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Cleavage of Poly(A)-Binding Protein by Poliovirus 3C Protease Inhibits Host Cell Translation: a Novel Mechanism for Host Translation Shutoff

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Cleavage of eukaryotic translation initiation factor 4GI (eIF4GI) by viral 2A protease (2Apro) has been proposed to cause severe translation inhibition in poliovirus-infected cells. However, infections containing 1 mM guanidine–HCl result in eIF4GI cleavage but only partial translation shutoff, indicating eIF4GI cleavage is insufficient for drastic translation inhibition. Viral 3C protease (3Cpro) cleaves poly(A)-binding protein (PABP) and removes the C-terminal domain (CTD) that interacts with several translation factors. In HeLa cell translation extracts that exhibit cap-poly(A) synergy, partial cleavage of PABP by 3Cpro inhibited translation of endogenous mRNAs and reporter RNA as effectively as complete cleavage of eIF4GI and eIF4GII by 2Apro. 3Cpro-mediated translation inhibition was poly(A) dependent, and addition of PABP to extracts restored translation. Expression of 3Cpro in HeLa cells resulted in partial PABP cleavage and similar inhibition of translation. PABP cleavage did not affect eIF4GI-PABP interactions, and the results of kinetics experiments suggest that 3Cpro might inhibit late steps in translation or ribosome recycling. The data illustrate the importance of the CTD of PABP in poly(A)-dependent translation in mammalian cells. We propose that enteroviruses use a dual strategy for host translation shutoff, requiring cleavage of PABP by 3Cpro and of eIF4G by 2Apro.

Infection of HeLa cells by human enteroviruses (poliovirus and coxsackievirus) or rhinovirus results in a nearly complete inhibition of cellular translation (26, 33). This inhibition was originally thought to result from cleavage of translation initiation factor 4GI (eIF4GI) by viral 2A protease (2Apro) and cellular proteases activated during infection (8, 22, 48). eIF4GI functions as a scaffolding protein by simultaneously binding eIF4E (cap binding protein), eIF4A (RNA helicase), and eIF3 (a factor tightly bound to 40S ribosomal subunit) (14). This complex recruits 40S ribosomal subunits to the cap group on the 5′ end of mRNA. Thus, cleavage of eIF4GI by 2Apro served as an attractive explanation for translation shutoff since its cleavage separated the eIF4G domains that bound to the mRNA cap (N-terminal domain) and the ribosome (C-terminal domain [CTD]) (25). However, cleavage of eIF4GI is only partially responsible for the translation shutoff during virus infections since infections modified with inhibitors of viral RNA replication (e.g., the use of 1 mM guanidine–HCl) resulted in only a 50% decline in translation despite complete eIF4GI cleavage (4, 34). Thus, additional events are required for complete host cell translation shutoff during infection. Two likely events are the cleavage of eIF4GII (a functional homologue of eIF4GI) and the cleavage of poly(A)-binding proteins (PABP), since the cleavage of each is blocked when viral RNA replication is inhibited (13, 18).

PABP and the poly(A) tail of eukaryotic mRNAs play an important role in stimulating translation initiation (17, 39). PABP is comprised of two functional domains, an N-terminal domain with four RNA recognition motifs (RRM) and a CTD (11, 31). eIF4GI simultaneously binds the N-terminal domain of PABP (RRM2) and eIF4E in a way that facilitates the circularization of mRNA (16, 43, 47). The eIF4E/eIF4G/PABP complex has been demonstrated to stimulate translation synergistically in yeast, plant, and mammalian systems (17, 39). The mechanism of this stimulation is unclear, but PABP binding was proposed to induce cooperative conformational changes in eIF4E and eIF4G that enhance the stability of initiation complexes on capped mRNAs (45).

The PABP CTD consists of a proline-rich region linked to a C-terminal globular domain containing a cleft that binds several translation factors (21). These include translation initiation factor eIF4B (cofactor of RNA helicase eIF4A) (5), PABP-interacting proteins Paip-1 (6) and Paip-2 (20), and eukaryotic release factor 3 (eRF3) (15). The PABP CTD also binds PABP to facilitate poly(A)-dependent oligomerization on poly(A) tails (23). Recently, a CTD point mutation that abolished binding to eRF3 and inhibited cap-poly(A)-dependent translation was reported (15, 44). Genetic studies suggested that the CTD of *Saccharomyces cerevisiae* PABP interacts with a 60S ribosomal protein (38) and that rabbit reticulocyte lysate (RRL) PABP could stimulate translation due to an enhancement of 60S and 40S ribosomal subunit joining (30). Thus, the CTD may function in ribosome assembly or recycling (44, 46).

Since PABP manifests multiple roles in translation, it is not surprising that viruses target PABP in an effort to manipulate cellular translation. Rotavirus mRNA transcripts are capped and nonpolyadenylated; however, they can compete with capped and polyadenylated cellular mRNAs for ribosomes.

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Rotavirus nonstructural protein 3 (NSP3) inhibits interactions between eIF4G and PABP, thus blocking circularization of cellular mRNAs and reducing translation efficiency (35). Since NSP3 binds the 3′ ends of rotavirus mRNA and eIF4G simultaneously, viral mRNA can still circularize to translate efficiently (35).

Joachims et al. and Kuyumcu-Martinez et al. have previously shown that both 2Apro and 3C protease (3Cpro) cleave PABP during enterovirus infection (18, 24). 2Apro cleaves at one site, and 3Cpro cleaves at three sites (two major and one minor). Cleavage at each site separates the CTD from the N-terminal RRM domains in PABP and likely inhibits CTD function in translation but may not interfere with mRNA circularization through PABP-eIF4G binding. Here we determined the effect of 3Cpro-mediated removal of the PABP-CTD on translation. We show that 3Cpro-mediated PABP cleavage significantly inhibited translation both in vitro and in vivo in a poly(A)-dependent manner but did not affect mRNA stability in vitro. Cleavage of PABP did not disrupt its interaction with eIF4GI. Importantly, addition of a cleavage-resistant mutant of PABP reversed 3Cpro-mediated translation inhibition. Kinetic analyses suggested that PABP cleavage affected a late step in translation separate from de novo initiation mediated by eIF4G. These results support a novel mechanism of translation inhibition employed by poliovirus 3Cpro that complements the effect of eIF4G cleavage by 2Apro.

MATERIALS AND METHODS

Plasmids. The 3Cpro coding region was cloned into the *Xba*I-*Hin*dIII sites of the tetracycline response plasmid pTRE2 (Clontech). pT7-Luc (Promega) containing the firefly luciferase (Luc) gene was obtained as the kind gift of P. Sarnow. This plasmid was linearized with *Hpa*I or *Bam*HI to produce polyadenylated (30 nucleotide) and nonpolyadenylated RNAs, respectively. A 3Cpro cleavage-resistant mutant of PABP (His-PABP Q537E) was generated using a QuickChange (Stratagene) mutation protocol with primers (IDT) (primer 1, 5′ GTC AAA GGT TCC TGA CCT TCT ACA TGA ACA GCA GGC TG 3′; primer 2, 5′ CAG CCT GCT GTT CAT GTA GAA GGT CAG GAA CCT TTG AC 3′) for site-directed mutation of pET28a-His-PABP. A stop codon was introduced into the PABP open reading frame at the 3Calt cleavage recognition site (codon 413) by the same method. Mutations were confirmed by sequencing, and the protein was expressed in BLR pLys DE3 (Stratagene).

Transfections. HeLa Tet-On cells (Clontech) were grown and induced by addition of doxycycline as specified by the supplier. Cells were cotransfected (using Lipofectamine Plus [Invitrogen] according to the manufacturer's recommendations) with pTRE2-3C, pTRE2, and pTRE2-luciferase (1 μg/30-mm-diameter dish) in 30-mm-diameter dishes. Transfection efficiency was assessed by cotransfecting pCMV-GFP. Cells were lysed in passive lysis buffer (Promega) prior to analyses. Newly synthesized proteins were pulse labeled for 30 min with ^{35}S-Trans Label (ICN) (50 μCi/ml) in Dulbecco's modified Eagle's medium lacking methionine and cysteine but containing 5% dialyzed calf serum. Protein was analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) with autoradiography and by phosphorimaging.

