A novel interaction of pokeweed antiviral protein with translation initiation factors 4G and iso4G: a potential indirect mechanism to access viral RNAs

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ABSTRACT

Pokeweed antiviral protein (PAP) is a ribosome inactivating protein recognized primarily for its ability to depurinate the sarcin/ricin loop of the large rRNA. Studies have demonstrated that PAP also depurinates other RNA templates, such as Human immunodeficiency virus-1 RNA and Brome mosaic virus RNAs. However, the mechanism by which PAP accesses viral RNAs is not known. Considering that PAP was shown recently to bind the m^7G of the cap structure, we speculated that PAP may interact with other factors involved in translation initiation. By far western analysis, we show that PAP binds specifically to eIF4G and eIFiso4G of wheat germ and analysis with truncation mutants of eIFiso4G indicates that a region of this protein, between amino acids 511 and 624, is required for PAP binding activity. The yeast two-hybrid system supports these results by showing reduced growth and α-galactosidase expression with truncation in this region of eIFiso4G. PAP binds m^7GTP-Sepharose and this interaction does not diminish the binding of PAP to purified eIFiso4G, indicating that a complex can form among the cap structure, PAP and eIFiso4G. We incubated PAP with uncapped and non-polyadenylated transcripts containing a 3′ translation enhancer sequence (TE) known to increase translation of the RNA in an eIF4F dependent manner. We show that in the presence of wheat germ lysate, PAP depurinates the uncapped and non-polyadenylated transcripts containing a functional wild-type 3′TE, but does not depurinate messages containing a non-functional mutant 3′TE. These results support our hypothesis that binding of PAP to eIF4G and eIFiso4G can provide a mechanism for PAP to access both uncapped and capped viral RNAs for depurination.

INTRODUCTION

The eukaryotic translation initiation factor eIF4G is a large cytosolic protein that serves as a scaffold for the binding and interaction of several initiation factors and other proteins that mediate the start of translation. eIF4G interacts with the cap-binding protein, eIF4E, and increases its affinity for the m^7G structure at the 5′end of the mRNA (1). Before the 40S ribosomal subunit contacts the mRNA, it is equipped with the ternary complex of eIF2, GTP and Met-tRNAi. eIF3 binds to this ribosomal subunit, and with the ternary complex, forms the 43S initiation complex. eIF3 also binds to eIF4G, and therefore, the 43S particle is brought to the 5′ end of the mRNA via interaction of initiation factors with eIF4G. The factors eIF4A and 4B, also bound to eIF4G, function to unwind RNA secondary structure and eIF1 and eIF1A enable scanning to the AUG codon [reviewed in Refs (2–4)]. Therefore, eIF4G is a central protein that mediates the connection of factors to the mRNA and the preparation of the mRNA for translation.

eIF4G can also serve as a central linking protein in cap-independent translation, whereby the 43S particle accesses the mRNA using an internal ribosome entry site [IRES, reviewed in Refs (5–7)]. For example, several picornaviruses, which rely on IRES-mediated translation of their RNAs, modify eIF4G to limit translation of capped cellular messages. The 2A protease of rhinovirus and the Lb protease of foot-and-mouth disease virus (FMDV) cleave eIF4G resulting in an amino terminal fragment containing the eIF4E binding site and the C-terminal region containing interacting sites for eIF3 and eIF4A (8,9). Hence, these viral proteins effectively uncouple cap-binding from ribosome interaction. The C-terminal fragment of eIF4G following proteolysis has been shown to directly bind domain 4 of the FMDV IRES, suggesting that eIF4G acts as a linker to recruit translation factors and the ribosomal

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small subunit in IRES-mediated initiation (10). The involvement of eIF4G in both cap-dependent and cap-independent initiation makes it a focus for proteins that regulate translation.

In this report, we describe a novel interaction between eIFiso4G of wheat germ and pokeweed antiviral protein (PAP), a ribosome inactivating protein synthesized by the pokeweed plant (*Phytolacca americana*). Like all ribosome inactivating proteins, PAP is an N-glycosidase that cleaves an adenine from the conserved sarcin/ricin loop of the large subunit ribosomal RNA (11,12). Depurinated ribosomes are unable to bind elongation factor 2, resulting in inhibition of translation and subsequent cell death [reviewed in Refs (13,14)]. The antiviral activity of many ribosome inactivating proteins has been attributed to their toxicity however, work with mutants of PAP expressed in tobacco has shown that antiviral activity can be separated from depurination of ribosomes (15). Moreover, studies have demonstrated that PAP depurinates viral RNAs such as Brome mosaic virus (BMV) and Human immunodeficiency virus *in vitro* (16,17) and limits virus multiplication *in vivo* without killing host cells (18,19). We have previously shown that PAP is able to bind the m^7G cap structure at the 5′ end of some viral messages and thus access them for depurination (16,20). However, this cap-dependent model does not explain the observed inhibitory effect of PAP on the replication of uncapped viruses such as influenza and poliovirus (21,22). We decided to investigate whether PAP may also be able to interact with translation initiation factors, and this work provides the first evidence that PAP binds to eIF4G and its isoform eIFiso4G. In wheat, two forms of eIF4G exist, which differ in size, 180 (eIF4G) and 86 kDa (eIFiso4G), and they bear only 30% identity at the amino acid level (23,24). We show that PAP binds specifically to each form, and by genetic and biochemical analyses, we present evidence that a region of the protein, between amino acids 511 and 624, is required for PAP binding activity. It is not known how PAP targets uncapped viral RNAs, however, its binding to either eIF4G or eIFiso4G may provide a mechanism to access uncapped and capped templates for depurination via these central scaffolding proteins.

