Efficient Cleavage of Ribosome-Associated Poly(A)-Binding Protein by Enterovirus 3C Protease

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Poliovirus (PV) causes a rapid and drastic inhibition of host cell cap-dependent protein synthesis during infection while preferentially allowing cap-independent translation of its own genomic RNA via an internal ribosome entry site element. Inhibition of cap-dependent translation is partly mediated by cleavage of an essential translation initiation factor, eIF4GI, during PV infection. In addition to cleavage of eIF4GI, cleavage of eIF4GII and poly(A)-binding protein (PABP) has been recently proposed to contribute to complete host translation shutoff; however, the relative importance of eIF4GII and PABP cleavage has not been determined.

At times when cap-dependent translation is first blocked during infection, only 25 to 35% of the total cellular PABP is cleaved; therefore, we hypothesized that the pool of PABP associated with polysomes may be preferentially targeted by viral proteases. We have investigated what cleavage products of PABP are produced in vivo and the substrate determinants for cleavage of PABP by 2A protease (2Apro) or 3C protease (3Cpro). Our results show that PABP in ribosome-enriched fractions is preferentially cleaved in vitro and in vivo compared to PABP in other fractions. Furthermore, we have identified four N-terminal PABP cleavage products produced during infection and have shown that viral 3C protease generates three of the four cleavage products. Also, 3Cpro is more efficient in cleaving PABP in ribosome-enriched fractions than 2Apro in vivo. In addition, binding of PABP to poly(A) RNA stimulates 3Cpro-mediated cleavage and inhibits 2Apro-mediated cleavage. These results suggest that 3Cpro plays a major role in processing PABP during virus infection and that the interaction of PABP with translation initiation factors, ribosomes, or poly(A) RNA may promote its cleavage by viral 2A and 3C proteases.

Enteroviruses are members of the family Picornaviridae and are etiologic agents responsible for many pathological syndromes. Enteroviruses cause drastic inhibition of host cell translation early in viral infection (22, 33, 56), an event referred to as host translation shutoff. Poliovirus (PV)-induced translation shutoff has been extensively studied, and it has been shown that during PV infection, cap-dependent host cell translation is inhibited. However, viral mRNA translation continues via a cap-independent mechanism facilitated by an internal ribosome entry site (IRES) element located in the viral mRNA. The mechanism of PV-induced shutoff of host cap-dependent translation was initially explained by the specific cleavage of an essential translation initiation factor, eIF4GI (formerly called p220), during PV infection in a reaction induced by viral 2A protease (2Apro) (23, 43, 49). eIF4GI is the largest component of the cap binding protein complex (called eIF4F) that also contains eIF4E (cap binding protein) and eIF4A (RNA helicase) (45, 51). eIF4GI is a multifunctional protein and is proposed to participate in translation initiation, mRNA deadenylation, inhibition of mRNA decapping, and mRNA maturation (4, 12, 16, 20, 27, 75).

Translation initiation is stimulated by the poly(A) tail-PABP complex through binding interactions between PABP and binding domains and thus blocking de novo binding of ribosomes to most cellular mRNAs (45, 51).

However, several lines of evidence suggest that eIF4GI cleavage is not sufficient to cause complete shutoff of host cell translation. First, there is a lag between eIF4GI cleavage and shutoff of host protein synthesis after PV infection (8, 23, 61). When eIF4GI cleavage first reaches completion, inhibition of host mRNA synthesis is only moderate. Moreover, in several studies using inhibitors of PV RNA replication, complete cleavage of eIF4GI occurred; however, host protein synthesis was inhibited only moderately (40 to 60%) (8, 10, 61). Furthermore, the expression of PV 2Apro in COS-1 cells resulted in substantial cleavage of eIF4GI, whereas protein synthesis was inhibited only threefold (19). Recently, cleavage of eIF4GII (30), which is a functional homolog of eIF4GI, and cleavage of poly(A)-binding protein (PABP) (38, 39) in virus-infected cells have been described. Virus-dependent cleavage of each factor was proposed to contribute to host translation shutoff phenotype; however, the relative importance of cleavage of either factor has not been determined.

PABP contains four highly conserved RNA recognition motifs (RRMs) in the N terminus and a less conserved proline-rich bridge connecting to a highly conserved carboxyl-terminal domain (CTD) (29, 31, 59, 64). PABP is now considered a multifunctional protein and is proposed to participate in translation initiation, mRNA deadenylation, inhibition of mRNA decapping, and mRNA maturation (4, 12, 16, 20, 27, 75).

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culture infective doses (TCID 50) was determined by incubation of serial dilutions. The supernatants were kept frozen at −70 °C for 24 h. Precipitates were sedimented by centrifugation at 2,000 g for 20 min; then pellets containing de novo initiation of new ribosomes and also by promoting reinitiation of terminating ribosomes on the same RNA (73). In higher eukaryotes, PABP also appears to indirectly stimulate translation initiation through its interaction with the translation factor PAIP-1 (18). PAIP-1 also interacts with eIF4A, and overexpression of PAIP-1 increases the rate of translation initiation (18).

PABP may play a key role in translation initiation by stimulating the utilization of the 60S ribosomal subunits to 48S preinitiation complexes at the last step of initiation. Experiments in yeast and in reticulocyte lysates support this hypothesis, and mutational analysis of S. cerevisiae PABP suggested that the C-terminal half of PABP may interact with 60S ribosomal proteins (57, 65, 66, 72).