In vitro transcription and RNA stability. Runoff mRNAs were capped using T7 RNA polymerase in the presence of m^7-GpppG. Some RNAs were radiolabeled with [^{32}P]GTP (2 μCi/50 μl) during in vitro transcription and were used to assess the concentration, integrity, and stability of transcripts following their translation. Following in vitro transcription, RNAs were purified on NucAway spin columns (Ambion). The stability of radiolabeled RNA in HeLa extracts was determined by removing samples from translation reactions at specific times and extracting the RNA immediately into Trizol reagent (Invitrogen). RNA samples were denatured in glyoxal-dimethyl sulfoxide, separated on a 1% (wt/vol) agarose gel, and transferred to a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech). Autoradiography using Kodak X-OMAT AR imaging film was performed overnight at −80°C. RNAs were visualized directly on the membrane after staining for 40 s in 0.03% methylene blue in 3 M NaOAc (pH 5.2), with subsequent destaining in water.

In vitro translation reactions. HeLa S3 cells were maintained in suspension cultures at a concentration of 7×10^5 to 9×10^5 cells/ml at 37°C with 5% CO_2 in Joklik's medium supplemented with 1% fetal calf serum and 9% calf serum. Cells were sedimented, washed in ice-cold Earle's salt solution, and resuspended in hypotonic lysis buffer containing 10 mM HEPES (pH 7.2), 10 mM KCl, 1.2 mM MgOAc, and 2.5 mM dithiothreitol. Cells were swollen on ice for 15 min and were then Dounce homogenized for 5 to 7 strokes until 80% lysis was achieved (according to microscopic examination results); then, nuclei were sedimented out at $2{,}000 \times g$ and lysates were further sedimented at $10{,}000 \times g$ for 5 min at 4°C before being frozen in liquid nitrogen. In vitro translation assays with HeLa lysates were typically performed (as previously described) (3) with 40-μl reaction mixtures containing a mix (l:1) of cell lysate-translation cocktail {final concentrations and quantities were 1.8 mM HEPES [pH 7.6], 25 μM amino acids minus methionine, 50 μg of creatine phosphokinase/ml, 3 mM 2-aminopurine, 25 mM creatine phosphate, 1 mM ATP, 0.5 mM GTP, 2 mM dithiothreitol, 90 mM KCl, 1.8 mM $Mg(OAc)_2$, 30 μCi of [^{35}S]methionine [where indicated]}. HeLa extracts were preincubated with 2Apro, 3Cpro, or buffer for 5 or 15 min at 30°C, as indicated in the figure legends. Translation cocktail was added to the extracts pretreated with proteases at the same time as exogenous RNA (100 to 300 ng) and incubated at 37°C. Incorporation of ^{35}S-Trans Label into translation products was analyzed by precipitation of proteins with trichloroacetic acid and scintillation counting. For rescue experiments with mutant His-PABP, equimolar concentrations (200 μM) of wild-type or His-PABP Q537E were preincubated with capped and polyadenylated or nonpolyadenylated luciferase RNA at room temperature for 10 min. RNA/PABP complexes were added to the non-nuclease-treated HeLa extracts pretreated with buffer or 3Cpro for 5 min at 30°C. After pretreatments with proteins or buffer for 5 min at 30°C, nuclease-treated RRLs (17.5 μl) were programmed with Luc RNA in the presence of ^{35}S-Trans Label (ICN) according to the protocol of the manufacturer.

Purified proteins and antibodies. CVB3 2Apro and poliovirus 3Cpro were expressed and purified as previously described (18). 3Cpro was inactivated by 20 min of incubation at 70°C. His-PABP, His-PABP Q537E, and truncated His-$PABP_{413}$ were purified using Talon resin (Clontech). Purified protein was dialyzed using 20 mM Tris-Cl (pH 8.0)–2 mM 2-mercaptoethanol–100 mM NaCl for 4 h at 4°C. Protein concentrations were determined using a Bradford protein assay, with quantities of bovine serum albumin (BSA) serving as standards. Polyclonal antisera against PABP (24), eIF4GI (29), and eIF4GII (12, 13) were used as described previously.

Luc assays. Luc activity was determined using Luc assay system reagents (Promega) according to the manufacturer's instructions, with photon emission levels measured using a Sirius luminometer (Berthold).

In vitro cleavage assays. Purified His-PABP and His-PABP Q537E proteins were incubated with 3Cpro or buffer for 3.5 h at 37°C. Proteins were analyzed (using a polyclonal anti-PABP antibody to assess the resistance of PABP to cleavage by 3Cpro) by immunoblotting with SDS–10% (wt/vol) PAGE.

Poly(A) agarose and m^7-GTP pulldown assays. eIF4GI-PABP complexes were isolated from mock- or poliovirus-infected cell lysates containing 0.5 mg of total protein with poly(A) agarose beads (Sigma) as described previously (24). After three washes in 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–1% Tween 20–0.3% NP-40–1 mM NaF, complexes were also isolated using m7-GTP-Sepharose beads (Amersham Pharmacia) containing 0.5 mg of total protein. Alternatively, 400 μl of *Escherichia coli* S10 extracts from control BLR(DH3) cells or cells expressing His-PABP or truncated His-$PABP_{413}$ (standardized for PABP expression concentration) was precipitated with 50% ammonium sulfate and then used to bind 100 μl of Talon resin (Clontech) in 5 mM imidazole and washed extensively with 5 mM imidazole in buffer containing 300 mM NaCl–20 mM Tris (pH 7.4). Talon beads bearing washed PABP or truncated PABP were used to bind complexes in HeLa lysates (100 μl). The beads were resuspended in SDS-PAGE buffer and incubated at 100°C for 2 min before immunoblot analysis.

RESULTS

Poliovirus 3Cpro inhibits endogenous mRNA translation in HeLa cell translation extracts independently of eIF4GI or eIF4GII cleavage. Kuyumcu-Martinez et al. have previously shown that poliovirus 3Cpro can cleave PABP at three locations within the CTD (resulting in the separation of RRM domain and CTD of PABP) (24). Here, we wanted to determine how this cleavage affected translation. For these studies we used non-nuclease-treated HeLa cell translation extracts to