MATERIALS AND METHODS

Expression and purification of initiation factors

The eIFiso4G gene of wheat and its truncation mutants (N52, N90, N136, N186, C462, C489, C511, C624 and C732) were cloned into pET-3d (Novagen) at the NcoI and BamHI sites (25). Vectors were transformed into *Escherichia coli* Rosetta(DE3)pLysS cells and induced with 0.5 mM IPTG for 3 h. eIFiso4G and its truncation mutants were purified from cell lysates with P-11 cellulose phosphate resin, essentially as described (26). Purified proteins were concentrated by filtration centrifugation with a 10 kDa cut-off filter (Amicon), washed twice with an equal volume of buffer B-50 (20 mM HEPES–KOH, pH 7.6, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA) and reconcentrated, and stored at −80°C.

The eIFiso4E gene of wheat was cloned into pET-3d (Novagen) at the NcoI and BamHI sites (26). The vector was transformed into *E.coli* BL21(DE3)pLysS cells and induced with 0.5 mM IPTG for 1.5 h. eIFiso4E was purified from cell-free extracts by incubation with m^7GTP-Sepharose (Amersham Pharmacia) essentially as described (26). Purified eIFiso4E was concentrated by filtration centrifugation with a 10 kDa cut-off filter (Amicon), washed twice with an equal volume of buffer V-50Ac (50 mM HEPES–KOH, pH 7.0, 50 mM KOAc, 10% glycerol, 1 mM DTT, 0.1 mM EDTA) and reconcentrated, and stored at −80°C. Purified proteins were visualized by separation in 12% SDS–PAGE and staining with Coomassie blue.

Far western assay

Purified proteins (50 nmol each) in blotting buffer (20 mM HEPES–KOH, pH 7.6, 1 mM DTT, 0.1 mM EDTA, 0.1 mM MgAc, 10% glycerol) were applied to nitrocellulose using a dot-blot apparatus (Bio-Rad). The membrane was blocked with 5% non-fat milk in blotting buffer for 2 h and then incubated with 4 μg/ml purified PAP in the same buffer for an additional 2 h. The membrane was probed with anti-PAP monoclonal antibody (1:500) and PAP was visualized using a chemiluminescence detection kit (NEN Life Science Products). Purified PAP (12 nmol) and ribosomes of *Saccharomyces cerevisiae* (10 nmol) were blotted as positive controls. BSA (75 nmol) and blotting buffer alone were used as negative controls.

Yeast two-hybrid analysis

The cDNA of eIFiso4E was cloned into the activating domain plasmid pGAD-424 and cDNAs of eIFiso4G or its truncation mutants were cloned into the binding domain plasmid pGBT-9/N (25). The cDNA of PAPx was cloned into the activating domain plasmid pAS2-1 (N. E. Tumer, Rutgers University). PAPx is a point mutant of wild-type PAP [E176V, (27)] and was used for transformation because it is not toxic to yeast growth. Plasmids were transformed into the yeast strain AH109 and plated onto SD -Leu, -Trp, -His, -Ade with 2% dextrose and X-α-Gal (5-bromo-4-chloro-3-indolyl α-D-galactopyranoside). Growth of colonies and presence of blue colour owing to *MEL1* (α-galactosidase) gene expression were compared with cells transformed with cDNAs of eIFiso4G and eIFiso4E. In addition, cells transformed with either pTD1 and pVA3 or pTD1 and pLAM5′ were used as positive and negative controls for interaction, respectively (Clontech Laboratories, Inc.).

Affinity pull-downs of PAP and eIFiso4G or truncation mutants with m^7GTP-Sepharose

Equal molar amounts of PAP and eIFiso4G or its truncation mutants (34 nmol) were mixed in 1 ml of buffer B-50 and added to m^7GTP-Sepharose beads equilibrated in the same buffer. Mixtures were rotated for 1 h at 4°C, and then beads were pelleted at 15 600× *g* for 2 min and washed three times with buffer B-50. Proteins were eluted with 1 mM m^7GTP in buffer B-50, separated on 12% SDS–PAGE and visualized with Coomassie blue staining.

