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Inhibition of Poliovirus-Induced Cleavage of Cellular Protein PCBP2 Reduces the Levels of Viral RNA Replication

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ABSTRACT

Due to their small genome size, picornaviruses must utilize host proteins to mediate cap-independent translation and viral RNA replication. The host RNA-binding protein poly(rC) binding protein 2 (PCBP2) is involved in both processes in poliovirus infected cells. It has been shown that the viral proteinase 3CD cleaves PCBP2 and contributes to viral translation inhibition. However, cleaved PCBP2 remains active in viral RNA replication. This would suggest that both cleaved and intact forms of PCBP2 have a role in the viral RNA replication cycle. The picornavirus genome must act as a template for both translation and RNA replication. However, a template that is actively being translated cannot function as a template for RNA replication, suggesting that there is a switch in template usage from translation to RNA replication. We demonstrate that the cleavage of PCBP2 by the poliovirus 3CD proteinase is a necessary step for efficient viral RNA replication and, as such, may be important for mediating a switch in template usage from translation to RNA replication.

IMPORTANCE

Poliovirus, like all positive-strand RNA viruses that replicate in the cytoplasm of eukaryotic cells, uses its genomic RNA as a template for both viral protein synthesis and RNA replication. Given that these processes cannot occur simultaneously on the same template, poliovirus has evolved a mechanism(s) to facilitate the switch from using templates for translation to using them for RNA synthesis. This study explores one possible scenario for how the virus alters the functions of a host cell RNA binding protein to mediate, in part, this important transition.

The picornavirus family is made up of small, single-stranded, positive-sense RNA viruses that cause a range of diseases. Poliovirus, coxsackievirus, and human rhinovirus (HRV) are some of the more well-studied members of this family, causing poliomyelitis, myocarditis, and the common cold, respectively. All picornaviruses have a similar genomic RNA structure that lacks a methyl guanosine cap on the 5' terminus. Additionally, the 5' noncoding region (NCR) is highly structured, with six RNA stem-loop structures that preclude canonical ribosome scanning from the 5' end of the template to the initiating start codon (1). Therefore, translation of the picornavirus genome is initiated in a cap-independent manner via an internal ribosome entry site (IRES). For poliovirus, coxsackievirus, and HRV, the IRES is comprised of stem-loop structures II to VI of the 5' NCR (2, 3). Ribosomes must be recruited to the IRES by a mechanism distinct from the canonical cap-dependent recruitment, although the mechanism is not yet understood. Canonical and noncanonical cellular translation factors aid in ribosome recruitment and translation initiation to translate the picornavirus genome into a single polyprotein that is subsequently processed to produce mature viral proteins. Among those proteins produced are the RNA-dependent RNA polymerase 3D and the viral proteinases 2A and 3C, as well as the 3C precursor 3CD, which cleave the polyprotein into precursor and mature viral proteins important for RNA replication, packaging, and release.

As translation and processing of the polyprotein proceeds, there is an accumulation of viral proteins important for RNA synthesis, leading to the formation of replication complexes and a switch from translation to RNA replication. As positive-sense RNAs, picornavirus genomes are able to serve as templates for both translation and RNA replication. Translation proceeds along

the template in the 5'-to-3' direction, whereas RNA replication is initiated at the 3' end and traverses the template to the 5' end. However, previous studies have shown that the viral polymerase cannot dislodge translating ribosomes (4). Furthermore, it has been shown that templates that are actively being translated cannot function as a template for RNA replication, suggesting that there must be a switch in template usage from translation to RNA replication (4, 5). Initiation of RNA synthesis produces negative-strand templates that are used for multiple rounds of positive-strand RNA synthesis. The resulting positive-strand RNAs are then used for subsequent rounds of translation and RNA replication or are packaged and released as virion RNA.

Due to their small genome size and limited coding capacity, picornaviruses must usurp the functions of host cell proteins for viral translation and RNA replication, directly benefiting the virus. Examples include poly(rC) binding protein 2 (PCBP2), polypyrimidine tract binding protein (PTB), and lupus autoantigen (La). PTB is a member of the hnRNP family and is known to shuttle from the nucleus to the cytoplasm (6). Previous studies

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have shown that PTB binds to stem-loop IV of the poliovirus 5' NCR and stimulates poliovirus translation (7–10). PCBP2 is involved in host cell mRNA stability and translational control of cellular mRNAs. During poliovirus infection, PCBP2 is important for both translation and RNA replication. PCBP2 binds to stem-loop IV of the poliovirus IRES and forms a ribonucleoprotein (RNP) complex necessary for synthesis of the viral polyprotein (11–14). Additionally, PCBP2 binds to stem-loop I of the 5' NCR, along with the viral proteinase 3CD, to form a ternary complex that is necessary for initiation of negative-strand RNA synthesis (13, 15–18).

