate up to 0.5% of total soluble protein (33). This compartmentalization prevents access to pokeweed ribosomes (34). It has been reported that PAP preferentially enters virus-infected cells. It is estimated that 80% of the RNA in healthy cells is ribosomal, but during viral infection the level of viral RNA increases significantly beyond the normal level of capped messages and approaches the levels of rRNA (16). In this situation, PAP may target capped viral RNAs. Nontoxic variants of PAP exhibit antiviral activity in vivo, suggesting that they do not target every capped RNA in the cell, but likely display specificity for viral RNAs or cellular messages that are involved in virus replication. RIPs are thought to be defense proteins; however, the mechanism by which they inhibit cell growth or pathogen infection is not well understood. The results reported here suggest that PAP may have a role in differentially regulating mRNA stability in vivo. Further studies will address the basis for the selectivity of this regulation and its role in the antiviral activity of PAP.

ACKNOWLEDGEMENTS:
We thank J. Dinman for plasmids used in this work, Drs. P. Day, J. Dinman, K. Hudak, E. Lam, S. Gunderson and A. Shatkin for helpful discussions and reading the manuscript. We also thank Dr. T. Kinzy for comments on the manuscript and the members of T. Kinzy’s lab for assistance with the yeast translation assays. This work was supported by a NSF grant MCB 9982498 to N. Tumer.

REFERENCES


FIGURE LEGENDS:

Figure 1. Diagram of wild type PAP and the PAP variants. The mature PAP protein is 262 amino acids in length. Twenty-two amino acids are cleaved from the N-terminus and 29 amino acids from the C-terminus during processing of the PAP-precursor to the mature protein. The amino acid changes and their positions are shown for each nontoxic variant, NT224 and NT538.

Figure 2. Growth of yeast expressing PAP and the PAP variants. Expression of PAP, PAP_{L71R}, PAP_{E176V}, PAP_{E176V} + anisomycin and vector control (VC) was induced by growing cells in SD-leu media containing 2% galactose. An OD_{600} reading was taken for growth measurement at the indicated times after induction.

Figure 3. Analysis of total translation in cells expressing PAP, PAP_{E176V} and PAP_{L71R}. Yeast harboring PAP, PAP_{L71R}, or PAP_{E176V} or vector control were grown in SD-leu,-met, 2% galactose for 4 hours (A) or 10 hours (B) to induce protein expression. At time zero, [\textsuperscript{35}S]methionine was added to cells growing on galactose that either express PAP (◊), or PAP_{E176V} (■), PAP_{L71R} (▲), or vector control (Ο) and incorporation was determined at the times indicated (in minutes). Each point was assayed in duplicate and the translation rates were determined from three separate experiments.

Figure 4. RNase protection of PAP, PAP_{E176V} and PAP_{L71R} expression in yeast. A. Expression of wild type PAP, PAP_{E176V}, and PAP_{L71R} was induced by growing cells in SD-leu containing 2% galactose. Total RNA (15µg) extracted from yeast cells harvested at 0, 2, 4, 6, 8 and 10 h post-induction was analyzed by RNase protection analysis. Positions of the PAP and U3 probes alone are indicated in lanes marked “PAP” and “U3.” The “tRNA” lane represents RNase protection using tRNA hybridized with both PAP and U3 probes. B. The mRNAs corresponding to PAP and U3 were quantified and the ratios of PAP mRNA to U3 snoRNA were plotted at various times after induction of expression.

Figure 5. RNase protection analysis of yeast cellular mRNAs. RNase protection analysis was carried out with probes specific for XRNI, LEU2, RPL3, and PGK1. Total RNA (10 µg) extracted from yeast cells expressing PAP or PAP_{E176V} at different times after induction was analyzed by RNase protection assay as described in Experimental
Procedures. The mRNAs corresponding to each gene were quantified and the ratios of \textit{XRN1}, \textit{LEU2}, \textit{RPL3} and \textit{PGK1} to \textit{CYH2} mRNA were plotted at various times after induction of expression.

**Figure 6. Immunoblot analysis of PAP and PAP\textsubscript{E176V} expression in yeast.** Total protein (7.5 µg) from each time point in Figure 4 was separated on 15% SDS-PAGE. Proteins were transferred to nitrocellulose and probed with polyclonal PAP serum (1:5000). Purified PAP (approximately 10ng) from pokeweed leaves was used as a standard. The immunoreactive species corresponding to mature PAP is indicated with an arrow. The blots were subsequently stripped and reprobed with anti-G6PD (1:5000) antibodies as loading controls.

**Figure 7. RNase protection of PAP\textsubscript{E176V} and PAP\textsubscript{E176V} + anisomycin expression in yeast.** A. Expression of PAP\textsubscript{E176V} was induced by growing cells in SD-leu containing 2% galactose. At 2 h post-induction, 15 OD\textsubscript{280} units of anisomycin (approximately 5µg/ml) per ml was added to one of two PAP\textsubscript{E176V} cultures. Total RNA (15µg) extracted from yeast cells harvested at 0, 2, 4, 6, 8 and 10 h post-induction was analyzed by RNase protection analysis as in Figure 4. B. The mRNAs corresponding to PAP and U3 were quantified and the ratios of PAP mRNA to U3 snoRNA were plotted at indicated times after induction of expression.

**Figure 8. Immunoblot analysis of PAP\textsubscript{E176V} and PAP\textsubscript{E176V} + anisomycin expression in yeast.** Total protein (7.5 µg) loaded from each time point in Figure 7 was separated on 15% SDS-PAGE. The amount of PAP protein expressed was determined as in Figure 6.