To test for complex formation with m^7GTP-Sepharose in a mixture of translation factors, 2 μg PAP was added to 500 μg wheat germ lysate (Promega) depleted of eIF4E/eIFiso4E and incubated with m^7GTP-Sepharose in 1 ml buffer B-50. Mixtures were incubated and proteins eluted from the

Sepharose as described above for incubations using purified proteins. Eluted proteins were analyzed by immunoblot for the presence of PAP or eIFiso4G, using polyclonal antibodies directed against each PAP or eIFiso4G (1:5000). Lysate (500 μg) was initially depleted of eIF4E/eIFiso4E by incubation with m⁷GTP-Sepharose in 1 ml of buffer B-50 with rotation for 1 h at 4°C. Beads were pelleted at $15\,600 \times g$ for 2 min and discarded. The supernatant was incubated an additional two times with m⁷GTP-Sepharose. Recombinant eIFiso4G (150 ng) was added to the final supernatant prior to incubation with PAP (2 μg) and additional m⁷GTP-Sepharose.

Depurination of uncapped RNA template in the presence of wheat germ lysate

The plasmids pGLO18 and pGLO28 [previously referred to as pLUC869 and pLUC869BF, respectively, (28)] encode luciferase flanked by the 5′-untranslated region (5′-UTR) and 3′-UTR of barley yellow dwarf virus (BYDV). pGLO28 differs from pGLO18 in that it contains a four-base duplication that fills the BamHI$_{4837}$ site within the translational enhancer element of the viral 3′-UTR. Plasmids were linearized with SmaI and used as template for generation of radiolabelled (α ³²P-CTP, 3000 Ci/mmol, Amersham) *in vitro* transcripts using T7 polymerase. Transcripts (1.3×10^5 c.p.m.) were added to a wheat germ *in vitro* translation system (Promega) in the presence or absence of 5 ng PAP in a total reaction volume of 25 μl. As a negative control for the presence of lysate, the volume of lysate (12.5 μl) was replaced by modified RIP buffer (60 mM KCl, 10 mM Tris–HCl pH 7.4, 2.5 mM MgCl$_2$). All samples were incubated at 30°C for 30 min prior to extraction of total RNA. The RNA was subsequently treated with aniline as described previously (15). Samples were solubilized in formamide buffer, separated on a 7 M urea/4.5% polyacrylamide gel, dried against filter paper and visualized by autoradiography.

RESULTS

### Expression and purification of translation initiation factors

Recombinant eIF4G, eIFiso4G and terminally truncated eIFios4G mutants used in this study were expressed and purified from *E.coli* Rosetta(DE3)pLysS cells using phosphocellulose (P11) chromatography. eIFiso4E was purified from BL21(DE3)pLysS cells using m⁷GTP-Sepharose. Purified proteins were separated by 12% SDS–PAGE and visualized by Coomassie blue staining (Figure 1). PAP used in this study was purified from pokeweed and was also included in this gel. The major protein products are seen in each lane, with few 'background' bands, indicating primarily intact and pure preparations of full-length eIFiso4E, eIF4G, eIFiso4G and its truncation derivatives.

In vitro binding of PAP to eIF4G, eIFiso4G and its terminally truncated mutants

To determine if PAP bound directly to eIF4G or eIFiso4G *in vitro*, far western analysis was conducted with purified proteins applied directly to nitrocellulose using a dot-blot apparatus. The nitrocellulose was incubated with purified PAP and interactions between eIF4G or eIFiso4G and PAP
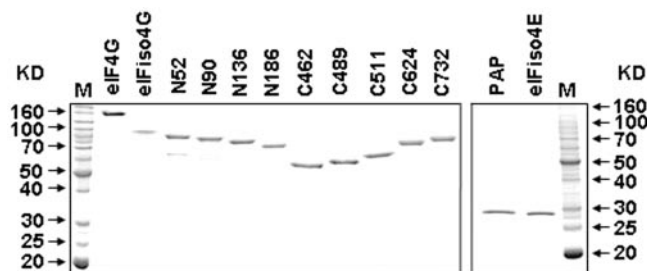
Figure 1. Separation of purified proteins. Recombinant eIFiso4E, eIF4G, eIFiso4G and its truncation mutants (1 μg) were separated by 12% SDS–PAGE and visualized with Coomassie blue stain. PAP (1 μg) was purified from pokeweed leaves. M refers to protein broad range molecular mass marker.
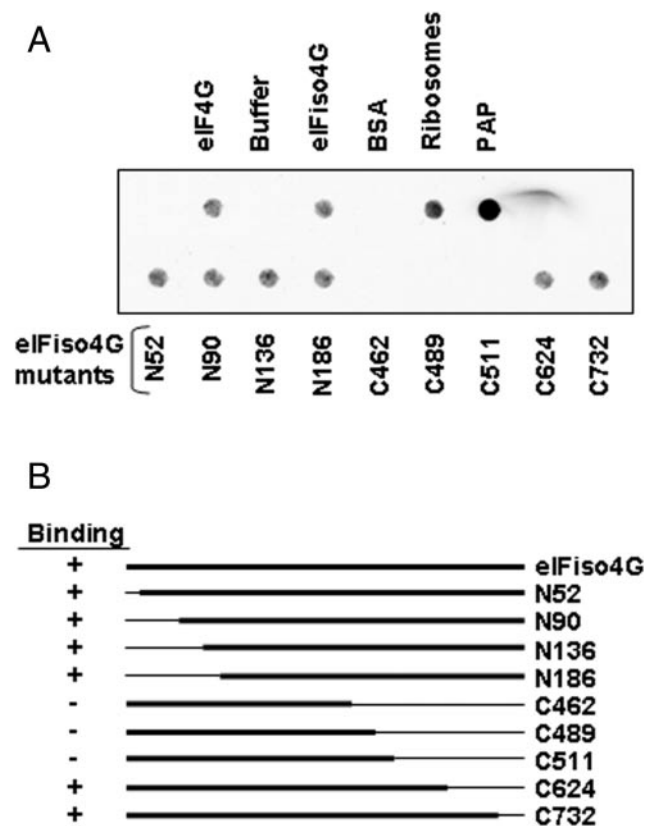
Figure 2. Far western analysis of PAP binding to eIFiso4G and its truncation mutants. (**A**) Equal molar concentration of purified proteins (50 nmol) was spotted onto nitrocellulose and incubated with purified PAP (4 μg/ml). PAP binding to proteins was tested by probing with PAP monoclonal antibody (1:500) and PAP was visualized by chemiluminescence. BSA (75 nmol) and buffer alone were used as negative controls and purified yeast ribosomes (10 nmol) were used as positive control for PAP binding. Purified PAP was applied as a positive control for antibody specificity. (**B**) Schematic drawing of full-length eIFiso4G and its truncation mutants. The full-length eIFiso4G is 788 amino acids. Thick lines represent relative sizes of each mutant. Thin lines indicate deleted regions from the full-length protein. The (+) sign denotes binding of the mutant form to PAP and (−) sign denotes no binding, as detected by the far western method.