PCBP2 is comprised of three hnRNP K homologous domains (called KH domains), which are common to a subset of a class of RNA binding proteins. Domains KH1 and KH3 have been shown to mediate the poly(rC) binding activity, with domain KH1 being the major RNA binding determinant (14, 19, 20). Domain KH2 is important for dimerization of the protein (21). Additionally, domain KH3 has been shown to be an important domain for the binding of PCBP2 to stem-loop IV to form the RNP complex necessary for translation (14). The KH3 domain is also required for binding to cellular protein SRp20, forming a complex that is proposed to play a role in the recruitment of translation initiation factors and the ribosome for viral translation (22). SRp20 belongs to the SR protein family and plays a role in host cell mRNA splicing and nucleo-cytoplasmic RNA export (23, 24). The function of SRp20 is usurped to benefit the virus by helping mediate efficient IRES-dependent translation (22).

Between domains KH2 and KH3, there is a linker region that has been shown to be an important determinant for binding of PCBP2 to stem-loop IV of poliovirus RNA (25). Previous studies have shown that PCBP2 is cleaved by the viral proteinase 3CD in the linker region between domains KH2 and KH3, removing the third KH domain. PCBP2 that lacks domain KH3 is unable to bind to stem-loop IV, leading to the inhibition of viral translation. However, the cleaved form of PCBP2 is still able to bind to stem-loop I to form a functional ternary complex with 3CD proteinase and thus remains active in RNA replication (26). Since RNA synthesis occurs in replication complexes, cleaved PCBP2 would be concentrated in these specified complexes, rather than occurring throughout the cell, to allow a switch to RNA replication. PCBP1, an isoform of PCBP, is thought to be generated from a retrotransposition of a fully processed PCBP2 mRNA and differs in the length of the linker region between domains KH2 and KH3 (25, 27). Previous work has shown that PCBP1 is able to interact with stem-loop I of the 5' NCR but cannot bind to stem-loop IV (14, 25). Additionally, when recombinant PCBP1 is incubated with purified poliovirus 3C or 3CD, the proteolysis of PCBP1 by 3CD proteinase occurs with an efficiency similar to that of PCBP2 cleavage (26). While PCBP1 can rescue RNA replication, it is not needed for viral translation as it cannot bind efficiently to stem-loop IV and is unable to rescue translation *in vitro* (14). Therefore, the focus of this paper is on PCBP2.

PCBP2, unlike PCBP1, is involved in both translation and RNA replication. We hypothesize that the cleavage of PCBP2 by viral proteinase 3CD helps, in part, to mediate the switch in template usage from translation to RNA replication. Cleavage of PCBP2 precludes stem-loop IV binding and, as this cleaved form is unable to bind stem-loop IV, inhibits translation, allowing the template to be cleared of ribosomes. The cleaved form of PCBP2 is still able to bind to stem-loop I and form a ternary complex with

3CD proteinase, initiating negative-strand RNA synthesis on the newly cleared template. In this paper, we describe experiments to test our hypothesis about the role of 3CD-mediated cleavage of PCBP2 in poliovirus replication. We report the generation of an uncleavable form of PCBP2 that is able to bind to both stem-loop I and stem-loop IV and function in translation. This genetically altered form of PCBP2 was used to demonstrate that cleavage of PCBP2 is important for poliovirus RNA replication.

MATERIALS AND METHODS

Infection of HeLa cells and lysate preparation. HeLa cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% newborn calf serum (NCS). Cells were infected with poliovirus or coxsackievirus B3 (CVB3) at a multiplicity of infection (MOI) of 20, and adsorption was carried out at room temperature for 30 min. DMEM with 8% NCS was added, and the cells were incubated at 37°C until the indicated time points. At specific times postinfection (0 to 7 h), cells were washed twice with phosphate-buffered saline (PBS) before the cells were scraped and collected to generate whole-cell lysate. Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0], 150 mM NaCl, 0.5% NP-40) on ice for 30 min. After incubation on ice, cell debris was pelleted, the resulting supernatant was collected, and total protein concentration of the whole-cell lysate was determined by Bradford assay. Total protein (100 µg) from lysates was analyzed by Western blotting.

Western blot analysis. Following SDS-PAGE, proteins were electroblotted to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in 5% milk in PBS with 0.1% Tween 20 for 1 h at room temperature. Mouse monoclonal antibody against PCBP2 (1:2,000 in PBS with 0.1% Tween 20), as described by Sean and coworkers, was used to detect full-length and cleaved forms of PCBP2 (25). The PVDF membrane was incubated with anti-PCBP2 antibody (1:2,000) for 1 h, followed by incubation with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) (Millipore) for 1 h. Protein bands were visualized using chemiluminescence (Thermo Scientific).