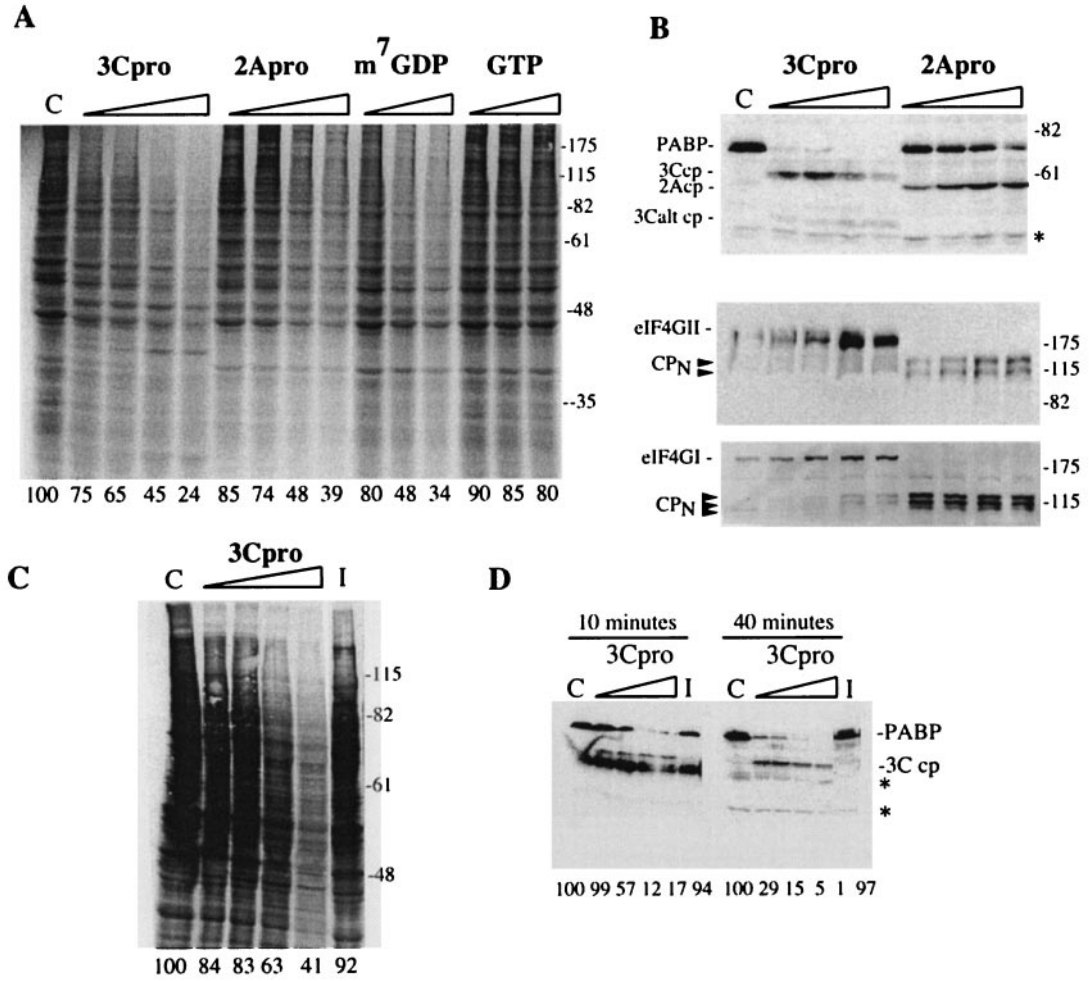

FIG. 1. Catalytically active 3Cpro inhibits cellular mRNA translation independent of eIF4GI and eIF4GII cleavage. (A) Autoradiogram of an SDS–10% (wt/vol) PAGE gel showing [^{35}S]methionine-cysteine incorporation into newly synthesized proteins in HeLa translation extracts that were untreated (C) or treated for 15 min with increasing amounts of 3Cpro (8, 16, 24, and 32 ng/μl), 2Apro (8, 16, 24, and 32 ng/μl), m^{7}GTP (0, 0.58, 0.85, and 1.16 mM), or GTP (0, 0.58, 0.85, and 1.16 mM). (B) Immunoblots of PABP, eIF4GI, and eIF4GII (from the lysates described for panel A) at the completion of translation. cp, intact PABP and PABP N-terminal cleavage products; CP_N, N-terminal fragment of eIF4G generated by 2Apro; *, nonspecific reactive bands. The migration of molecular-weight markers is shown on the right. (C) Autoradiogram of an SDS-PAGE gel after 30 min of labeling with ^{35}S-Trans Label in HeLa translation extracts treated with buffer (C), active 3Cpro (10, 20, 40, or 80 ng/μl), and inactive 3Cpro (I) (80 ng/μl). (D) Immunoblot of intact and cleaved PABP from the same lysates as described for panel C. Percentages of cleavage of PABP compared to the results seen with control extracts are shown below the panel. Numbers below the autoradiograms indicate percent translation compared to the results seen with untreated controls. In each case, data have been reproduced at least three times.

preserve competition for ribosomes from endogenous mRNAs, and retain cap-poly(A) synergy. As reported previously, poly(A) tails on mRNA can stimulate a 5- to 10-fold increase in translation in these extracts compared to the results seen with RNA lacking poly(A) tails (3) (see Fig. 5). We preincubated viral proteases with translation extracts before testing the effects on translation of endogenous HeLa mRNA (Fig. 1). Preincubation with GTP served as a negative control and did not affect translation appreciably. Cap analogue (m^{7}GDP) sequesters eIF4E and inhibited translation in a dose-dependent manner, as reported previously (40). As expected, preincubation with 2Apro resulted in rapid and complete cleavage of eIF4GI and eIF4GII (Fig. 1B). Interestingly, this treatment resulted in only partial translation inhibition in HeLa translation lysates, in similarity to the results observed for virus-infected cells in which viral RNA replication is blocked with 1 mM guanidine or other agents (4, 34).

When 3Cpro was preincubated with HeLa lysates, endogenous mRNA translation was inhibited in a dose-dependent manner. Surprisingly, 3Cpro inhibited translation as effectively as 2Apro even though there was no cleavage of eIF4GI or eIF4GII. Upon examination of PABP by immunoblotting after the 90-min incubation period, 2Apro had partially cleaved PABP, generating the 2Apro cleavage product previously described (2Acp) (18). 3Cpro resulted in more efficient cleavage of PABP than 2Apro and created two N-terminal PABP cleavage products (3C cp and 3Calt cp) (24). Partial cleavage of PABP correlated with significant reduction of translation (e.g., 65% of control levels when 16 ng of 3Cpro/μl was added); however, complete cleavage of PABP did not result in com-

plete translation inhibition, in similarity to the results seen with complete eIF4GI cleavage. Thus, cleavage of PABP by 3Cpro inhibited translation as effectively as cleavage of eIF4GI and eIF4GII by 2Apro. Further, 3Cpro significantly inhibited endogenous mRNA translation in the HeLa cell translation system independently of eIF4GI and eIF4GII cleavage. These data suggest that cleavage of eIF4G or PABP alone is insufficient for severe inhibition of endogenous HeLa mRNAs and that both events may be required.

To address whether the protease activity of 3Cpro was required for translation inhibition, HeLa translation extracts were preincubated with active or heat-inactivated 3Cpro for 10 min prior to translation. Enzymatically active 3Cpro inhibited translation in a dose-dependent manner (Fig. 1C); however, heat-inactivated 3Cpro did not affect translation levels at the highest concentrations tested. Only active 3Cpro, and not inactive 3Cpro, resulted in PABP cleavage in lysates and generated 3C cleavage products (Fig. 1D). As seen previously in the immunoblot results, the signal intensities of 3C cleavage products, particularly the 3Calt cp, are significantly lower than those seen with intact PABP and do not increase consistently with time, suggesting that the cleavage products are unstable in lysates (24). These results suggest that the protease activity of 3Cpro is required for its ability to inhibit translation.

PABP is only partially cleaved during poliovirus infection, and 3Cpro specifically targets the pool of PABP associated with polysomes but does not target non-ribosome-associated PABP (24). Thus, we were interested in determining whether partial PABP cleavage was sufficient to significantly inhibit translation in HeLa cell lysates. The results of many in vitro experiments showed that significant translation inhibition was observed with cleavage of only a portion of PABP. Figure 1C shows that lower amounts of 3Cpro (10 ng/μl) cleaved only trace amounts of PABP after 10 min and yet endogenous translation was inhibited by about 16% at the end of the experiment. Higher levels of PABP cleavage resulted in a higher level of translation inhibition, and yet translation of endogenous mRNA was never inhibited completely (see Fig. 3, 4, and 6). The data suggest that partial PABP cleavage might be sufficient to significantly inhibit translation; however, total PABP cleavage is insufficient to completely inhibit endogenous mRNA translation.

3Cpro inhibits exogenous RNA translation in non-nuclease-treated HeLa extracts. To determine whether translation of exogenous RNA can be inhibited by 3Cpro in HeLa cell extracts, polyadenylated Luc RNA was tested in similar reactions. Luc RNA translation was markedly reduced in lysate pretreated with 3Cpro compared to that seen with untreated lysate (more so than endogenous mRNA translation) (Fig. 2B). Marked (approximately 70%) translation inhibition occurred despite cleavage of less than half the PABP in the lysates after 35 min of incubation (Fig. 2C). Equimolar concentrations of 2Apro inhibited translation only 50 to 60% despite complete cleavage of eIF4GI, as seen previously. When lysates were pretreated with both 2A and 3Cpro, translation levels dropped lower than the levels achieved with either protease alone (approximately 95%). The results of complete cleavage of eIF4GI by 2Apro and incomplete PABP cleavage were very similar to what is observed in poliovirus-infected cells (13, 18, 24). These data suggest that partial cleavage of

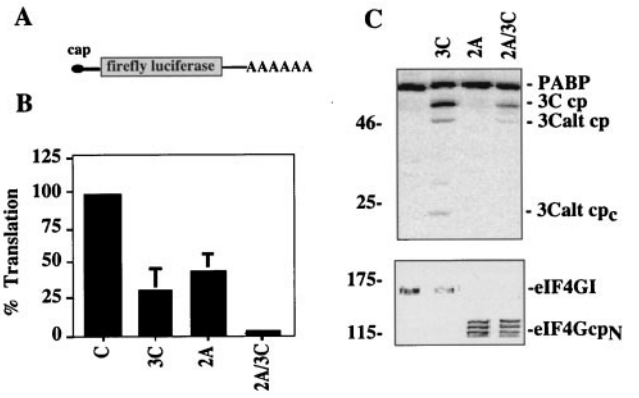

FIG. 2. 3Cpro inhibits exogenous RNA translation in non-nuclease-treated-treated HeLa extracts. (A) Schematic of Luc RNA used for translation reactions in HeLa extracts. (B) Luc activity after 30 min of translation in HeLa extracts preincubated for 5 min at 25°C with buffer (C), 3Cpro (12 ng/μl), 2Apro (12 ng/μl), or 3Cpro and 2Apro. Error bars represent SD of the results of three experiments performed in duplicate. (C) Immunoblots of PABP and eIF4GI from one of the same extracts. The identities of intact proteins and their cleavage products are as indicated, and migration of markers is shown on the left.