were detected by probing with a PAP-specific monoclonal antibody. Importantly, the direct application of protein to nitrocellulose allowed for the test of interactions without denaturation of the proteins. The results in Figure 2A indicate

that PAP binds directly to either eIF4G or eIFiso4G. As a positive control, purified yeast ribosomes were blotted on the nitrocellulose because we have previously shown that PAP binds to ribosomal protein L3 (29). Negative controls for binding were the application of BSA and buffer alone to the nitrocellulose. Both the controls yielded validating results.

To investigate the region of eIFiso4G involved in binding to PAP, equal molar amounts of N- and C-terminal truncation mutants of eIFiso4G were tested by the same method. Figure 2A shows that PAP bound to the N-terminal truncation mutants and to the C732 and C624 truncation mutants, but not to the C-terminal mutants with larger deletions, namely C511, C489 and C462. Improper folding of the eIFiso4G derivatives was not likely, given that these truncation mutants were used to characterize the binding site of eIFiso4E to eIFiso4G (25). Our results, summarized in Figure 2B, suggest that PAP binds to a central region of eIFiso4G and that the portion between amino acids 511 and 624 is required for binding activity.

In vivo interaction between PAP and eIFiso4G

To test whether the *in vitro* interaction of PAP and eIFiso4G could also be demonstrated *in vivo*, yeast two-hybrid vectors encoding the full-length eIFiso4G or its truncation mutants were expressed in the yeast strain AH109 with a vector encoding the non-toxic mutant PAPx. Interaction on SD -Leu, -Trp, -His, -Ade, 2% dextrose plates was assessed by growth and α-galactosidase activity (intensity of blue colour) compared with the positive control, cells transformed with plasmids encoding the full-length eIFiso4G and the cap-binding protein eIFiso4E. In addition, cells transformed with either pTD1 and pVA3 or pTD1 and pLAM5′ were used as positive (+) and negative (−) controls for interaction, respectively. The results indicate that PAP interacts with full-length eIFiso4G and with all N-terminal truncation mutants and with the C732 and C624 mutants. However, little growth and α-galactosidase activity could be detected in cells expressing both PAP and the C-terminal truncation mutants C511, C489 and C462 (Figure 3A). These *in vivo* data correlate well with the *in vitro* data (Figure 3B) and corroborate the importance of the central region of eIFiso4G for PAP binding activity.
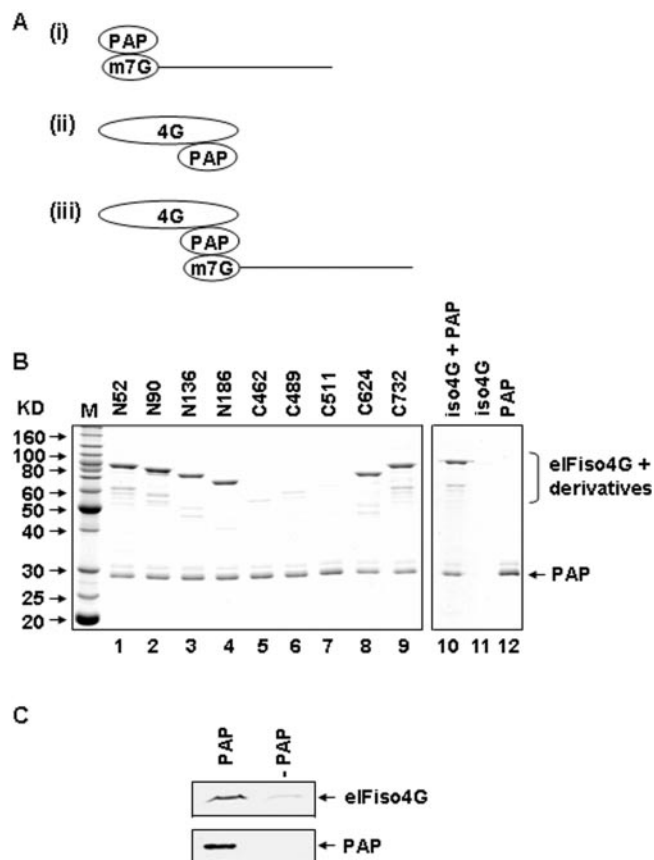
eIFiso4G-PAP-m⁷G complex formation