HRV16 3CD construct. Synthetic oligonucleotide primers used were as follows: HRV16-3CD-Nde(+) (5'-AGCCATATGGGTCCAGAAGAATTT-3'), HRV16-3CD-BamHI(-) (5'-GCCGGATCCCTATTAAGAATTTTCATACATT-3'), HRV16-3CDµ10(+) (5'-ATCATACTCACTGAACAAGCAGCCCAAATTCAAATCTCTAAAC-3'), HRV16-3CDµ10(-) (5'-GTTTAGAGATTTGAATTTGGGCTGCTTGTTTCAGTGAAGTATGAT-3'). Synthetic oligonucleotide primers HRV16-3CD-Nde(+) and HRV16-3CD-BamHI(-) were used to generate PCR fragments containing HRV type 16 (HRV16) amino acids 5155 to 7085, flanked by NdeI or BamHI upstream or downstream, respectively. The restriction sites were then used to clone HRV16 3CD into the pET15b vector through ligation. Site-directed mutagenesis of the newly synthesized pET15b HRV16 3CD plasmid was then used as a template for site-directed mutagenesis to alter the 3C/3D self-cleavage site such that recombinant 3CD could not be self-cleaved into 3C and 3D. The synthetic oligonucleotide primers used were HRV16-3CDµ10(+) and the reverse complement, HRV16-3CDµ10(-). The resulting plasmid, pET15b HRV16 3CDµ10, was used for protein expression.

Protein purification. Purification of hexa-histidine-tagged pET22b PCBP2, wild-type PCBP2, or uncleavable PCBP2 (QS→ID) (i.e., an uncleavable form of the PCBP2 protein with the glutamine-serine changed to an isoleucine-aspartic acid), was carried out as previously described (17, 26). Briefly, PCBP2 was expressed in BL21(DE3) (Rosetta) cells. The cells were grown at 37°C to an absorbance at 600 nm (A_{600}) of 0.6, and then protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) for 3 h at 25°C. Cells were resuspended in lysozyme buffer without lysozyme (10 mM EDTA, 25 mM Tris-HCl [pH 8.0], 4.5% glucose) and lysed by sonication for five cycles consisting of 15 s followed by 1 min on ice. The soluble fraction following sonication was precipitated with ammonium sulfate (20% [wt/vol]). The resulting pellet

fraction was dialyzed overnight in I-60 buffer (20 mM Tris-HCl [pH 8.0], 250 mM NaCl, 60 mM imidazole, 10% glycerol) with 0.5% NP-40. The fraction was then subjected to Ni²⁺ ion-based affinity chromatography (GE Healthcare). The column was washed with I-60 buffer, and the protein was eluted with I-200 buffer (20 mM Tris-HCl [pH 8.0], 250 mM NaCl, 200 mM imidazole, 10% glycerol). Hexa-histidine-tagged 3CD viral proteinase (poliovirus or HRV16) was purified as previously described (17, 28), with a few modifications. Briefly, 3CD was expressed in BL21(DE3) (Rosetta) cells, and the cells were grown at 37°C to an A₆₀₀ of 0.4. Recombinant protein expression was induced by the addition of IPTG (1 mM) for 3 h at 25°C. Cells were resuspended in buffer A (20 mM Tris-HCl [pH 8.0], 25 mM NaCl, 5% glycerol, 1 mM dithiothreitol [DTT]) and lysed by one passage through a French pressure system (Amicon) at 8,000 lb/in². The lysate was then subjected to centrifugation for 20 min at 13,800 × g using a Beckman Coulter centrifuge and JA-17 rotor, and the resulting supernatant was discarded. The pellet was washed three times in buffer A, omitting DTT for the last wash. To extract protein from the pellet fraction, the pellet was resuspended in high-salt I-30 buffer (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 30 mM imidazole, 10% glycerol) and incubated on ice for 30 min. The resuspended pellet was then subjected to centrifugation for 20 min at 13,800 × g, as described above. The resulting supernatant was subjected to Ni²⁺ ion-based affinity chromatography (GE Healthcare). The column was washed with high-salt I-30 buffer, and the protein was eluted with high-salt I-200 buffer (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 200 mM imidazole, 10% glycerol).

PCBP2 *in vitro* cleavage assays. The PCBP2 *in vitro* cleavage assays were carried out as previously described (26), with some minor modifications. Briefly, recombinant purified PCBP2 and 3CD proteinase were incubated at a 1:1 molar ratio in cleavage buffer (20 mM HEPES [pH 7.4], 1 mM DTT, 150 mM potassium acetate [KOAc]) for 4 h at 30°C. The cleavage products were analyzed by SDS-PAGE and Western blotting.