Besides playing a direct role in translation initiation, PABP is a very important determinant in mRNA stability. PABP binds to the poly(A) tail of mRNA with high affinity primarily via its RRM 1 and RRM 2 domains (13, 44, 52, 59). Both human and yeast PABP bind eIF4G via the RRM 1 and 2 domains (13, 36, 40). Additionally, the PABP CTD has been suggested to mediate oligomerization of PABP on mRNA and contribute to the multiple, regularly spaced organization of the PABP on the poly(A) tail (44, 67). Recently, the importance of this domain in mRNA stability and translation initiation has been shown through an intriguing report demonstrating the interaction of the eukaryotic polypeptide chain-releasing factor (eRF3/GSPT) with the PABP CTD (35). Multimerization of PABP on the poly(A) tail was inhibited by its interaction with eRF3, leading to enhanced nuclease activity and poly(A) shortening. Thus, interaction of eRF3/GSPT with PABP may play important roles in the degradation of mRNA.

We have shown previously that the PABP CTD is targeted during PV infection by viral proteases 2A and 3C. Therefore, the cleavage of PABP and specific removal of the CTD may inhibit the reinitiation of translation on mRNA or 60S ribosomal subunit joining and may contribute to translational shut-off during PV infection. In this report we examine the cleavage of PABP by 2A and 3C proteases. We show that during infection, PABP is processed at four sites in the CTD, three of which are recognized by 3Cpro. Furthermore, we show that ribosome-associated PABP is preferentially cleaved by PV 3Cpro in vitro and in vivo.

**MATERIALS AND METHODS**

Cells and virus infection. HeLa S3 cells were grown in spinner culture in Joklik's minimal essential medium supplemented with 9% bovine calf serum−1% fetal calf serum and penicillin-streptomycin (Gibco). PV type 1 (Mahoney) was transfected in vitro and in vivo.

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Virus infections of HeLa cells were done at a multiplicity of infection (MOI) of 3 to 15 in serum-free medium. Serum was added to 7% final concentration at 30 min postinfection (pi), and the cells were harvested at various time points, pelleted, resuspended in 2 cell volumes of lysis buffer (10 mM KCl, 2.5 mM diithiothreitol, 1.2 mM magnesium acetate, 20 mM HEPES-KOH; pH 7.4), and then incubated on ice for 10 min. The cells were lysed by 25 strokes of a Dounce homogenizer, and the lysate was clarified by centrifugation at 10,000 × g for 10 min in total phosphate buffer (S0).

HeLa cell fractionation. For fractionation of the HeLa cells into further compartments, S0 lysate was centrifuged at 200,000 × g for 1 h with a 70 Ti rotor in a Beckman ultracentrifuge. The supernatant was retained as the non-ribosome-associated fraction (S200). The pellet was resuspended in 1 cell volume of lysis buffer containing 1 M KCl to dissociate ribosome-associated proteins and resedimented at 200,000 × g for 45 min. The supernatant was dialyzed and used as the crude translation initiation factor extract (RSW). The pellet was resuspended in lysis buffer and used as the ribosome-enriched fraction (ribosome). All fractions were used in assays as substrates for PABP.

Production of PABP and viral proteases. Radiolabeled PABP was synthesized by in vitro translation reactions in reticulocyte lysate (Promega) under standard translation conditions recommended by the manufacturer. PABP RNA was transcribed in vitro from PV 2A-PABP and purified prior to use as described previously (38). His-tagged PABP was generated from a pTrcHis vector (Invitrogen) by expression in E. coli. Growth of transformants was induced by induction of recombinant protein were carried out at 30°C. His-PABP was purified on Talon resin (Clontech) under denaturing conditions. CVB3 2Apro was subcloned from the vector pCVB3-20 (15), produced in Escherichia coli, and purified as described previously (38, 47). PV 3Cpro was expressed in E. coli and purified as described previously (38).

PV 3Cpro's affinity and specificity to ribosome-bound PABP. We have previously shown that the PABP CTD is targeted during PV infection by viral proteases 2A and 3C. Therefore, the cleavage of PABP and specific removal of the CTD may inhibit the reinitiation of translation on mRNA or 60S ribosomal subunit joining and may contribute to translational shut-off during PV infection. In this report we examine the cleavage of PABP by 2A and 3C proteases. We show that during infection, PABP is processed at four sites in the CTD, three of which are recognized by 3Cpro. Furthermore, we show that ribosome-associated PABP is preferentially cleaved by PV 3Cpro in vitro and in vivo.

**MATERIALS AND METHODS**

Cells and virus infection. HeLa S3 cells were grown in spinner culture in Joklik's minimal essential medium supplemented with 9% bovine calf serum−1% fetal calf serum and penicillin-streptomycin (Gibco). PV type 1 (Mahoney) was grown and purified as previously described (11). For preparation of virus stocks, coxackie B3 virus (CVB3) was grown in HeLa cells for 24 h; then virus was precipitated from cell supernatants by inclusion of 2.2% NaCl and 7% polyethylen glycol 6000, followed by stirring at 4°C for 24 h. Precipitates were sedimented by centrifugation at 2,000 g for 20 min; then pellets containing concentrated virus were resuspended in Dulbecco's minimal essential medium. Virus suspensions were kept frozen at −70°C. Virus titer (measured as 50% tissue culture infective doses [TCID50]) was determined by incubation of serial dilutions of virus with HeLa cells in 96-well plates and inclusion of liquid Dulbecco's minimal essential medium overlay supplemented with 2% serum. The number of
proteases, or the proteins were solubilized with SDS-PAGE sample buffer, separated by SDS-PAGE, and analyzed by autoradiography.

**Determination of PABP cleavage sites.** A glutathione S-transferase–PABP fusion protein was generated and purified as previously described (3) and cleaved with 3C protease (equimolar ratio of substrate to protease) for 16 h. The cleaved PABP fragments were electrophoresed in an SDS–12% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes. The Coomassie blue-stained protein bands were excised and subjected to microsequencing by the University of Oklahoma Health Sciences Center Molecular Biology Research Facility.