PAP has been shown previously to bind m^7GTP-Sepharose [Figure 4A (i)] and modeling studies suggest that binding would occur at the enzymatic active site of PAP, similar to the binding of adenine (16). Our present data indicate that PAP can also bind to eIFiso4G [Figure 4A (ii)]. To determine if PAP could simultaneously form a complex with both eIFiso4G and the cap structure [Figure 4A (iii)], equal molar amounts of PAP and eIFiso4G were incubated with m^7GTP-Sepharose beads and eluted with 1 mM m^7GTP. Eluted samples were separated by 12% SDS–PAGE and visualized by Coomassie blue staining (Figure 4B). The presence of both PAP and eIFiso4G in the eluted fraction indicates that PAP was able to form a tertiary complex with full-length eIFiso4G (Figure 4B, lane 10). Moreover, the data show that the binding site of m^7GTP to PAP does not overlap the binding site of eIFiso4G to PAP. Importantly, eIFiso4G did not bind in the absence of PAP (Figure 4B, lane 11), indicating
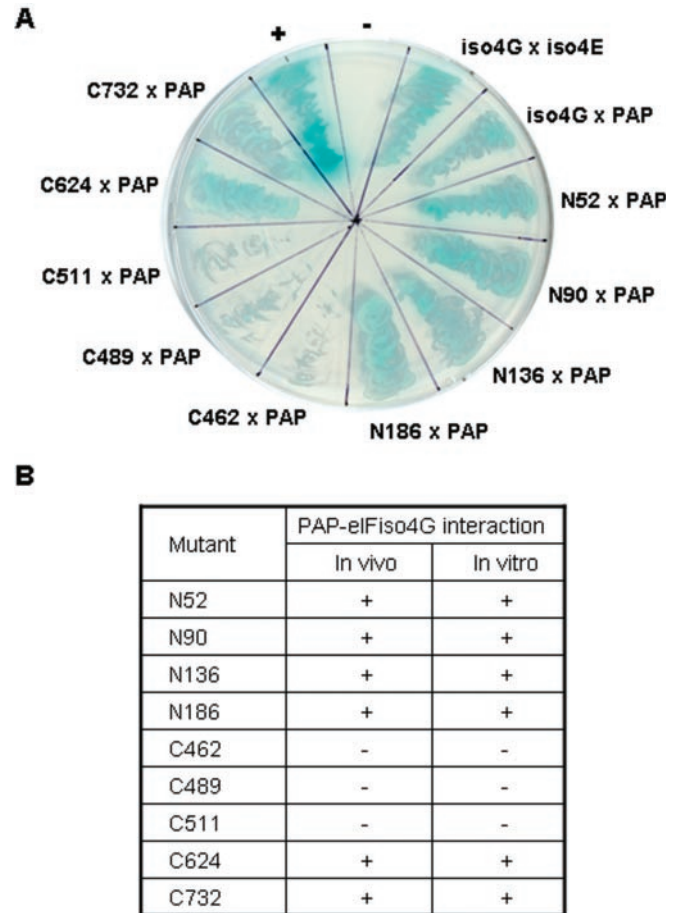
Figure 3. (**A**) Yeast two-hybrid interactions between PAPx, eIFiso4G and its truncation mutants. Activation domain plasmids encoding PAPx and binding domain plasmids encoding full-length eIFiso4G or its truncation mutants were transformed into the yeast strain AH109 and plated onto SD -Leu, -Trp, -His, -Ade with 2% dextrose and X-α-Gal. Representative colonies were re-plated onto the same medium to indicate relative growth and the presence of blue colour. Cells transformed with cDNAs of full-length eIFiso4G and eIFiso4E were plated as a positive control for interaction. In addition, cells transformed with either pTD1 and pVA3 or pTD1 and pLAM5′ were used as positive (+) and negative (−) controls for interaction, respectively. (**B**) Summary comparison of *in vivo* and *in vitro* binding data for PAP and eIFiso4G mutants. *In vivo* data were from results of panel A and *in vitro* data were from Figure 2.