HeLa cell S10 preparation. Preparation of HeLa cell S10 cytoplasmic extract was carried out by following the procedure described previously (14). Depletion of PCBP from HeLa cell S10 cytoplasmic extracts using a poly(rC) column was performed as previously described (29). To sequester PCBP2 from S10 cytoplasmic extracts, 400 nM poly(rC) RNA (Thermo Scientific) was incubated with HeLa cell S10 cytoplasmic extract for 30 min at 30°C.

***In vitro* translation and RNA replication.** The coupled *in vitro* translation and RNA replication assays were carried out essentially as described previously (14), with some alterations. Briefly, the translation-replication mixture (50 μl) contained 60% HeLa S10 cells (by volume), 250 ng poliovirus virion RNA, and all-4 buffer lacking CTP (1 mM ATP [Pharmacia Biotech], 0.25 mM GTP, 0.25 mM, 30 mM creatine phosphate, 60 mM KOAc, 20 mM HEPES [pH 7.4], 4 mg/ml creatine kinase). The negative control contained 2 mM guanidine hydrochloride (GuHCl), which is known to inhibit poliovirus negative-strand RNA synthesis (30, 31). Poly(rC) and recombinant wild-type PCBP2 or PCBP2 (QS→ID) were added to the indicated concentrations (see Fig. 5 and 6). Following poly(rC) RNA incubation with HeLa cell cytoplasmic extract, virion RNA, buffer, and recombinant protein were added to the reaction mixture. The reaction mixtures were then split into two portions, with 10 μl for translation and the remaining 40 μl for RNA replication. Ten μCi [³⁵S]methionine (PerkinElmer) was added to the translation reaction, and it was incubated for 4 h at 30°C, supplemented with 2× Laemmli sample buffer (LSB), boiled, and subjected to SDS-PAGE followed by fluorography. The replication mixture was incubated at 30°C for 4 h before 25 μCi [³²P]CTP (PerkinElmer) was added, and the reaction mixture was incubated for an additional 2 h at 34°C. The RNA replication product was then purified using an RNeasy kit (Qiagen) and separated on a 1.1% agarose gel. Images of both translation and RNA replication were based on scans of phosphor screens performed using a Personal Molecular Imager FX (Bio-Rad) and analyzed with Quantity One software (Bio-Rad). Quantity One software was used to determine the optical density per mm² for the band of interest. The VP3 band was analyzed for

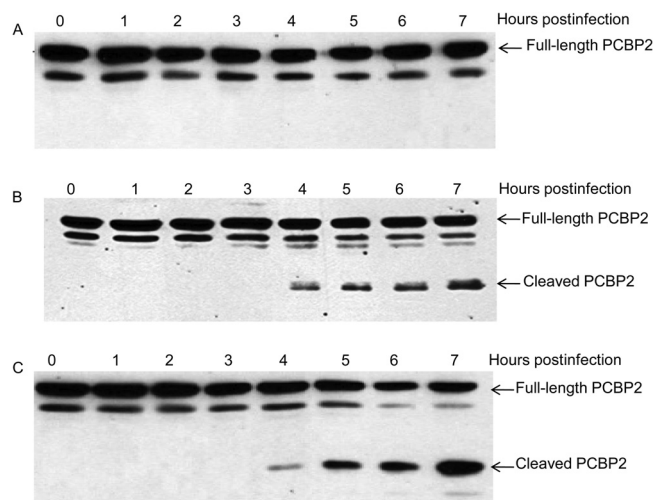


FIG 1 Cleavage of PCBP2 during poliovirus and coxsackievirus infection. HeLa cells were mock infected (A), infected with poliovirus (B), or infected with coxsackievirus B3 (C) at an MOI of 20. NP-40 lysate was collected from 0 to 7 h postinfection, and equal amounts of total protein were analyzed for PCBP2 cleavage by Western blotting, probing with a monoclonal antibody against PCBP2. Arrows to the right of the gel image indicate full-length and cleaved PCBP2.

translation, and the single-stranded RNA (ssRNA) band was evaluated for RNA synthesis. The fold change with the addition of wild-type PCBP2 or PCBP2 (QS→ID) was determined as a ratio relative to the optical density per mm² for poly(rC), with poly(rC) set to 1.