**RESULTS**

**Distribution of PABP in subcellular fractions.** Our previous PABP studies utilized a monoclonal anti-PABP antibody that did not recognize any cleavage products of PABP produced by viral protease. To be able to analyze PABP cleavage products, we developed a new polyclonal anti-PABP serum. Figure 1A shows that this antisem reacts with recombinant His-tagged PABP and also reacts with the 55-kDa N-terminal cleavage product produced by cleavage of His-PABP with 2Apro. This antisem also recognized native human PABP (see Fig. 3B, lane 1) in cytoplasmic extracts of HeLa cells; however, it was found to cross-react with some HeLa proteins, particularly a strongly cross-reactive moiety that migrates in gels near 36 kDa and other bands ranging in size from 40 to 65 kDa (see Fig. 3B).

In HeLa cells, PABP is a very stable and abundant cytoplasmic protein (29); however, very little information is available concerning its subcellular distribution within the cytoplasmic compartment. For this reason, we fractionated HeLa total cytoplasmic extract (S10) into S200 (non-ribosome-associated fraction), RSW (crude translation initiation factor extract), and ribosome (polyribosome-enriched) fractions. As a fractionation control, eIF4GI immunoblotting was performed, since eIF4GI fractionates in S200 and RSW fractions but not salt-washed ribosome fractions. Figure 1B shows the distribution of PABP and eIF4GI in each subcellular fraction. Interestingly, a large proportion (37%) of total cytoplasmic PABP was not associated with ribosomes or translation factors and remained in the S200 fraction. About 28% of PABP could be stripped from ribosomes by high-salt treatment and cofractionated with eIF4G and other translation initiation factors in the RSW fraction. The remaining 35% of total cellular PABP was tightly associated with ribosomes and was likely bound in poly(A)/mRNPs and polysomes. Thus, more than a third of total PABP was not associated with the translation apparatus or translation factors, and the overall distribution of PABP was different from that of eIF4GI. This experiment was repeated several times with similar results. Interestingly, almost half of the eIF4GI in HeLa cells is also non-ribosome associated. The presence of eIF4GI in nonribosomal compartments has been noted previously; however, we consistently see larger proportions of eIF4GI in S200 fractions than previously reported by others (24).

**Identification of PABP cleavage products produced in vivo.** Previously, we showed that PV 2Apro and CVB3 2Apro cleaved PABP at the junction between amino acids (aa) 486 and 487 (38). At least two additional cleavage products produced by PV 3Cpro were identified in our studies; however, the 3Cpro cleavage sites were not mapped. Thus, microsequencing of the small cleavage products (8 and 22 kDa) generated by in vitro cleavage of glutathione S-transferase–PABP was performed to identify two additional 3Cpro cleavage sites, termed 3C and 3Calt (Fig. 2). The predicted size of the N-terminal fragments produced by cleavage at these sites was consistent with the in vitro cleavage products observed previously (38) and in this study (see below). Cleavage of PABP at the 3C site (aa 537 and 538) is expected to generate a 61-kDa N-terminal fragment, and cleavage at the 3Calt site is expected to generate a 46-kDa N-terminal fragment. We did not directly sequence a third, minor 3Cpro site; however, based on migration in gels of an immunoreactive cleavage product (see Fig. 5) and a radiolabeled cleavage product (see Fig. 8) at 49 kDa, the third 3Cpro site likely occurs at the amino acid residues 434WTAQ/G437. This site contains a consensus 3Cpro sequence and would generate cleavage products consistent in molecular size to those observed. No apparent 3Cpro cleavage recognition site is located anywhere near the region in RRM 3 that would be appropriate for production of the same-size fragments. Fur-
ther, 3Cpro cleavage of His-PABP (N-terminal His tag) and subsequent immunoblot analysis with anti-His antibody detected N-terminal PABP cleavage fragments consistent with cleavage at all three of the sites listed above (data not shown). The 3Cpro site at aa 537 and 538 contains a Q/G scissile bond similar to those cleaved by 3C pro in the viral polyprotein. Surprisingly, the 3Calt cleavage occurs between glutamine (412) and threonine (413) residues. It is unusual to find threonine at the P1/H1 position, since most other 3Cpro sites identified thus far contain glycine at P1/H. P V3Cpro recognizes glutamine-glycine pairs in the viral polyprotein but does not cleave at all Q-G pairs available (17, 58). The presence of alanine or a hydrophobic residue in the P4 position is also favored by 3Cpro as a determinant for cleavage (7). This unusual 3Calt cleavage site does contain Ala at the P4 position.

Taken together, the data identify four viral protease cleavage sites on the C-terminal half of PABP; however, 2Apro and 3Cpro cleave PABP at distinct locations. Thus, every viral-protease-mediated cleavage event on PABP results in separation of the CTD or portions of the CTD from the RNA-binding domain. Cleavage of the RRM 2 domain that interacts with eIF4G1 has not been observed.