Mutant	PAP-eIFiso4G interaction	
	In vivo	In vitro
N52	+	+
N90	+	+
N136	+	+
N186	+	+
C462	−	−
C489	−	−
C511	−	−
C624	+	+
C732	+	+

that PAP is responsible for 'bridging' the formation of the eIFiso4G-PAP-cap complex. For the eIFiso4G mutants, all except C462, C489 and C511 were able to form the trimeric complex (Figure 4B, lanes 1–9). These results are consistent with the PAP-binding properties defined for eIFiso4G *in vitro* and *in vivo* (Figures 2 and 3), respectively. Collectively, the results indicate that PAP can simultaneously bind to both eIFiso4G and m^7GTP to form a hetero-trimeric complex [Figure 4A (iii)].

To determine if a similar complex could form using a mixture of translation factors that would more closely mimic *in vivo* conditions, PAP was added to wheat germ lysate which was then incubated with m^7GTP-Sepharose beads. Following bead sedimentation, any bound proteins were eluted with 1 mM m^7GTP and analyzed by immunoblot for the presence of PAP or eIFiso4G. To exclude the possibility of cap-4E-4G complex formation, lysate depleted of eIF4E and

Figure 4. (**A**) Schematics of (i) PAP- m^{7}Gppp interaction shown previously; (ii) PAP-eIFiso4G interaction shown in this study and (iii) potential trimeric complex of eIFiso4G-PAP-m^{7}Gppp (investigated in **B**). (**B**) Complex formation of PAP and eIFiso4G or truncation mutants with m^{7}GTP-Sepharose. Equal molar concentrations of PAP and eIFiso4G or its truncation mutants (34 nmol) were incubated with m^{7}GTP-Sepharose. Bound proteins were eluted with 1 mM m^{7}GTP and separated by 12% SDS–PAGE. Eluted proteins were visualized by Coomassie blue staining. Controls for binding to beads were PAP alone and eIFiso4G alone. (**C**) Complex formation of PAP and eIFiso4G in wheat germ lysate. Wheat germ lysate depleted of eIF4E and eIFiso4E and replenished with eIFiso4G was incubated with m^{7}GTP-Sepharose, with or without the prior addition of PAP. Bound proteins were eluted with 1 mM m^{7}GTP and analyzed by immunoblot for the presence of PAP or eIFiso4G using a polyclonal antibody (1:5000) to each protein.

eIFiso4E was used for incubation. Immunoblot analysis of eIF4E and eIFiso4E depleted wheat germ lysate showed a corresponding depletion of eIFiso4G (data not shown). Therefore, recombinant eIFiso4G was added back to the lysate depleted of wheat eIFiso4G, at a concentration that mimicked endogenous levels (30). PAP addition to 4E-depleted/4G-replenished wheat germ lysate and subsequent incubation with m^{7}GTP-Sepharose indicated the presence of both PAP and eIFiso4G in the eluate (Figure 4C). Therefore, PAP bound to the cap structure and formed a complex between the cap and eIFiso4G in wheat germ lysate.

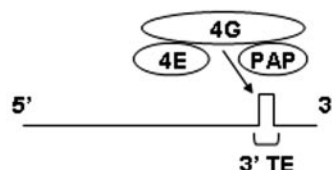### Effect of a lysate component on the depurination activity of PAP

We have shown previously that PAP is able to bind directly to m^{7}GTP [Figure 5A (i)]. In addition, PAP could also indirectly access capped mRNAs by binding to eIFiso4G. This
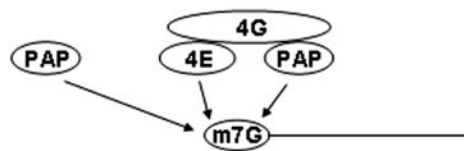

Figure 5. Models illustrating how PAP may access RNA substrates. (**A**) In the cap-dependent model, (i) PAP may access RNAs either through direct binding of PAP to the cap structure or (ii) via complex formation between eIF4G/eIFiso4G, eIF4E/eIFiso4E and cap. (**B**) In the cap-independent model, PAP may bind to eIF4G/eIFiso4G (a component of eIF4F/eIFiso4F) and gain access to uncapped, non-polyadenylated RNAs containing a 3′TE associated with eIF4F/eIFiso4F.