Electrophoretic mobility shift assays. Mobility shift assays were carried out by following previously described methods (14, 32). Briefly, [³²P]UTP-labeled RNA corresponding to the stem-loop I or stem-loop IV probe was transcribed from linearized plasmid. Increasing amounts of recombinant PCBP2 and/or 3CD proteinase were incubated in RNA binding buffer (25 mM KCl, 0.05 mM HEPES-KOH [pH 7.4], 2 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT, 3.7% glycerol [vol/vol]), with 1 mg/ml tRNA, 0.5 mg/ml bovine serum albumin (BSA; New England Biolabs), 8 U RNasin (Promega), and diluted probe (0.1 nM final concentration for the 10-μl reaction). After incubating for 10 min at 30°C, 50% glycerol was added for a 12.5% final concentration and the reaction mixtures were subjected to nondenaturing electrophoresis on a 5% polyacrylamide gel containing glycerol in 0.5× Tris-borate-EDTA (TBE). Images were generated from scans of phosphor screens performed using a Personal Molecular Imager FX and analyzed with Quantity One software.

RESULTS

Cleavage of PCBP2 during poliovirus and coxsackievirus infection. Previous studies have shown that PCBP2 cleavage products accumulate over the course of poliovirus infection (26). To determine if PCBP2 cleavage could be observed in cells infected by other picornaviruses, HeLa cell lysates were collected over the course of infection by poliovirus or coxsackievirus, and PCBP2 cleavage was analyzed by Western blotting, probing with a monoclonal antibody that is specific for PCBP2. During mock infection, only full-length PCBP2 was detected, migrating around 39 kDa (Fig. 1A). There is also a faster-migrating PCBP2-related species that could be an alternative form of PCBP2, perhaps with different posttranslational modifications. During either poliovirus (Fig. 1B) or coxsackievirus B3 (Fig. 1C) infection, cleaved PCBP2 is detected by 4 h postinfection. We estimate that the cleavage product has a molecular mass of approximately 28 kDa and that it

represents PCBP2 lacking the KH3 domain. The appearance of cleaved PCBP2 at 4 h postinfection corresponds with an increase in titer, as determined by plaque assay, suggesting that PCBP2 cleavage may be important for more efficient RNA replication (data not shown). Therefore, these data suggest that PCBP2 cleavage is important for infection with both poliovirus and coxsackievirus.

PCBP2 cleavage by viral proteinase 3CD. Previous work has demonstrated that poliovirus 3CD proteinase is responsible for cleaving PCBP2 during infection and that while full-length PCBP2 can bind to stem-loop IV, cleaved PCBP2 cannot (26). PCBP2 is cleaved by the viral proteinase 3CD when bound to stem-loop IV or when unbound with a similar efficiency (data not shown) (33). This suggests that PCBP2 can be cleaved when bound to stem-loop IV, when bound to stem-loop I, or when unbound, leaving the question of how cleavage of PCBP2 exerts its effect on the viral RNA replication cycle.

To further characterize the importance of the observed cleavage during the course of viral infection, a PCBP2 construct with a cleavage site mutation was utilized. Previous studies have shown that poliovirus 3CD preferentially cleaves at glutamine-glycine sites, with an aliphatic residue in the P4 position (34, 35). 3CD also cleaves at glutamine-serine sites, as found in the linker region of PCBP2 (26). Therefore, an uncleavable form of the PCBP2 protein with a double-amino-acid substitution at the cleavage site within the linker region was generated by changing the glutamine-serine to an isoleucine-aspartic acid (QS→ID) at amino acid positions 253 and 254, respectively, as shown in Fig. 2A (26). This mutated form of PCBP2 was then utilized in a cleavage assay to ensure that it was unable to be cleaved by the viral proteinase 3CD. The ability of recombinant 3CD derived from poliovirus or HRV16, a closely related picornavirus, to cleave the purified, recombinant mutated PCBP2 (QS→ID) was tested in an *in vitro* cleavage assay. Recombinant wild-type PCBP2 or PCBP2 (QS→ID) was incubated with poliovirus or HRV16 3CD proteinase, and cleavage was analyzed by Western blotting. In the absence of viral proteinase, wild-type PCBP2 or PCBP2 (QS→ID) is not cleaved and the full-length 39-kDa band is observed (Fig. 2B, lanes 1 and 4, respectively). There is also a faster-migrating PCBP2 with an apparent approximate molecular mass of 35 kDa that is present with wild-type PCBP2 or PCBP2 (QS→ID) alone (lanes 1 and 4), suggesting that it could be a truncated form of PCBP2 produced during expression in *Escherichia coli*, as no posttranslational modifications could be occurring. This form of PCBP2 is present when incubated with poliovirus 3CD proteinase (lanes 2 and 5) but is absent in the presence of HRV16 3CD proteinase (lanes 3 and 6). It is known that HRV16 3CD proteinase has less stringent requirements for cleavage site recognition, suggesting that the altered form of PCBP2 has a secondary cleavage site that is recognized by HRV16 3CD but not by poliovirus 3CD (34). With the addition of either poliovirus or HRV16 3CD, the 28-kDa PCBP2 cleavage product is observed (Fig. 2B, lanes 2 and 3). However, when PCBP2 (QS→ID) was incubated with either poliovirus or HRV16 3CD proteinase, only full-length PCBP2 was observed (Fig. 2B, lanes 5 and 6). Our results demonstrate that both poliovirus and HRV16 3CD have the ability to cleave wild-type PCBP2 and that the mutated version of PCBP2 (QS→ID) is uncleavable by the viral proteinase 3CD.