We previously reported that PABP was cleaved in vitro by both 2Apro and 3Cpro; however, use of a monoclonal antibody prevented detection of cleavage products in vivo (38). To determine if our new polyclonal antibody recognized PABP cleavage products produced by 2Apro or 3Cpro in HeLa extracts, we first performed in vitro cleavage reactions with each protease. Immunoblot analysis of HeLa S10 incubated with 2Apro detected intact PABP plus one major immunoreactive band. This band comigrated in SDS-PAGE gels with the 55-kDa cleavage product produced by incubation of 2Apro with radiolabeled PABP (Fig. 3) and was consistent with the previously characterized 2Apro large cleavage fragment (N-terminal domain) (38). In addition, immunoblot analysis detected two putative 3Cpro cleavage products that comigrated with the radiolabeled 3C and 3Calt cleavage products generated in vitro. Upon development of more sensitive chemiluminescent immunoblot methods, a third 3Cpro cleavage product was also detected that migrated slightly slower than the 3Calt cleavage product. This cleavage product, designated 3Calt (Fig. 3), was inconsistently detected after in vitro cleavage reactions and may be unstable or may be a minor cleavage product.

To determine which cleavage products were produced during PV infections, we used immunoblots to compare cleavage products produced in vivo with those generated in vitro by 2Apro and 3Cpro. Figure 3B shows that immunoreactive bands comigrating with 3C, 2A, and 3Calt cleavage products were detected in infected-cell lysate; however, the latter was very faint. Further analysis demonstrated that a significant amount of the 3Calt cleavage product was produced; however, it appeared to be unstable in vivo and often stained with less intensity than the 2Apro cleavage product, possibly due to loss of antibody epitopes retained in the larger 2Apro cleavage product (see below). In summary, three major N-terminal cleavage products of PABP were detected in PV-infected cells, two of which were produced by 3Cpro, not 2Apro. A fourth, minor cleavage product (3Calt') was also observed in many immunoblots.

Kerekatte et al. utilized a different polyclonal PABP antiserum to detect three potential cleavage PABP products in lysates from CVB3-infected cells, yet only a single 2Apro cleavage product was identified (39). To determine which cleavage products were produced in both PV and CVB3 infections, we performed infections in HeLa cells with both viruses and immunoblotted cell extracts by using our new polyclonal antiserum.
Figure 4 shows that CVB3 infection produced two cleavage products. The slower-migrating product comigrated exactly with the 2A<sup>pro</sup> cleavage product, as reported previously (39). The cleavage product that migrates faster in gels than the 2A<sup>pro</sup> cleavage product, which was previously unidentified, comigrated with the PV 3C<sub>alt</sub> cleavage product and is likely produced by CVB3 3C<sup>pro</sup>. The 3C cleavage product observed in PV infections has not yet been observed in immunoblots of whole cytoplasmic extract from CVB3-infected cells. Although high-sensitivity immunoblotting with CVB3-infected cell extracts has not yet been observed in immunoblots of whole cytoplasmic extract from CVB3-infected cells. Although high-sensitivity immunoblotting with CVB3-infected cell extracts has not yet been performed, this product may not be produced or may be produced in lesser amounts than in PV-infected cells. The lack of 3C cleavage product may be partly compensated by an increased production of 3C<sub>alt</sub> cleavage product in CVB3 infection (Fig. 4, compare lanes 5 and 10).

Evidence from immunoblots and immunoprecipitation of truncated PABP polypeptides suggests that the major epitopes recognized by this polyclonal antiserum are located in the C-terminal half of PABP (data not shown). Thus, the presence of weaker 3C<sub>alt</sub> bands than 2A<sup>pro</sup> bands does not indicate that the 3C<sub>alt</sub> product (which is lacking most of the CTD) is present at lower levels than the 2A cleavage product. In this experiment, PABP cleavage occurred slightly earlier in CVB3 than PV infection. However, CVB3 and PV titers were not obtained by the same methods, and rigorous comparative infections using carefully matched MOIs have not been performed. In summary, these data suggest that PABP is processed by 2A<sup>pro</sup> and 3C<sup>pro</sup> in both PV and CVB3 infections.

Ribosome-associated PABP is preferentially cleaved by viral proteases. Previous reports showed that in PV- or CVB3-infected cells only 25 to 35% of cytoplasmic PABP is cleaved at times when host cell translation shutoff is complete (38, 39). Therefore, we hypothesized that the pool of PABP associated with polysomes may be preferentially targeted for cleavage by PV proteases. To investigate this, in vitro cleavage reactions were performed with equal cell equivalents of HeLa subcellular fractions as PABP substrates, each supplemented with equal concentrations of PV 3C<sup>pro</sup> or CVB3 2A<sup>pro</sup>. Figure 5 shows immunoblot analysis of typical in vitro cleavage reactions with each fraction using polyclonal PABP antibody. Control lanes for each fraction reveal a strong PABP band migrating at 75 kDa and one or two background bands representing serum cross-reactivity with other HeLa proteins (lanes 1, 4, 7, and 10). Some of the cross-reacting proteins appear to be concentrated in RSW and ribosome-enriched fractions. When 2A<sup>pro</sup> was incubated with S10 total cytoplasmic extract, only partial cleavage of PABP occurred, the 55-kDa 2A cleavage product appeared, and a faint new 2A<sub>alt</sub> cleavage product that migrated faster than 55 kDa was detected (Fig. 5). When 3C<sup>pro</sup>
was incubated with S10, PABP was partly cleaved and 61-kDa 3C and 46-kDa 3Calt cleavage products became apparent. A new faint cleavage product seen in some experiments migrating at 49 kDa was also detected (3Calt').