PABP might inhibit exogenous mRNA translation very efficiently and that 3Cpro can inhibit translation more effectively than 2Apro in non-nuclease-treated HeLa lysates.

PABP cleavage by 3Cpro specifically inhibits poly(A)-dependent translation independent of mRNA stability. PABP/poly(A)-dependent translation stimulation is demonstrated most effectively in non-nuclease-treated yeast or HeLa extracts when there is competition with endogenous mRNAs for ribosomes (3, 36, 42). In such systems, the inhibitory effects of PABP depletion were manifest only on poly(A)-containing mRNA and thus were poly(A) specific. Similarly, we wanted to determine whether 3Cpro was inhibiting translation in a poly(A)-specific manner to test whether 3Cpro interfered with cap-poly(A) synergy or other mechanisms not involving PABP. Figure 3A shows that preincubation of lysates with 3Cpro resulted in dose-dependent translation inhibition when polyadenylated reporter RNA was used. A 40% inhibition of Luc translation was induced by very low amounts of 3Cpro and plateaued near 50% until very high levels of protease were added. Inactivated 3Cpro (10 ng/μl) did not cleave PABP (Fig. 1C) and (like BSA) did not inhibit translation of poly(A) Luc RNA. When nonpolyadenylated Luc RNA was examined, the overall translation rates were consistently four- to fivefold lower than those seen with polyadenylated RNA, demonstrating that poly(A) stimulation was functional in the lysates. Interestingly, addition of 3Cpro had no inhibitory effect on translation levels when RNAs contained no poly(A) (Fig. 3B). This suggests that 3Cpro-mediated translation inhibition is specific for polyadenylated mRNAs.

PABP also functions in mRNA stability by oligomerizing on poly(A) and interfering with exonuclease-mediated degradation. Thus, it was possible for PABP cleavage to affect PABP-RNA binding affinity and decrease mRNA stability rather than translation. Figure 3D shows the effect of 3Cpro on the stability of polyadenylated and nonpolyadenylated Luc RNA. Over

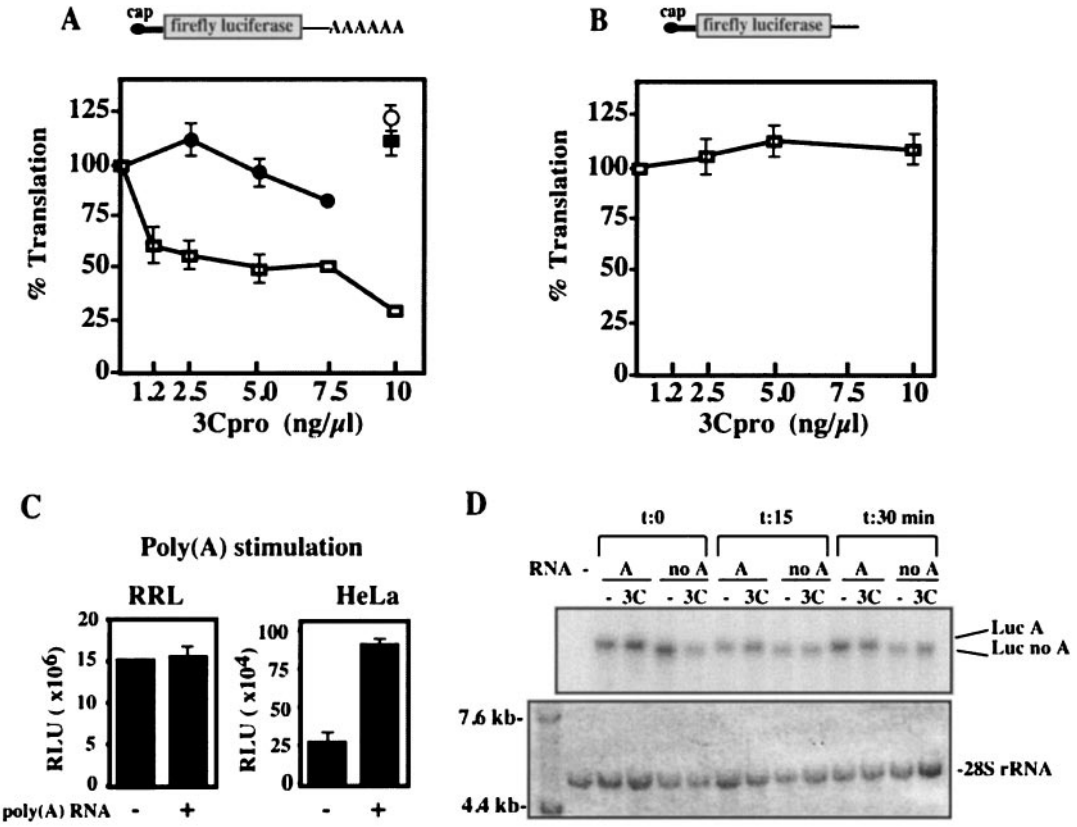

FIG. 3. PABP cleavage by 3Cpro specifically inhibits poly(A)-dependent translation and does not affect RNA stability. (A) Luc activity (expressed in light units set to 100% of control reactions) generated in HeLa translation reactions programmed with polyadenylated RNA. Reaction mixtures contained buffer or active or inactive 3Cpro (10 ng/μl) or BSA (10 ng/μl). Symbols: open square, 3Cpro in HeLa lysate; open circle, inactive 3Cpro in HeLa lysate; closed square, BSA in HeLa lysate; closed circle, 3Cpro in RRL. Error bars indicate SD of the results from three individual experiments. For this experiment, 100% translation was equivalent to 2.56×10^5 relative light units (RLUs). (B) Luc activity generated in HeLa translation reactions programmed with nonpolyadenylated RNA. Symbols and data analysis are as described for panel A. (C) Comparison of levels of stimulation of translation of polyadenylated versus nonpolyadenylated Luc RNA in nuclease-treated RRL (left panel) and in non-nuclease-treated HeLa (right panel) extracts. (D) Autoradiogram of nylon membrane showing the stability of [^{32}P]GTP-labeled nonpolyadenylated (Luc no A) or polyadenylated Luc (Luc A) RNAs in buffer or 3Cpro-treated HeLa translation extracts. The lower panel shows the methylene blue stain of ribosomal RNAs (28S rRNA) on the same membrane used as a loading control.

the span of the translation assay (30 min), polyadenylated and nonpolyadenylated mRNAs were equally stable in comparison to 28S rRNA. Also, the addition of 3Cpro did not affect the stability of either polyadenylated or nonpolyadenylated RNA in vitro. This suggests that 3Cpro-mediated PABP cleavage specifically inhibits poly(A)-dependent translation by mechanisms independent of mRNA stability.

Nuclease-treated RRL is widely used for the translation of RNAs in vitro; however, it has been previously demonstrated that exogenous RNA does not require poly(A) for efficient translation in this system. In contrast, the non-nuclease-treated HeLa lysates showed poly(A)-mediated stimulation increases that ranged from 3- to 10-fold (3) (Fig. 3C). Thus, the effect of the presence of 3Cpro on translation of exogenous mRNA in RRL was assessed. Figure 3A (filled circles) shows that the addition of increasing concentrations of 3Cpro had only a modest effect on the translation of capped and polyadenylated Luc RNA. In addition, concentrations (2.5 ng/μl) of 3Cpro which inhibited translation nearly 50% in non-nuclease-treated extracts did not reduce translation in RRL. These data suggest that cleavage of PABP by 3Cpro does not significantly affect translation in nuclease-treated extracts, supporting previous results demonstrating that 3Cpro specifically affects poly(A)-dependent translation.

Restoration of 3Cpro-mediated translation inhibition by the addition of exogenous PABP. To further implicate PABP as the focus of 3Cpro-mediated translation inhibition, we supplemented 3Cpro-treated lysates with recombinant His-PABP to test whether 3Cpro-mediated translation inhibition could be reversed. As seen before, increasing concentrations of 3Cpro inhibited reporter RNA translation in a dose-dependent manner in HeLa extracts (Fig. 4A). Addition of His-PABP alleviated this inhibition in extracts pretreated with low concentrations of 3Cpro but showed a progressively reduced ability to rescue translation as higher levels of 3Cpro were added. It is possible that His-PABP was cleaved by the high concentrations of 3Cpro in these extracts or that 3Cpro might have cleaved another factor involved in translation initiation. To address these questions, we generated a 3Cpro-resistant cleavage mutant of PABP.