Functional analysis of uncleavable PCBP2. To be functional in translation or RNA synthesis, PCBP2 (QS→ID) must efficiently

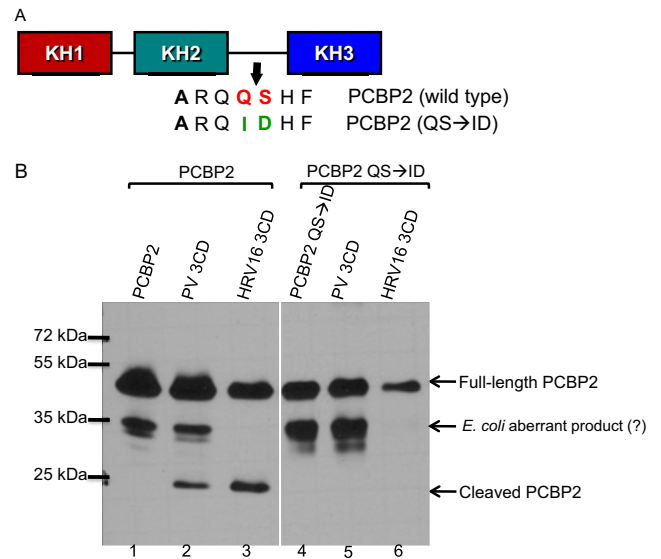


FIG 2 PCBP2 cleavage site mutations and cleavage by picornavirus proteinase 3CD. (A) To generate an uncleavable version of PCBP2 (QS→ID), the P1 and P1' positions at the cleavage site were mutated from the wild-type glutamine-serine (Q-S; red type) to an isoleucine-aspartic acid (I-D; green type). The K-homologous domains (KH1, KH2, and KH3) of PCBP2 are depicted as colored rectangles. (B) The cleavage of wild-type and mutated PCBP2 (2 μ M) was tested in an *in vitro* cleavage assay with recombinant poliovirus or HRV16 3CD (2 μ M) and analyzed by Western blotting, probing with a monoclonal antibody against PCBP2. Lane 1 is wild-type PCBP2, with added poliovirus and HRV16 3CD in lanes 2 and 3, respectively. Uncleavable PCBP2 (QS→ID) was incubated alone (lane 4), or with poliovirus (lane 5) or HRV16 (lane 6) 3CD. Arrows to the right of the gel image indicate full-length and cleaved PCBP2, and molecular mass markers are indicated on the left of the gel image. The unmarked species migrating around 35 kDa is an altered species of PCBP2 that can be recognized and cleaved by HRV16 3CD but not poliovirus 3CD.

form the RNP complexes required for translation or RNA replication. Therefore, the ability of PCBP2 (QS→ID) to bind stem-loop I and stem-loop IV RNA, which is required for RNA replication and translation, respectively, was tested. The ability of PCBP2 (QS→ID) to form an RNP complex with stem-loop IV was analyzed by incubating radiolabeled poliovirus stem-loop IV RNA with increasing amounts of recombinant wild-type PCBP2 (Fig. 3A, lanes 2 to 4) or PCBP2 (QS→ID) (Fig. 3A, lanes 5 to 7). As expected, wild-type PCBP2 could efficiently shift the electrophoretic mobility of stem-loop IV RNA, indicating RNP complex formation. PCBP2 (QS→ID) was also seen to bind to stem-loop IV RNA, as indicated by the shifted probe. The shifted complex observed with PCBP2 (QS→ID) had a slightly different electrophoretic mobility than the complex formed with wild-type PCBP2 (Fig. 3A, compare lanes 4 and 7). The slightly altered electrophoretic mobility could be due to altered folding of the mutated protein, contributing to a different migration pattern.