**Figure 9. Analysis of rRNA depurination during PAP induction.** Yeast cells expressing wild type PAP were resuspended in SD-leu plus galactose to induce PAP expression. Immediately prior to galactose addition, an aliquot was pelleted to serve as a raffinose control (Raf). Upon 1 hour of galactose induction, a second aliquot was collected (Gal), and either glucose alone (+Glucose) or glucose plus cycloheximide (100µg/ml final concentration) was added (+Glucose+CHX). At the times indicated (hours) aliquots were removed for depurination (B and D) and immunoblot analysis (A and C).
Figure 10. Analysis of rRNA depurination during induction of PAP and PAP<sub>L71R</sub>. A. Schematic representation of dual-oligo primer extension assay. Two different end-labeled primers (depurination primer and 25S control primer) are annealed to rRNA and reverse transcribed. The resulting fragments represent extension products that have stopped prematurely at the depurination site (indicated with an asterisk) and extension products that have stopped at the 5’ end of the 25S rRNA. B. The primer extension products for PAP and PAP<sub>L71R</sub> representing the extent of depurination and the amount of total 25S rRNA present at the times indicated (hours) from the same samples assayed in Figure 4 are resolved on a denaturing polyacrylamide gel. Ribosomes treated in vitro with purified PAP or buffer alone are designated as PAP and no PAP, respectively. Primers were also extended separately as marked in the first two lanes. C. The extent of depurination in Figure 10B was quantified by calculating the ratio of the depurination fragment to the 25S rRNA fragment and this ratio was expressed as a percent of the depurination observed in vitro. The quantification was repeated twice more with similar results. D. Comparison of the temporal pattern of rRNA depurination (Figure 10C) and PAP mRNA abundance (Figure 4B).
### Table I: Translation rates of PAP and PAP variants

<table>
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<th>PAP or PAP variant</th>
<th>Translation Rate (% of control)(^a)</th>
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<tr>
<td>PAP</td>
<td>27.4 ± 3.0%</td>
</tr>
<tr>
<td>PAP(_{E176V})</td>
<td>88.7 ± 2.9%</td>
</tr>
<tr>
<td>PAP(_{L71R})</td>
<td>33.8 ± 0.7%</td>
</tr>
<tr>
<td>PAP(_{E176V}) + Anisomycin(^b)</td>
<td>35.0 ± 5.0%</td>
</tr>
</tbody>
</table>

\(^a\) Translation rates were determined at 4 hours post induction by measuring the slope of the methionine incorporation curve shown in Figure 3A and expressed as a percent of the translation rate for the vector control. The rates were confirmed by at least two independent experiments.

\(^b\) Approximately 5µg/ml of anisomycin was added to cells expressing PAP\(_{E176V}\) at 2 hours post induction and translation was analyzed at 4 hours as above.
Table II: Comparison of the effects of PAP and PAP variant expression in yeast

<table>
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<tr>
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<th>PAP</th>
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</table>
Figure 1
Parikh B.A., et al

RNA Binding
Active Site
Lipid Binding

Signal peptide
(22 a.a.)

LYVM
R(71)
NT538

EAAR
V(176)
NT224

C-terminal extension
(29 a.a.)
Figure 2
Parikh B.A., et al
Figure 3
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4 Hours Post-Induction
- PAP
- PAP<sub>E76A</sub>
- PAP<sub>L71R</sub>
- Vector Control

10 Hours Post-Induction
- PAP
- PAP<sub>E76A</sub>
- PAP<sub>L71R</sub>
- Vector Control
Figure 4

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A. 

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B. 

![Graph showing PAP/U3 ratio over time for different RNA samples.](image)

- **A.** Diagram showing gel electrophoresis of RNA samples labeled PAP, PAP<sub>E176V</sub>, and PAP<sub>L71R</sub> at different time points post-induction.

- **B.** Bar graph showing PAP/U3 ratio over time for the same RNA samples. The ratio is calculated and plotted at intervals of 0, 2, 4, 6, 8, and 10 hours post-induction.
Figure 5
Parikh B.A., et al
Figure 6
Figure 7

Parikh B.A., et al

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**A.**

![Image of gel electrophoresis](image)

- PAP
- U3

**B.**

![Bar graph of PAP/U3 ratio](image)

- PAP/E176V
- PAP/E176V + Anisomycin

Hours Post-Induction:

0, 2, 4, 6, 8, 10
Figure 8
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[Image of Western Blot showing protein bands for PAP and G6PD under different conditions]
Figure 9
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A.

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<th>+ Glucose</th>
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PAP →

G6PD →

B.

<table>
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Depurination →

C.

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PAP →

G6PD →

D.

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Depurination →
Figure 10
Parikh B.A., et al

A.  
5' 25S Control primer Depurination site Depurination primer 3' 25S rRNA

B.  
25S Control primer Depurination primer

C.  
% of in vitro depurination

Hours post-induction

0 2 4 6 8 10

0.0% 10.0% 20.0% 30.0% 40.0% 50.0%
Figure 10
Parikh B.A., et al

D.

![Graph showing PAP and PAP_{L71R} mRNA depurination over time.](image-url)
Pokeweed antiviral protein regulates the stability of its own mRNA by a mechanism that requires depurination, but can be separated from depurination of the α-sarcin/ricin loop of rRNA

Bijal A. Parikh, Chris Coetzer and Nilgun E. Tumer

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