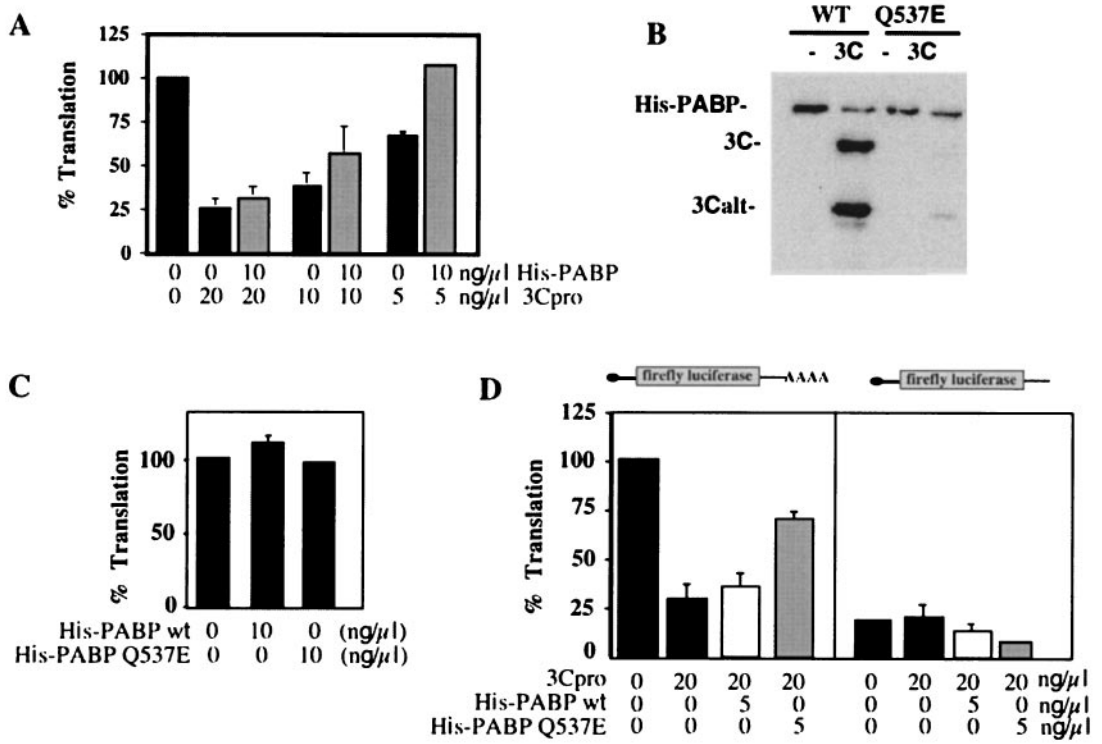

FIG. 4. Restoration of polyA-dependent translation by addition of exogenous PABP. (A) Luc RNA translation in HeLa extracts treated with buffer or 3Cpro and supplemented with His-PABP. Black bars represent the percentages of translation (light units relative to buffer control levels) in buffer- or 3Cpro-treated extracts. Gray-shaded bars indicate Luc translation levels after the addition of His-PABP. (B) Immunoblot of wild-type (WT) or mutant (Q537E) His-PABP left untreated or treated with 3Cpro. Cleavage fragments of PABP are indicated (3C cp and 3Calt cp). (C) Luc RNA translation in extracts incubated with wild-type (wt) or Q537E PABP. (D) Translation of polyadenylated or nonpolyadenylated RNA in buffer- or 3Cpro-treated extracts (black bars) supplemented with wild-type (wt) PABP (empty bars) or Q537E PABP (dark-gray-shaded bars). Data in panels A, C, and D represent the means ± SD of the results from three individual experiments.

Mutation of the glutamine at position 537 to glutamic acid (Q537E) in PABP was designed to eliminate its cleavage by 3Cpro at the 3C site and was confirmed to be effective, as no 3C cp was produced upon incubation with protease (Fig. 4B). Surprisingly, this mutation also blocked cleavage at the second 3Cpro cleavage site (i.e., the 3Calt site) by an approximately 10-fold reduction. Addition of wild-type and mutant His-PABP-Q537E to untreated HeLa translation extracts did not stimulate Luc-poly(A) RNA translation levels, showing that the mutation did not introduce an unforeseen function that altered translation (Fig. 4C). Equimolar concentrations of wild-type or mutant His-PABP were incubated with either polyadenylated or nonpolyadenylated Luc RNA. This RNA/protein complex was used to supplement extracts pretreated with buffer or 3Cpro. As seen before, addition of wild-type His-PABP only slightly stimulated translation in extracts treated with high concentrations of 3Cpro (Fig. 4D). However, the same concentration of His-PABP-Q537E significantly restored translation of polyadenylated Luc RNA (Fig. 4D). Since His-PABP-Q537E was not cleaved during the incubation it was able to restore translation at lower concentrations (only 5 ng/μl) than wild-type PABP (10 ng/ml) and was effective compared with the results seen with higher concentrations of 3Cpro. Neither the wild type nor His-PABP-Q537E stimulated translation of nonpolyadenylated RNA in 3Cpro-treated extracts (Fig. 4D, right panel). Collectively, the results suggest that cleavage-resistant PABP restores poly(A)-dependent translation after inhibition by 3Cpro but does not affect non-poly(A)-dependent translation. Further, the data support the hypothesis that 3Cpro inhibits translation specifically through cleavage of PABP.

eIF4GI-PABP interaction is not disrupted during poliovirus infection. After cleavage of eIF4GI and PABP in infected cells, their N-terminal cleavage products can still potentially interact via intact binding domains. To determine whether these fragments still interacted as expected, mock- or poliovirus-infected cell lysates were incubated with poly(A)-agarose (Fig. 5A) or m^7GTP-Sepharose (Fig. 5B), respectively, to affinity purify PABP and eIF4G. Immunoblotting results for complexes isolated with poly(A)-agarose showed that PABP was retained and likely interacted with both intact eIF4GI (Fig. 5A, lane 4) and N-terminal cleavage fragments of eIF4GI with equivalent efficiency (lane 5). PABP cleavage products did not bind poly(A) as efficiently as intact PABP (compare Fig. 5A, lane 5, to input lane 2), suggesting that PABP cleavage might negatively affect PABP oligomerization or binding affinity to poly(A). However, PABP cleavage is never complete in virus-infected cells and interactions may be primarily mediated through the presence of uncleaved PABP in the lysate. Thus, reverse affinity purification with m^7GTP was performed; the