To analyze the ability of the uncleavable PCBP2 (QS→ID) to function in RNA replication and form a complex with stem-loop I, radiolabeled poliovirus stem-loop I RNA was incubated with recombinant wild-type PCBP2 or PCBP2 (QS→ID). An electrophoretic mobility shift was observed with both the wild-type or uncleavable forms of PCBP2, indicating RNP complex formation (Fig. 3B, lanes 3 and 4 and lanes 7 and 8, respectively). Increasing amounts of recombinant poliovirus 3CD were then added to analyze ternary complex formation, which is required for initiation

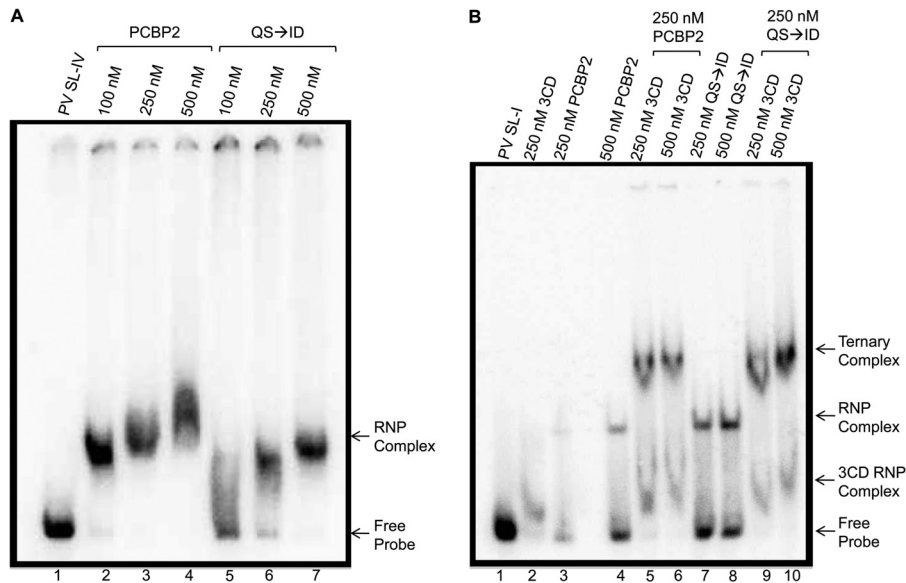


FIG 3 RNA binding of cleavage-resistant PCBP2. Radiolabeled poliovirus stem-loop RNA was used in mobility shift assays to determine the ability of the cleavage-resistant PCBP2 to bind to stem-loop I or IV derived from the 5' NCR. (A) Radiolabeled poliovirus stem-loop IV (SL-IV) was incubated with increasing amounts of PCBP2 (lanes 2 to 4) or uncleavable PCBP2 (QS→ID) (lanes 5 to 7). (B) Radiolabeled poliovirus stem-loop I (SL-I) was incubated with poliovirus 3CD alone (lane 2) or with PCBP2 alone (lanes 3 and 4) or PCBP2 (QS→ID) alone (lanes 7 and 8). To analyze ternary complex formation, increasing amounts of poliovirus 3CD were added to wild-type (lanes 5 and 6) or uncleavable (lanes 9 and 10) PCBP2. The arrows to the right of the gel image indicate probe alone (Free Probe), RNP complex formation, and ternary complex formation.

of negative-strand RNA synthesis. A supershifted product, indicating ternary complex formation, was observed with the PCBP2 wild type (Fig. 3B, lanes 5 and 6) and with uncleavable PCBP2 (QS→ID) (Fig. 3B, lanes 9 and 10). Importantly, the two forms of the protein form a ternary complex with a similar affinity. Therefore, the uncleavable form of PCBP2 was able to form an RNP complex with stem-loop IV and a ternary complex with stem-loop I and 3CD proteinase. This suggests that uncleavable PCBP2 can form the complexes necessary for both viral translation and RNA replication.

To analyze the ability of uncleavable PCBP2 (QS→ID) to rescue IRES-dependent translation, HeLa cell cytoplasmic extracts were depleted of PCBP by a poly(rC) affinity column (29). Translation experiments were carried out utilizing purified poliovirus virion RNA and [³⁵S]methionine labeling of the synthesized viral proteins (Fig. 4). As a positive control for the level of viral translation, HeLa cell cytoplasmic extract was mock depleted (lane 1), and a high level of viral translation was observed. When HeLa cytoplasmic extracts were PCBP depleted, the level of viral translation was significantly decreased (lane 2). As expected, the addition of wild-type PCBP2 was able to rescue translation in PCBP-depleted HeLa cytoplasmic extracts to near wild-type levels of translation (lanes 3 and 4). In agreement with the ability of PCBP2 (QS→ID) to bind to stem-loop IV, uncleavable PCBP2 was able to rescue translation in PCBP-depleted HeLa cytoplasmic extracts (Fig. 4, lanes 5 and 6). From these experiments, we conclude that the uncleavable version of PCBP2 is able to function similarly to wild-type PCBP2 in poliovirus translation, as shown with both *in vitro* translation assays and mobility shift assays with stem-loop IV. Additionally, we predict that PCBP2 (QS→ID) is functional in viral RNA replication because it is able to bind stem-loop I and form a ternary complex with an affinity similar to that of wild-type PCBP2.