Interestingly, PABP in S200 fractions was resistant to cleavage in vitro since neither 2Apro or 3Cpro were able to cleave this form of PABP or produce any detectable cleavage fragments (Fig. 5, lanes 5 and 6). In contrast, PABP in both RSW and ribosome-enriched fractions could be efficiently cleaved by 2Apro and 3Cpro. 2Apro cleavage did not result in accumulation of the 61-kDa 3C cleavage product may be masked in this fraction, the 3Calt fragment was detected; however, the presence of this secondary cleavage product accumulates only in infections carried out at higher multiplicities (38, 39) (compare Fig. 6 and 4). In addition, in enhanced-sensitivity chemiluminescence immunoblots, an additional immunoreactive band (M_r ~ 25,000) which we called CPc was apparent. The apparent molecular mass of this putative cleavage product matched the expected molecular mass of the C-terminal cleavage product released by cleavage at the 3Calt site. Potential transfer of dominant PABP antibody epitopes to this putative C-terminal cleavage product is consistent with sporadic and weak immunostaining of the N-terminal 3Calt fragment that we have observed. In higher-multiplicity infections, which sustained more robust PABP cleavage, CPc was sometimes not observed, and this band was not observed in in vitro cleavage reactions. This lack of detection of CPc could be due to further cleavage of CPc at the 2Apro or 3C sites it contains, producing smaller nonimmunoreactive fragments.

When the extent of PABP cleavage was examined in the subcellular compartments after 6 h of infection, total cytoplasmic PABP (S10 fraction) was only modestly reduced (22%). However, the PABP in the ribosome-enriched fraction was extensively cleaved (76%) and the PABP in RSW fraction was degraded substantially (34%). In contrast, PABP in the S200 fraction was not significantly degraded in vivo. Therefore, the preferential targeting of ribosome-associated PABP by PV proteases was observed in vivo as well as in vitro.

Examination of the types of cleavage fragments produced in various compartments in vivo revealed the 3C cleavage product was observed in total lysate but not in the ribosome-enriched fraction, similar to in vitro results (Fig. 5). Here it was observed in the RSW fraction. The 2A cleavage product and the 3Calt cleavage product were produced in both RSW and ribosome-enriched fractions; however, the 3Calt' cleavage product appeared in only the RSW fraction. The putative 25-kDa CPc was observed in both ribosome-enriched and RSW fractions. The increased staining intensity of the CPc and lack of 3C cleavage product in the ribosome-enriched compartment suggest that most processing of polysome-associated PABP oc-
curred at the 3Calt site. Collectively, the data show that PV proteases target the ribosome-associated PABP pool most aggressively. In addition, four types of PABP N-terminal cleavage products are produced, and these data suggest that 3Cpro plays a much larger role in the total PABP processing in vivo than previously recognized. It is likely that three of these four cleavage products were also previously detected in CVB3-infected cells by using a different peptide-derived anti-PABP serum. In that case, the authors identified only the 55-kDa 2Apro cleavage product and not the 46-kDa (3Calt) product or the weak 49-kDa (3Calt/H11032) product that appeared in their immunoblots. An 61-kDa 3C cleavage product was not visible with this anti-serum (39).

We performed additional infections to determine if any correlation exists between cleavage of ribosome-associated PABP and host translation shutoff. In these experiments, nearly all cleavage of eIF4GI occurred between 2 and 3 h p.i. At the 3-h time point, only 7.7% of eIF4GI was still intact, yet translation rates were reduced only approximately 50%. This unlinkage between eIF4GI cleavage and translation shutoff has been observed before in infections containing guanidine-HCl or performed at low temperature (8, 10, 61). At 3 h p.i., very little cleaved PABP was detected, clearly showing that eIF4GI cleavage precedes significant PABP cleavage. By 4.5 h p.i., host translation shutoff was complete and over 50% of ribosome-associated PABP was cleaved. This correlates the completion

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**FIG. 6.** Ribosome-associated PABP is preferentially cleaved during PV infection. (A) Enhanced cleavage of ribosome-associated PABP. HeLa S3 cells were mock infected or infected with PV (MOI = 3) and harvested at the indicated time points. Harvested HeLa cells were fractionated into S200, RSW, and ribosome-enriched fractions. Aliquots of these fractions were subjected to SDS-PAGE and immunoblotted with anti-PABP serum. The percent reduction of intact PABP (percent cleavage) relative to controls is indicated below the panels. Numbers on the left show the migration of molecular weight markers (in thousands). (B) Correlation of cleavage of ribosome-associated PABP with translation shutoff. Additional HeLa infections were performed, cells were pulse-labeled with [35S]methionine, and cell lysates were analyzed by SDS-PAGE with autoradiography or immunoblotting. Host translation levels were quantitated from three sections of autoradiographs not containing viral proteins. PABP- and eIF4GI-specific immunoblotting was also performed, blots were scanned, and data were quantitated. Levels of intact PABP in mock-and PV-infected cells and levels of 2A cleavage product (CP) and C-terminal cleavage product (CPc) released by 3Cpro cleavage were determined. Levels of intact eIF4GI in cell lysates were determined by immunoblot analysis to be 100, 7.7, and 0% of mock control at 2, 3, and 4.5 h p.i., respectively. Densitometry of scanned data was performed with NIH Image 1.62. Three separate experiments are represented.
(or second stage) of translation shutoff with significant cleavage of ribosome-associated PABP. Examination of the appearance of PABP cleavage products in the ribosome pools demonstrated that 2A cleavage product was first detectable at 3 h p.i. yet did not accumulate further during infection. The C-terminal cleavage product released by 3Cpro was not usually detectable at 3 h p.i., however, it rapidly accumulated to high levels by 4.5 h p.i. This suggests that the kinetics of cleavage of ribosome-associated PABP by 2Apro and 3Cpro during the course of infection may be different.