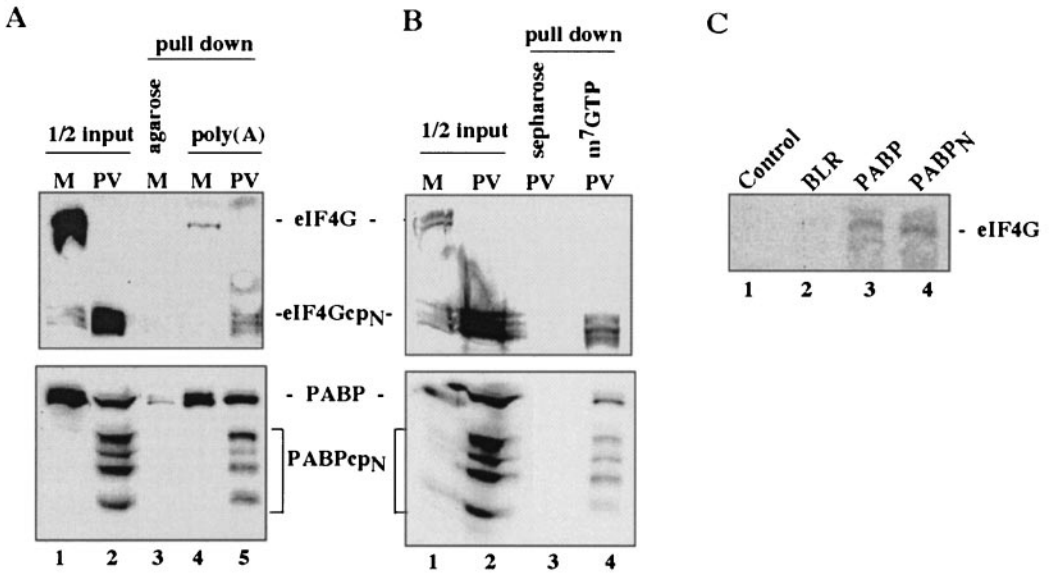

FIG. 5. PABP-eIF4GI interactions are not abolished during poliovirus infection. (A) Immunoblot analysis of eIF4G (upper panel) and PABP (lower panel) in complexes isolated from mock (M)- or poliovirus (PV)-infected HeLa lysates by the use of poly(A) agarose. (B) Immunoblot analysis of eIF4GI (upper panel) or PABP (lower panel) in complexes isolated from mock (M)- or poliovirus (PV)-infected HeLa lysates by the use of m^7GTP-Sepharose. Cleavage fragments of PABP or eIF4GI (i.e., PABP cp_N and eIF4G cp_N) are indicated. (C) Immunoblot analysis of eIF4GI in complexes isolated from uninfected HeLa lysates by the use of His-PABP or the N-terminal fragment of His-PABP truncated at the 3Calt cleavage site (amino acid 413) by insertion of a stop codon (His-$PABP_{413}$). Recombinant proteins were expressed in BLR(DE3) bacterial cells and purified on Talon beads before being used in pulldown assays. BLR indicates bacterial lysate from nontransformed cells (used as a negative control); the control was HeLa lysate incubated alone with Talon beads.

results showed that cleaved eIF4GI was isolated in a complex together with intact or cleaved forms of PABP (Fig. 5B, lane 4). Finally, recombinant His-PABP or His-PABP truncated at the 3Calt cleavage site His-$PABP_{413}$ was used to bind eIF4GI. The results show that both forms of PABP were equally efficient at pulling down intact eIF4GI (Fig. 5C). The results suggest that after eIF4GI and PABP cleavage during poliovirus infection, the interaction between their N-terminal cleavage products remains largely intact.

Kinetic analysis of translation in 3Cpro-treated HeLa cell lysates. We took advantage of the large linear scale and sensitivity of Luc assays to more closely analyze how 3Cpro affected the kinetics of Luc synthesis in translation extracts. Figure 6A shows the kinetics of Luc accumulation in mock- and protease-pretreated RRL extracts as taken at 3-min intervals. Figure 6B shows similar levels of Luc accumulation in mock-treated control and pretreated HeLa translation extracts. As previously reported (44), it took 7 to 8 min for Luc RNA to undergo initiation, ribosome transit through the open reading frame, and release of the 61-kDa Luc product in the in vitro translation systems. Thus, there was a consistent 7-min lag before Luc activity was detected in control translations followed by a linear accumulation of Luc (Fig. 6A and B). Throughout these experiments, pretreatment of lysates with either 2Apro or 3Cpro failed to alter the time required for Luc translation products to appear; thus, neither protease was found to affect ribosome transit rates. Pretreatment of lysate with 0.3 μg of 2Apro for 5 min before RNA and an energy cocktail were added (to initiate translation) caused a large reduction in Luc translation in both RRL and HeLa extracts. Immunoblot analysis confirmed that >95% of HeLa eIF4GI was cleaved during the 5-min pretreatment period (data not shown). Thus, it was expected that destruction of eIF4G before translation was started would have this drastic effect (since all Luc RNAs added to the lysate are required to load ribosomes de novo via cap-dependent initiation mechanisms). In contrast, pretreatment of nuclease-treated RRL extracts with 0.3 μg of 3Cpro had only a minor effect on translation, reflecting the lack of cap-poly(A) synergy in this system. However, 3Cpro preincubation resulted in an approximately 50% decrease in translation in HeLa extracts in which cap-poly(A) synergy is preserved (Fig. 6B).

We next tested the effect of the time of addition of proteases relative to the state of polysome formation on Luc RNA. Since de novo ribosome initiation, transit, and termination are first completed within 8 min, it therefore takes approximately 7 to 8 min for Luc RNA polysomes to become maximally loaded with ribosomes and 4 min to become half-loaded with ribosomes (Fig. 6F). During the first 8 min of the assay, all ribosome initiation must be de novo (since no ribosomes have reached the termination codon); at time points after 8 min, however, ribosome initiation can occur via de novo or recycling mechanisms. What is the effect of cleavage of eIF4GI or PABP after polysomes are formed versus the effect seen before polysome formation? Fig. 6C shows that when 2Apro is used to pretreat HeLa lysates before RNA is added, accumulation of Luc is drastically inhibited compared to the results seen with mock-treated control translation reactions and continues to decline throughout the incubation period. The higher-level initial translation probably reflects incomplete eIF4GI cleavage when RNA was added (0-min time point) that progresses to total cleavage after the 4-min time point. When 2Apro was