could occur by PAP binding to eIFiso4G that is associated with eIFiso4E, which is bound to the cap, i.e. a PAP-eIFiso4G-eIFiso4E-capped RNA complex [Figure 5A (ii)]. However, this would be difficult to demonstrate given the direct binding of PAP to m^{7}GTP. Alternatively, eIFiso4G-dependent PAP access to RNA could occur in a cap-independent manner. BYDV is a positive-sense RNA virus that does not have a cap or poly(A) tail. However, its efficient translation is stimulated by a 3′ translation enhancer RNA sequence (3′TE) contained within its 3′-UTR (31). Addition of excess enhancer sequence inhibited translation of the uncapped RNA in a wheat germ system, and inhibition was reversed by addition of elongation factor 4F, suggesting that the 3′TE mediates eIF4F-dependent translation (32). More recently, it has been reported that the 3′TE binds to eIF4F and eIFiso4F (33). We suggest that PAP is able to access uncapped RNAs by binding to eIF4F and eIFiso4F (Figure 5B) and that transcripts containing the 3′-UTR and 3′TE of BYDV would thus be depurinated upon binding of PAP. To test this concept in a translationally relevant context, which more closely mimics the cellular environment, PAP activity was assessed using a wheat germ lysate system. Following incubation of PAP in wheat germ lysate with uncapped transcripts containing a functional or non-functional 3′TE, total RNA was recovered and incubated with aniline as described previously (15). Figure 6 illustrates that incubation of transcripts containing the wild-type 3′TE with PAP in the presence of lysate, and subsequent treatment with aniline resulted in fragmentation of the uncapped RNA. This fragmentation was not observed with the same transcripts recovered from incubation lacking lysate, indicating that a lysate component, presumably eIF4F or eIFiso4F (33), enhanced depurination of the transcript by PAP. In contrast, incubation of PAP in lysate with an mRNA containing a defective 3′TE, which is unable to mediate
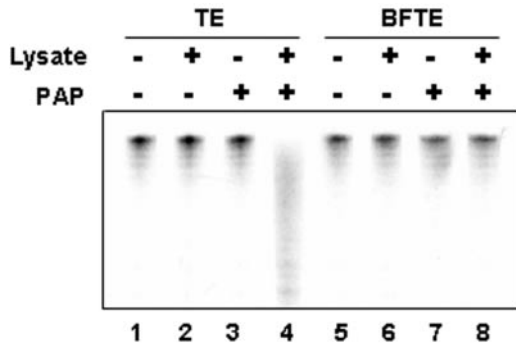
Figure 6. Effect of wheat germ lysate on the depurination activity of PAP. PAP (5 ng) was incubated with 3×10^5 c.p.m. of ^{32}P-labelled transcript containing either the wild-type (TE) or mutant (BFTE) 3'-UTR of BYDV. Transcripts were incubated in the presence or absence of wheat germ lysate and subsequently treated with aniline. As a negative control, transcripts were incubated without PAP. RNAs were separated on 7 M urea/4.5% acrylamide gel and visualized by autoradiography.

cap-independent translation, did not result in depurination (Figure 6, lanes 7, 8). These results are consistent with the concept that PAP gains access to the RNA by binding to eIF4G/eIFiso4G in the eIF4F/eIFiso4F complex associated with the 3'TE.

DISCUSSION

In this report we show that PAP binds directly to eIF4G and eIFiso4G. The yeast two-hybrid and far western analyses with truncation mutants of eIFiso4G indicate that a central region of the protein is required for binding to PAP. The binding site of PAP on eIFiso4G was partially defined in this work, showing the portion between amino acids 511 and 624 as critical for PAP binding activity. Comparison of the amino acid sequence alignment between eIFiso4G and eIF4G indicates that the region of eIFiso4G required for PAP binding activity is conserved in both proteins (2). PAP has been shown previously to bind m^{7}GTP and we demonstrate here that PAP is able to form a complex between the cap structure and eIFiso4-G in wheat germ lysate. In addition, we show that PAP is able to depurinate uncapped transcript in the presence of wheat germ lysate. The transcript contained the 3'TE sequence of the UTR of BYDV, which has been shown previously to be translated in an eIF4F dependent manner (32). Transcripts containing a mutant form of the translation enhancer sequence that are not affected by the presence of eIF4F were also not depurinated by PAP, supporting the hypothesis that PAP may access uncapped templates for depurination by binding to eIF4G or eIFiso4G.

Complex formation among PAP, eIFiso4G and the cap structure indicates that the binding sites for each do not overlap. Previous modeling studies showed that the active site of PAP can accommodate the binding of m^{7}GDP similar to the binding of an adenine (16). Therefore, it is unlikely that eIFiso4G binds the active site of PAP. Testing the ability of wild-type PAP to support translation in a lysate system depleted of eIFiso4E and eIF4E was not attempted because of the translation inhibition that would result from ribosome

depurination by PAP. Moreover, the non-depurinating active-site mutant PAPx (27) does not efficiently bind m^{7}GTP (unpublished data). However, PAPx was still able to bind eIFiso4G *in vivo*, further suggesting that the interaction of PAP with eIFiso4G is independent of its binding to cap.

Complex formation at the cap structure suggests that PAP may compete with eIF4E or eIFiso4E for binding to the cap. We have previously performed *in vitro* competition assays in which m^{7}GTP-Sepharose was incubated with equal or increasing molar concentrations of either PAP or eIFiso4E. Results of this experiment indicated that PAP does not compete with eIF4E or eIFiso4E for binding to cap analogue (unpublished data). Recently, a novel cap-binding protein bearing sequence homology to eIF4E and eIFiso4E was characterized from *Arabidopsis thaliana* (34). Similar to PAP, this protein has a higher affinity for m^{7}GTP than other wheat cap-binding proteins and also does not compete with eIF4E or eIFiso4E for cap-binding (16,34). The role of the novel cap-binding protein *in vivo* is not known, however, given that it does not support the translation of all mRNAs, this protein may sequester and prevent the translation of some messages. Even though PAP bears no sequence homology to eIF4E or eIFiso4-E, its binding to cap and eIF4G or eIFiso4G suggests that it may interfere with or alter the translation of particular messages.