Ribosome-associated PABP is cleaved more efficiently by 3Cpro than 2Apro. It is unclear why two viral proteases cleave PABP, but their different cleavage specificities may target different conformations or functions of PABP which exist in cells. Thus, we were interested in whether these two proteases have different cleavage efficiencies when processing ribosome-associated PABP. Figure 7 shows an immunoblot of ribosome-associated PABP incubated with equivalent, increasing concentrations of 2A and 3C proteases. The cleavage products are labeled according to their molecular masses. The data show that at equivalent concentrations (e.g., 0.1 μg/ml) (Fig. 7, lanes 2 and 5), 3Cpro cleaves the ribosome-associated PABP more efficiently than 2Apro. As seen previously, in vitro cleavage of ribosome-associated PABP by 3Cpro generates only the 46-kDa alternative cleavage product, not the 61-kDa cleavage product. The 25-kDa CPc was not observed, again possibly because of further processing or degradation of this fragment into smaller nonimmunoreactive cleavage products in vitro.

Since the ribosome fraction contains polysomes, we wanted to determine if either protease specifically targeted poly(A)-bound PABP. PABP binds poly(A) RNA in an oligomerized and repeating configuration via interactions that involve the CTD (44, 67). Since the cleavage sites we have mapped are all located in different regions of the PABP CTD, we hypothesized that 2A and 3C proteases would display differential cleavage activity on PABP-containing poly(A) RNAs. Therefore, we bound radiolabeled PABP to poly(A) agarose and compared the cleavage of bound PABP and unbound PABP by 2A and 3C proteases. Figure 8A shows that only approximately one-third of labeled PABP bound to the poly(A) agarose, even though RNA binding sites were calculated to be in excess of PABP molecules in the lysate. To exclude the possibility that poly(A) agarose was saturated with unlabeled reticulocyte PABP, the unbound fraction was incubated with an excess of fresh poly(A) agarose. Again, only about a third of the PABP bound to the beads. This binding step was repeated a third time with similar results. Thus, an apparent equilibrium ratio between bound and unbound PABP of roughly 1 to 2 was achieved in every step. The reason for this resistance to binding is unknown, but it may be due to conformational differences or phosphorylation status of the radiolabeled PABP. The failure of all PABP to bind poly(A) RNA was not due to free poly(A) RNA in the lysate, since the extracts were nuclease treated prior to translation and the input PABP RNA did not contain a poly(A) tail. These results indicate that unbound and bound PABP fractions used in the subsequent experiments (Fig. 8B and C) were enriched only for the desired pool of PABP.

When poly(A)-bound PABP and unbound PABP were compared as substrates for viral proteases, 3Cpro was again more active in cleaving PABP than 2Apro (Fig. 8B and C). Surprisingly, 2Apro showed very poor cleavage of poly(A)-bound PABP and more efficient cleavage of unbound PABP. In contrast, 3Cpro displayed the highest cleavage efficiency when PABP was associated with RNA. 3Cpro also exhibited more cleavage activity on either form of PABP (Fig. 8C). Both 3C and 3Cal cleavage sites were used; however, PABP cleavage at the 3Cal site was more predominant. Binding of PABP to RNA may also influence which cleavage site is utilized by 3Cpro, since binding stimulated cleavage at the 3Cal and 3Calt sites (Fig. 8B, lanes 5 and 6). When equal concentrations of both proteases were coincubated with unbound PABP, 2Apro cleavage predominated over that by 3Cpro (Fig. 8B, lane 8). Conversely, when poly(A)-bound PABP was incubated with both proteases, 3Cpro cleavage products were more predominant. Together, these results suggest that binding of PABP to poly(A) RNA may cause a conformational change that makes PABP more susceptible to cleavage by 3Cpro than 2Apro. Further, these results show that 3Cpro is more effective in cleaving poly(A)-bound PABP, which is concentrated in the ribosome fraction.

**DISCUSSION**

This report describes the identification of three cleavage sites in PABP that are recognized by 3C protease and provides evidence that PABP is cleaved at four sites in PV-infected cells, including the previously characterized 2Apro cleavage site (38). Surprisingly, we have found that 3Cpro plays a dominant role over 2Apro in the processing of native HeLa PABP, particularly ribosome-associated PABP. The importance of 3Cpro-mediated cleavage was initially underestimated in our own work, since most initial experiments used soluble recombinant His-PABP, which is more susceptible to 2Apro cleavage, as a substrate. Further, since early reports demonstrated that 3Cpro did not cleave eIF-4GI (48) whereas 2Apro did (43, 49), little attention has been focused on potential roles of 3Cpro in the host translation shutoff mechanism. The new data presented here suggest that 3Cpro may contribute to the execution of
translation shutoff in PV infection via targeted inactivation of ribosome-associated PABP. Investigators who have expressed 2Apro in cells have reported extensive inhibition of cellular translation (5, 19, 69), concluding that expression of 2Apro alone was sufficient for the translation shutoff phenotype.

However, translation inhibition resulting from overexpression of 2Apro (which also will cleave PABP) does not disprove a significant role for 3Cpro in translation regulation during natural virus infections in which both proteases are expressed. 3C protease exists in infected cells in two forms, the precursor 3CD and the fully processed 3Cpro. Both forms of the enzyme are active proteases with largely overlapping, but not identical, substrate preferences in cleavage of the viral polyprotein (76). Furthermore, 3CD is more abundant than 3Cpro in virus-infected cells. Thus, it is important to recognize that 3CD may be a physiologically significant form of 3Cpro-protease that cleaves PABP in vivo. Experiments are under way to determine if significant differences exist between 3C and 3CD in PABP cleavage site specificity.