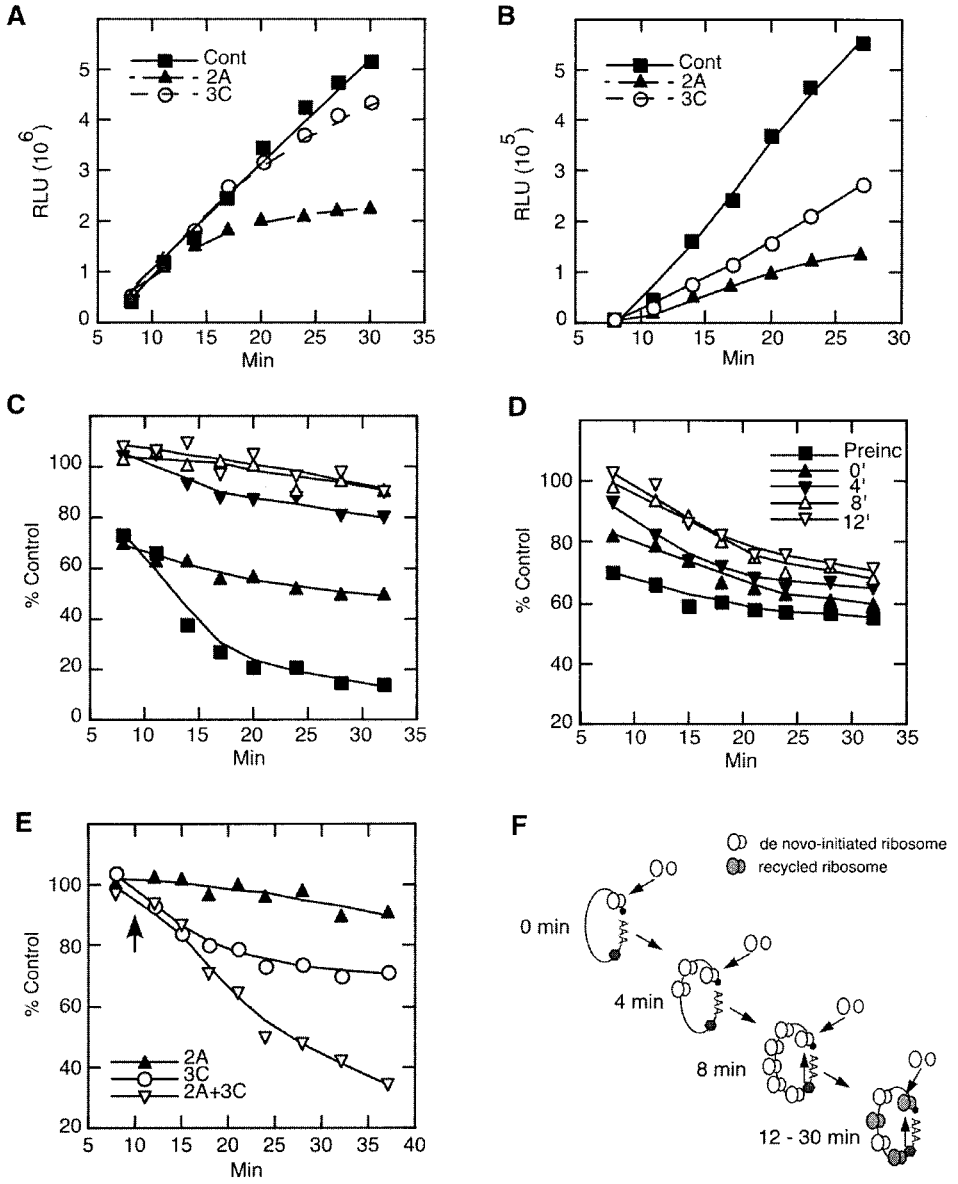

FIG. 6. Kinetics of 3Cpro inhibition of translation in vitro. (A) The graph indicates the accumulation of Luc (expressed in RLUs) in nuclease-treated RRL translation extracts pretreated for 5 min at 30°C with buffer (Cont), 5 ng of 3Cpro/μl, or 5 ng of 2Apro/μl before capped and polyadenylated Luc RNA was added and the incubation was shifted to 36°C (time point 0). At each time point, samples were transferred to Luc assay buffer and immediately assayed for Luc. (B) The graph indicates Luc accumulation in experiments in which HeLa extracts were similarly pretreated with 2Apro or 3Cpro and then translation cocktail and capped and polyadenylated Luc RNAs were added to start translation reactions (time point zero). (C) 2Apro (5 ng/μl) was used to pretreat HeLa lysate or was added at 0, 4, 8, or 12 min after the addition of Luc RNA and translation cocktail. The graph shows the accumulation of Luc RLUs plotted as percentages of the translation in protease-treated lysates compared to the results seen with mock-treated control lysates. The legend (depicted in panel D) is applicable to both panels C and D. (D) 3Cpro (5 ng/μl) was used to pretreat HeLa lysate or was added at 0, 4, 8, or 12 min after the addition of Luc RNA and translation cocktail. (E) HeLa translation extracts were supplemented with 2Apro (5 ng/μl) or 3Cpro (5 ng/μl) or both at the 11-min time point (arrow), and translation was allowed to continue. (F) Translation schematic.

added to reaction mixtures at 0 or 4 min or later, interestingly, translation was inhibited to markedly lesser degrees. When 2Apro was added to reaction mixtures at 8 or 12 min (when full polysomes had formed), 2Apro showed little ability to inhibit translation (even through 38 min of incubation). This was more than enough time to allow complete eIF4GI cleavage and polysome runoff (approximately 12 to 14 min).

The lack of drastic translation inhibition with preformed polysomes after eIF4GI cleavage is likely due to ribosome recycling and suggests that this reaction is not dependent on the presence of intact eIF4GI. In contrast, 3Cpro was able to inhibit translation when added at early or late time points. Preincubation with 3Cpro reduced initial Luc accumulation by 30%, and accumulation increased to nearly 50% by the end of

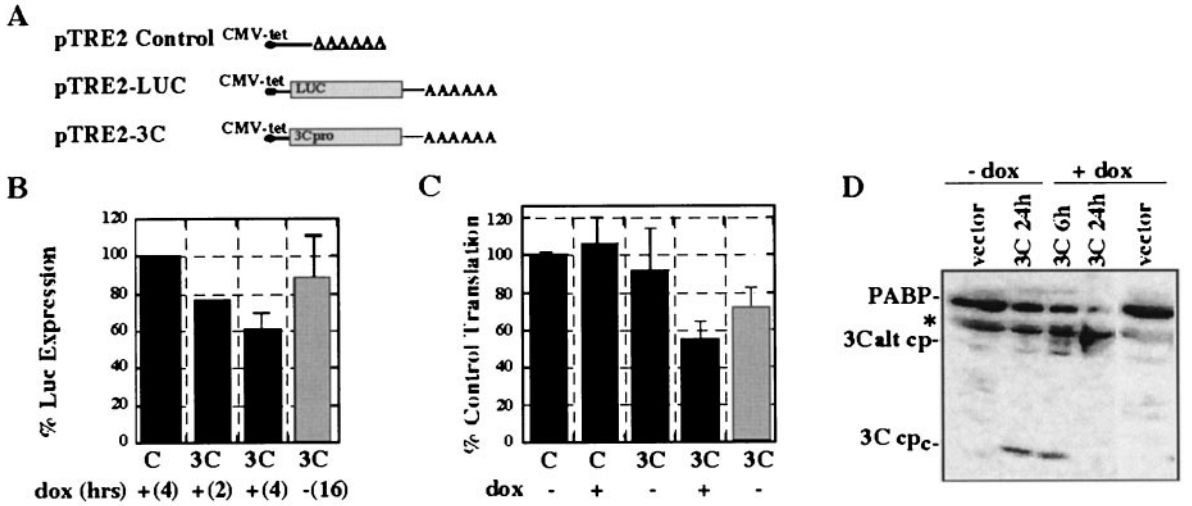

FIG. 7. Expression of 3Cpro inhibits translation in HeLa cells. (A) Schematic of DNAs cotransfected into HeLa Tet-On cells. (B) The graph shows levels of Luc expression in cells transiently transfected with pTRE-luc and cotransfected with control pTRE2 vector (C) or pTRE2-3C. Data are expressed as percentages of Luc expression in cells transfected with control pTRE2 vector. At 4 h posttransfection, cells were induced with doxycycline for 2 or 4 additional hours and then cell lysates were harvested and analyzed for Luc activity. Both 2- and 4-h control Luc RLU levels were set to 100% in the graph to calculate percentages of reduction in translation after 3Cpro expression. Black bars indicate cells induced with doxycycline for 4 h, and gray-shaded bars indicate cells left uninduced for 16 h before harvesting and analysis. (C) Graph showing [^{35}S]methionine-cysteine incorporated into newly synthesized proteins in doxycycline-induced or uninduced HeLa Tet-On cells transfected with pTRE2 control vector (C) or pTRE2-3C (3C). Black bars indicate cells treated for 4 h posttransfection or left untreated; gray-shaded bars indicate cells left untreated for 16 h. (D) Immunoblot analysis of PABP in HeLa Tet-On cells transfected with control vector or pTRE2-3C DNA that were either induced with doxycycline (+dox) or left uninduced (−dox). Cleavage products of PABP (3Calt cp and 3Calt cp$_c$) are indicated. Transfection rates determined with an enhanced green fluorescent protein expression vector and immunofluorescence analysis were 85 to 90% in the experiments represented here. Data represent the means ± SD of three individual experiments.

the assay. When added at later time points, 3Cpro had apparent diminishing effects on total Luc accumulation; however, this was partly due to the fact that before the enzyme was added to assays at later time points, more Luc had already been translated. However, addition of 3Cpro at 12 min (when polysomes were fully loaded) still resulted in a decline in total translation of approximately 30%. The kinetics also showed that the effect of 3Cpro addition at any time point does not progress to more that a 50% inhibition of translation. Figure 6E shows that when viral proteases were added to fully loaded polysomes (at 12 min), the presence of both 2Apro and 3Cpro was required to significantly shutdown translation reactions.

Taken together, these data suggest that 3Cpro might inhibit de novo translation initiation slightly; however, inhibition by 3Cpro increased later in the assays, as the reporter RNA encountered the opportunity to undergo ribosome recycling. This decline may also result from continued 3Cpro cleavage of PABP; however, immunoblot analysis showed rapid cleavage of about 10% total PABP cleavage by 5 min of incubation which increased only slightly after continued incubation (data not shown). Importantly, 2Apro was unable to effectively inhibit translation when added to preformed polysomes; however, 3Cpro was more effective. Last, the inhibitory effect of 2Apro and 3Cpro on polysome translation were additive, suggesting that each affects different steps in the translation initiation-recycling process (Fig. 6E). These data imply that 3Cpro might inhibit a late translation process that occurs after cap recognition, scanning, and elongation.

Expression of 3Cpro inhibits translation in HeLa cells. Plasmids containing the 3Cpro coding region and Luc genes were cotransfected into HeLa cells to assess whether 3Cpro inhibited translation in vivo (Fig. 7A). Previously, stable expression of 3Cpro was shown to be toxic to cells and to induce apoptosis (2). To circumvent this problem, we used transient inducible expression of 3Cpro for only short periods (2 to 4 h). Further, we measured the effects on translation early (when Luc accumulation was linear in cells [data not shown] and reflected translation rates more accurately). We typically achieved high-efficiency (85 to 90%) transfection of Tet-On-3Cpro expression vector (pTRE2-3C) into a tetracycline-inducible HeLa cell line. Following induction of 3Cpro, Luc synthesis was significantly reduced by 4 h postinduction (Fig. 7B). In addition, translation of endogenous cellular mRNAs declined after 3Cpro induction (doxycycline treatment) (Fig. 7C) but not in cells bearing control vectors. We attempted to directly assay 3Cpro in cell lysates via immunoblotting; however, 3Cpro accumulation was not detectable (data not shown). However, evidence of 3Cpro expression was obtained from immunoblot detection of specific PABP cleavage fragments (3Calt and 3C cp$_c$) in cell lysates (Fig. 7D, second and third lanes from left). Importantly, only a small fraction of total PABP was cleaved in cells that experienced a 40% decline in translation. PABP 3Cpro cleavage products were also detectable in uninduced cells, indicating leaky expression. Interestingly, induced cells contained PABP 3C cp at 6 h of postinduction but not after 24 h of induction. Taken together with the finding of a lack of accumulation of 3Cpro itself, this finding shows that is possible that 3Cpro limits its own production in this system via translation inhibition or induction of apoptosis. These data support previous in vitro results and demonstrate that 3Cpro expres-

sion can inhibit both Luc expression and cellular mRNA translation in vivo.