Apart from enhancing the binding of eIF4E to m^{7}GTP, eIF4G has been shown to bind directly to capped and uncapped RNAs. For example, the central domain of eIF4G has been cross-linked to a structural element of the EMCV IRES upstream of the initiation codon and the presence of eIF4G stimulated the cross-linking of eIF4A to the IRES (35). Moreover, yeast eIF4G contains an RNA recognition motif characteristic of RNA-binding proteins (36) and eIF4F exhibits RNA-binding activity independent of sequence (37). Recently, a nucleic acid binding domain of wheat eIFiso4G has been characterized to span amino acids 170–201 and to bind both single and double-stranded sequences (38). eIF4G of wheat germ has been shown to enhance the IRES-mediated translation of naturally uncapped tobacco etch virus RNA (39), and the central domain of mammalian eIF4G can also support the translation of uncapped versions of normally capped mRNAs (40,41). Therefore, several reports illustrate how eIF4G or eIFiso4G is able to bind mRNAs and viral RNAs, and is principally involved in translation of these messages, even without the participation of the cap-binding protein eIF4E (42,43).

The mechanism by which PAP selects RNAs for depurination is not known. Figure 5 illustrates two models for PAP access to capped or uncapped cellular and viral messages. The first model shows a cap-dependent mechanism whereby PAP accesses RNAs by binding directly to the cap, independently of eIF4F/eIFiso4F [Figure 5A (i)]. We have shown previously that PAP binds the cap structure *in vitro* and specifically depurinates capped BMV RNA transcripts as opposed to uncapped BMV RNA templates (20). A single molecule of PAP would not likely bind simultaneously to cap and an adenine within the sequence of the RNA, given that both would bind to the active site of PAP, rather, PAP would bind to cap or the RNA. The binding of PAP to cap opens the possibility that PAP competes with eIFiso4E for binding to the 5' end of capped RNAs. Competition assays using increasing

concentrations of either PAP or eIFiso4E were unable to show increased binding of either protein to m^{7}GTP-Sepharose (unpublished results). However, complex formation seen in wheat germ lysate supports the simultaneous binding of PAP to cap and eIFiso4G. An alternative cap-dependent mechanism is illustrated in Figure 5A (ii), whereby PAP accesses RNAs by binding to eIF4F/eIFiso4F, which binds to cap. In this scenario, PAP may also bind to cap via eIF4G/eIFiso4G, independently of eIF4E/eIFiso4E. Primary dependence on the presence of a cap does not completely explain PAP substrate selection, given that PAP reduces the infectivity of potato virus Y (44), which is uncapped, and the accumulation of influenza virus and poliovirus, which are also uncapped (21,22). Figure 5B illustrates a cap-independent mechanism, whereby PAP may access uncapped templates via the association of the RNA with translation initiation factors such as eIF4F/eIFiso4F. For example, the 3′TE of BYDV has been shown to increase the level of translation in an eIF4F/eIFiso4F dependent manner (32). In this study, we have shown that PAP depurinates RNA containing the 3′TE in the presence of wheat germ lysate. However, no depurination was observed of RNA containing a functionally deficient 3′TE that does not associate with eIF4F/eIFiso4F. Given that the 3′TE has been reported to bind eIF4F and eIFiso4F (33), our results suggest that the presence of eIF4F/eIFiso4F may allow the depurination of some uncapped RNAs. Therefore, binding of PAP to eIF4G or eIFiso4G may provide PAP access to either capped or uncapped templates.

The binding of PAP to eIF4G or eIFiso4G would position PAP in close proximity to RNAs about to be translated. Many viral proteins sequester eIF4G or eIFiso4G during infection to ensure the preferential translation of viral messages as opposed to cellular mRNAs. For example, both rotavirus and influenza virus RNAs encode non-structural proteins that bind to their respective viral RNAs and also to the N-terminal region of eIF4G (45,46). Complex formation between non-structural proteins and eIF4G specifically favours viral RNA translation, either by preventing the binding of poly(A)-binding protein to eIF4G as with rotavirus, or by selectively recruiting eIF4G to the 5′ end of influenza virus RNA. Therefore, the ability of PAP to bind eIF4G or eIFiso4G suggests that during virus infection, PAP would have greater access to viral RNAs than cellular mRNAs. The preferential depurination of viral RNAs during infection is supported by previous results of mammalian cell cultures incubated with PAP, that cite a decline in virus titre without toxicity to the host cells (19,22). In this report, we show that PAP binds eIF4G and eIFiso4G, both *in vivo* and *in vitro*, and analyses using truncation mutants indicate that a central portion of the protein is required for this binding activity. Interaction of PAP with eIF4G or eIFiso4G would place PAP in close proximity to RNA substrates for depurination. Future studies will focus on determining the extent to which this novel mechanism for substrate selection contributes to the antiviral activity of PAP *in vivo*.

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