At the time when host translation is initially shut off during high-MOI infections, only a portion of HeLa cell PABP (approximately 25%) is cleaved (38, 39). This leads to questions concerning the functional significance of PABP cleavage in the mechanism of host translation shutoff. We demonstrate here that a significant proportion of total cytoplasmic PABP (37%) is not associated with the translation apparatus, and interestingly, this fraction was not cleaved by viral proteases. Conversely, the viral proteases specifically targeted and cleaved ribosome-associated PABP with the highest efficiency. PABP in RSW fractions was cleaved at different sites and with different efficiencies than PABP in ribosome fractions. Further, binding of PABP to poly(A) RNA enhanced cleavage by 3Cpro but inhibited cleavage by 2Apro. Proteases are excellent sensors of changes in protein conformation; thus, these data suggest that PABP exists in multiple conformations contingent upon its subcellular localization. Such conformational alterations are likely caused by PABP interacting with different protein or RNA binding partners in different types of holoenzyme and mRNP complexes. In some complexes, direct steric hindrance from protein binding partners may also mask protease cleavage sites. The data provided here suggest that enterovirus proteases have evolved to efficiently cleave two forms of PABP: 2Apro targets non-RNA-bound PABP (possibly associated with other initiation factors), whereas 3Cpro targets PABP bound to mRNA. The fact that both proteases encoded in the small enterovirus genome evolved to target PABP is striking and lends support to the functional importance of PABP cleavage in the viral replication cycle. It is also important to consider that the loss of intact PABP may not be as important as creation of PABP CTD cleavage fragments, which may function as dominant-negative regulators of translation.

Cleavage of eIF4GI is required but not sufficient for host translation shutoff. Virus infections supplemented with guanidine or other drugs that block viral RNA replication result in complete cleavage of eIF4GI, yet cellular translation is abated only 50% (8, 10, 61). This led to the suggestion that an addi-
tional event, missing or blocked in guanidine-supplemented infections, was required for complete translation shutoff. Two nonexclusive cleavage events have been shown to be deficient in guanidine-supplemented infections which are excellent candidates for the "missing required event" in the shutoff mechanism; (i) cleavage of eIF4GII (the functional homolog of eIF4GI) and (ii) cleavage of PABP (30, 38). Cleavage of eIF4GII correlates with translation shutoff in experimental systems. However, examination of unique eIF4GII and eIF4GII caspase-3 cleavage products (53, 54) derived from in vitro cleavage of purified eIF4F suggests that only a small portion of total eIF4G in eIF4F complexes is made up of eIF4GII (10 to 15%, the bulk being eIF4GI) (data not shown). Thus, the low level of eIF4GII in cells may be insufficient to catalyze continued translation at 50% of normal rates after complete eIF4GI cleavage has occurred. Conversely, the limited 25% cleavage of total PABP initially reported (38) masked the more efficient cleavage of ribosome-associated PABP that occurs during infection (this study). In addition, we hypothesize below that targeted cleavage of only the 3'-terminal PABP oligomerized on mRNA is sufficient to have a major impact on translation.

Finally, we have evidence that addition of 3Cpro alone to HeLa translation lysates causes a significant inhibition of translation (unpublished data). Thus, limited cleavage of PABP, properly targeted, may be quite significant, but not enough is known about PABP functions to firmly define the relative role of PABP cleavage in the mechanism of host translation shutoff.

It is important to note that PABP cleavage at all four cleavage sites results in removal of all or part of the PABP CTD. Why would removal of the PABP CTD be so important to enteroviruses? A large segment of the CTD is proline rich, suggesting a flexible or extended conformation. The extreme C-terminal 74 aa fold into a globular domain with five a-helices which form a binding cleft that binds PAIP-1, PAIP-2 (which function in 5'-3' interactions), and eRF3 (which functions in ribosome termination) (41, 42). The larger CTD may be involved in interactions with other several proteins and these interactions may be disrupted by cleavage during virus infection. The larger CTD has also been shown to interact with PABP (oligomerization) and the 60S ribosomal subunit (ribosome joining at initiation or reinitiation) (2, 32, 35, 44, 57, 67). These multiple interactions suggest that the PABP CTD may play important and varied roles in translation initiation and termination, including attraction and binding of 60S ribosomal subunits during de novo initiation. Interactions between PABP and 60S ribosomal subunits may be even more important during ribosome reinitiation, which may constitute half of all translation in vivo (1). Such ribosome reinitiation may also be partly mediated via 5'-3' interactions between PABP and eIF4G or PAIP-1. The existence of multiple binding partners for the PABP CTD also underscores the possibility that cleaved or released CTD may function as a dominant negative translation inhibitor.

The exact structure or configuration of PABP as it is bound on poly(A) RNA is not known; however, the footprint of PABP occupies about 25 nucleotides, and multiple PABP molecules are oligomerized on poly(A) tails longer than 50 nucleotides [up to eight or nine on poly(A) RNA of 200 nt] (68). In such a configuration, the PABP molecule on each end of the oligomer would exist in unique conformations (not having a PABP binding partner on one side), whereas the PABP molecules in the center would be similar to each other in confor-

FIG. 9. Schematic of proposed configuration of free and poly(A)-bound PABP and preferred substrates for viral proteases. PABP is depicted with four globular RRM domains and an extended flexible C-terminal tail containing a highly conserved globular domain at its tip (C terminus). RRM 2, which binds eIF4G, is hatched. Directional arrangement of poly(A) RNA-bound PABP was determined by X-ray structure (21). The CTD is shown interacting with neighboring PABP, although the actual site of binding interaction is not known. Arrows indicate enterovirus protease sites mapped on PABP, and arrow thickness shows relative cleavage efficiency. Binding of the CTD of PABP to adjacent PABP may sterically block binding of eIF4GII to all but the 3'-terminal PABP moiety. The CTD of 3'-terminal PABP is in a unique configuration and may be free to interact with the 60S ribosome (binding site hatched), eIF4B, PAIP-1, PAIP-2, and eRF3 (14, 42).
mation (Fig. 9). It is likely that the 3′-terminal PABP moiety has an unpaired CTD (not bound to a neighboring PABP moiety) that would be uniquely free to interact with eRF3, PAIP-1, eIF4B, or 60S ribosomal subunits. In this configuration, cleavage of only the 3′-terminal PABP CTD would disrupt the ability of mRNA to interact with ribosomal subunits. Thus, significant disruption of translation may result from targeted cleavage of only a small subset of total cytoplasmic PABP. Further, since the terminal PABP must exist in a unique conformation not shared with adjacent PABP moieties and 3C protease is affected by the conformational state of PABP, it is possible that the 3′-terminal PABP moiety is preferentially targeted for cleavage. A series of experiments is under way to specifically test this hypothesis.