DISCUSSION

Poliovirus induces a dramatic translation inhibition in infected cells that has been extensively studied, and yet the exact mechanisms remain elusive. Translation inhibition was thought to be caused by cleavage of eIF4GI, which thereby blocks assembly of eIF4F and 40S ribosome subunits on the 5′ cap structure of host mRNA. However, later work showed that eIF4GI cleavage was not sufficient for host translation shutoff (since cleavage of eIF4GI resulted in only a twofold decline in translation) (4, 34). Similarly, in this work 2Apro cleaved all eIF4Gs, including eIF4GII, and yet failed to inhibit translation more than twofold in lysates (Fig. 1). Since eIF4GI comprises 90% of eIF4G in HeLa cells, eIF4GII may play a smaller role in the translation shutoff mechanism than proposed previously (13). Interestingly, although 2Apro partially cleaves PABP it does not efficiently cleave polysome-bound PABP (24), which may lessen its impact on translation.

Here we show that 3Cpro plays a major role in translation regulation that was previously unknown and that it affects a different step in translation than 2Apro. This is the first report to show that 3Cpro inhibits translation in the absence of any other viral proteins. The importance of 3Cpro in translation inhibition may have been underestimated in the poliovirus field, since it did not cleave eIF4GI (27) whereas 2Apro did (22, 28). Further, early reports indicated that expression of 2Apro can cause strong inhibition of translation in cells (7, 41) although the confounding effects of translation inhibition caused by 2Apro-induction of apoptosis (10) were not known.

Our data show that 3Cpro inhibits translation by removal of the CTD of PABP and that this selectively inhibits poly(A)-dependent translation. Further, the inhibitory effects of 3Cpro on translation can only be effectively measured in non-nuclease-treated translation extracts. In contrast, 2Apro is selective for capped RNA and equally inhibits polyadenylated and non-polyadenylated capped mRNAs in nuclease-treated translation extracts (data not shown).

Only about one-third of total HeLa cell PABP is cleaved in virus-infected cells at the time complete translation shutoff is attained (18, 19), thus raising questions of the functional significance of PABP cleavage in translation shutoff. However, we have recently shown that a large pool of PABP is not associated with the translation apparatus (40% of total) and that this pool is resistant to 3Cpro cleavage. Conversely, polysome-associated PABP (35% of the total) was cleaved efficiently by 3Cpro (24) and another PABP pool associated with initiation factors was also cleaved by 3Cpro but less efficiently. Here we show that partial cleavage of PABP in the absence of any eIF4GI and eIF4GII cleavage can have a significant impact on cellular translation in vitro and in vivo (Fig. 1 to 3). Interestingly, translation inhibition was significant when only a portion of PABP was cleaved and yet total degradation of PABP did not block translation more than two- to threefold. This result supports earlier reports that PABP is not required for translation but may stimulate its efficiency (17). Also, the PABP CTD is dispensable for cell viability in yeast (37).

It is unknown what specific translation mechanism(s) is inhibited by 3Cpro cleavage and removal of the CTD, although the functions are poly(A) dependent and may occur late in translation. After cleavage by 3Cpro, the remaining N-terminal domain of PABP contains four intact RRM domains to facilitate binding to eIF4G and poly(A) RNA (23). Here we show interactions between eIF4G and PABP N-terminal cleavage fragments are retained after cleavage, and it was previously shown that recombinant fragments of PABP containing RRM1-2 can bind eIF4G (16). Thus, unlike the results seen with rotavirus-infected cells, translation inhibition in poliovirus-infected cells may not involve opening the closed-loop structure of mRNA. Other investigators have shown that mutations within PABP that blocked interactions with eIF4G did not affect the viability of yeast and concluded that eIF4G-PABP interaction is important but not essential for the survival of yeast. Similarly, these mutations did not affect poly(A)-dependent translation; thus, other mechanisms were proposed to be responsible for the control of poly(A)-dependent translation (32).

Similarly, 3Cpro cleavage of PABP does not seem to drastically inhibit de novo assembly of 40S ribosomes on the cap structure. 2Apro-mediated eIF4G cleavage effectively blocked this step and had an additive inhibitory effect on translation with PABP cleavage. When HeLa lysates were pretreated with 3Cpro, the levels of very first Luc that was released by 8 min were already reduced 30% compared to those of the controls. However, instead of 3Cpro inhibiting translation initiation steps, this 30% inhibition could also result from defects in ribosome termination via interference with interactions between eRF3 and PABP. Since the shapes and slopes of the 3Cpro inhibition curves were similar whether 3Cpro was added before or after polysome formation (Fig. 6D), it is likely that 3Cpro is affecting a late step in translation (more so than de novo initiation). Further, 3Cpro-PABP cleavage inhibition of translation complemented the inhibition of eIF4F formation on cap structures by 2Apro (Fig. 6E), suggesting that different translation steps are targeted by 3Cpro and 2Apro. Further, PABP and eIF4GI N-terminal fragments can still interact and stimulate eIF4G-eIF4E complex formation on capped RNA through induced conformation changes (45).

So how does 3Cpro cleavage of PABP affect translation? The C-terminal 74 amino acids of CTD form a globular domain with a cleft that binds PAIP-1, PAIP-2, eIF4B, and eRF3 (5, 21). These multiple interactions suggest that the CTD of PABP plays important and differing roles in translation initiation and termination. However, the impact of these interactions on translation remains unclear. A recent report indicated that a point mutation within the CTD that abolishes interactions with eRF3 results in inhibition of cap-poly(A)-dependent translation (44). The data suggested that the interactions between eRF3 and PABP might be important in translation, possibly allowing ribosomes to recycle rather than to facilitate the initial formation of 80S complexes. Cleavage of PABP by 3Cpro at any of the three cleavage sites removes the CTD that interacts with eRF3 and eIF4B. Thus, it is intriguing to speculate that the removal of CTD by 3Cpro inhibits ribosome recycling on mRNA. It is also possible that release of the CTD creates a dominant-negative inhibitor of translation that functions at the recycling stage. Indeed, we have preliminary un-

published data from studies of expression of the CTD in vivo that may support this hypothesis.

Since viral RNA contains poly(A) tails that have been shown to synergistically enhance internal ribosome entry site (IRES)-dependent translation (3), 3Cpro cleavage may also inhibit viral RNA translation. Our recent results support this hypothesis, since viral IRES-mediated translation is also inhibited by 3Cpro in a poly(A)-dependent manner (unpublished data). Thus, there may be mechanisms that allow discrimination between host and viral polyadenylated RNAs in cells such that host mRNAs are inhibited first (before viral RNAs) during infection. Such mechanisms may involve compartmentalization or regulated production of 2Apro and 3Cpro from precursors. Further, the source of PABP that bind poly(A) tails of the rapidly expanding pool of nascent viral mRNAs may be drawn from the large cellular pool of cleavage-resistant PABP (24). Translation and RNA replication initially occur on the same viral RNA template in virus-infected cells; thus, the viral polysome must be cleared of initiating or recycling ribosomes before RNA replication can begin. Although other mechanisms have been implicated in this process, cleavage of PABP by 3Cpro may play a key role and is presently being investigated (1, 9).

In summary, neither 3Cpro cleavage of PABP nor 2Apro cleavage of eIF4GI and eIF4GII completely inhibits translation in the HeLa translation system. Thus, 2Apro cleavage of eIF4G may be needed to block de novo translation initiation, 3Cpro cleavage of PABP may be required to interrupt ribosome recycling, and the net effect of both processes may be required for total translation inhibition. Our data also suggest that targeted removal of the CTD of PABP by 3Cpro is part of a dual strategy employed by poliovirus to shut down host cell translation during infection.

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