The recently reported interaction of the PABP CTD with eRF3 is particularly intriguing (35). eRF3 is a release factor that binds to eRF1 complexed with ribosomes on termination codons and facilitates release of 60S ribosomes from mRNA. Interaction of PABP with eRF3 was associated with 3′-directed poly(A) tail shortening, suggesting that the 3′-terminal PABP moiety was dissociated from the poly(A) tail by interacting with eRF3. This provides evidence that the 3′-terminal PABP moiety, not the others, has an unoligomerized CTD (Fig. 9). This unencumbered 3′ CTD would be free to interact with the binding site on 60S ribosomal subunits (63). Thus, after cleavage of this specific 3′-terminal CTD by 3C\textsuperscript{pro} or 2A\textsuperscript{pro}, reinitiation of translation could be inhibited. Cleavage of the CTD or its truncation has been reported to lessen the affinity of the RRM domains with RNA; however, this does not result in immediate release of bound PABP, since only RRMs 1 and 2 are sufficient for RNA binding (21, 44). Our data indicate that the 3C\textsuperscript{alt} N-terminal cleavage product can still efficiently bind to poly(A) agarose (data not shown).

Why would 2A\textsuperscript{pro} also target PABP? Recently, PABP has been shown to increase the efficiency of translation even though it is not bound to poly(A) mRNA (63). Further, PABP has been shown to dissociate from mRNA during the down-regulation of translation associated with cell differentiation (34). Thus, PABP has multiple functions in translation initiation and may cycle on and off mRNA molecules. In the present study we show that 2A\textsuperscript{pro}, rather than 3C\textsuperscript{pro}, preferentially cleaves unbound PABP. Thus, PV and CVB3 may use two viral proteases with different substrate specificities to cleave two unique pools of PABP that function in translation.

What could be the effect of PABP cleavage on viral mRNA translation? We predict that PABP cleavage would down-regulate viral translation and cellular translation similarly. Viral mRNA does not contain a cap structure but does have a poly(A) tail. Since both intact and 2A\textsuperscript{pro}–truncated eIF4G can directly bind the viral IRES, viral RNA can experience 5′–3′ interactions via the eIF4G-eIF4A-PAIP-PABP axis. This has been inferred from the demonstration that IRES-poly(A) interactions synergistically enhance translation of poliovirus mRNA (6). Furthermore, PABP enhances cap-independent translation (60, 62). Thus, like cellular mRNAs, viral mRNA is likely circularized via 5′–3′ interactions. Therefore, ribosome reinitiation on viral mRNA would also require the same interaction between a 3′ CTD and the 60S subunit that we propose is disrupted by protease cleavage. Before RNA replication can ensue in virus-infected cells, translation on the viral template must be blocked to allow 3D RNA polymerase to transverse the same template in the opposite direction from transiting ribosomes. Recently, it has been shown that the PV polymerase precursor 3CD mediates the switch from translation to RNA replication through binding to the cloverleaf structure at the 5′end of the viral RNA (25). This binding interaction stimulates the binding of polycytidine binding protein 2 (PCBP2) to the same cloverleaf structure and disrupts IRES-mediated translation initiation. This effect would be predicted to disrupt de novo translation initiation on viral mRNA; however, it may not disrupt ribosome reinitiation on viral poly-somes. We hypothesize that viral mRNA is maintained in a closed-loop configuration; thus, 3CD binding to the cloverleaf also positions this protease close to PABP on the poly(A) tail. Therefore, 3CD binding to the cloverleaf may stimulate cleavage of the 3′ PABP CTD on viral mRNA and disrupt ribosome reinitiation. This would promote ribosome runoff and convert the viral template to a replication-competent state. Experiments are under way to test this hypothesis. It is important to note that translation inhibition would be strictly regulated by the supply and concentration of 3CD, which must be produced first by translation (25, 26). As viral RNA replication begins and produces more viral mRNA, the levels of 3CD would become insufficient to inhibit new viral translation on these templates until more 3CD is produced via new translation reactions. Further, poly(A) tails on nascent plus-strand RNA would bind PABP from some source within the cell, possibly from the large pool of uncleaved PABP in S200 fractions (Fig. 6). This intact PABP could bind nascent RNA and facilitate new rounds of translation until more 3CD or 2A\textsuperscript{pro} was produced, which would cleave the additional PABP. Thus, a balance between translation and RNA replication could be maintained during the viral growth cycle.

In conclusion, we have shown that ribosome-associated PABP is preferentially cleaved during PV infection, generating four cleavage products. In addition, 3C\textsuperscript{pro} is the dominant protease in processing of ribosome-associated PABP in vivo and in vitro. Further investigation of differential cleavage of PABP pools by 2A and 3C viral proteases may provide important information about the emerging multiple functions of PABP in translation and potential roles of these cleavage events in translation control during infection.

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