***In vitro* translation and RNA replication in the presence of wild-type or uncleavable PCBP2.** Since the uncleavable PCBP2 (QS→ID) can function in both translation and RNA replication, the mutated protein can be used to address what role PCBP2 cleavage plays in the poliovirus replication cycle. To answer this question, an *in vitro* translation/RNA replication assay was utilized. To circumvent potential complications inherent with depleting cytoplasmic extracts of PCBP, HeLa cell S10 cytoplasmic extract was incubated with poly(rC) RNA to bind and sequester PCBP2 present in the extract, thereby inhibiting translation. Following the preincubation, recombinant PCBP2 or uncleavable PCBP2 (QS→ID) was added in increasing amounts, along with poliovirus virion RNA. Translation was analyzed by the incorporation of [³⁵S]methionine to label synthesized viral proteins. As shown in Fig. 5A, translation of poliovirus virion RNA (lane 1) produces viral proteins, both mature and precursor forms, labeled by the incorporation of [³⁵S]methionine. In the presence of poly(rC) RNA, levels of translation are decreased (lane 3) as expected because PCBP2 is sequestered in the cytoplasmic extract and unable to form an RNP complex with stem-loop IV. With the addition of either wild-type PCBP2 (lanes 4 to 6) or uncleavable PCBP2 (QS→ID) (lanes 7 to 9), levels of translation are increased above translation levels with poly(rC) alone. Additionally, the levels of translation with added wild-type PCBP2 or PCBP2 (QS→ID) are similar, as seen by the equal amounts of viral proteins synthesized. Translation can be quantified and compared based on levels of viral proteins produced, such as the structural protein VP3 (Fig. 5A; quantified in Fig. 5B). The data shown are representative of results of two independent experiments. Normalization of translation levels with the addition of recombinant protein to levels of translation with poly(rC) RNA, but without added protein, allows a more direct comparison of levels of viral translation (Fig. 5B). With the addition of increasing amounts of

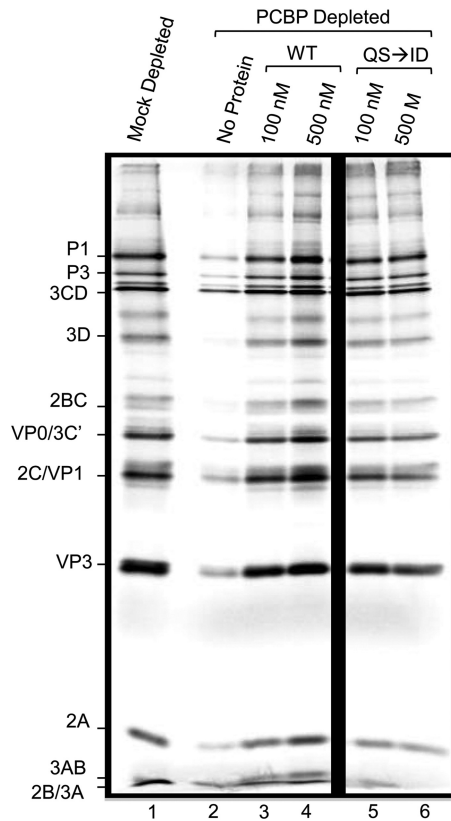


FIG 4 Functional analysis of uncleavable PCBP2 (QS→ID) during poliovirus translation. Poliovirus virion RNA was translated *in vitro* in PCBP-depleted HeLa cytoplasmic extracts in the presence of [³⁵S]methionine. Poliovirus proteins were resolved by SDS-PAGE and detected by autoradiography. Translation reactions were carried out in mock-depleted (lane 1) or PCBP-depleted (lanes 2 to 6) HeLa cytoplasmic extracts. No added recombinant protein (lane 2), 100 nM or 500 nM recombinant wild-type PCBP2 (lanes 3 and 4), and 100 nM or 500 nM purified recombinant PCBP2 (QS→ID) (lanes 5 and 6) were analyzed for rescue of viral translation levels. Viral proteins are indicated to the left of the gel. WT, wild type.

PCBP2, either the wild type or PCBP2 (QS→ID), the levels of translation were enhanced above levels seen with poly(rC) depletion of PCBP. Importantly, levels of translation appear to be similar with the addition of either wild-type PCBP2 or PCBP2 (QS→ID), indicating that there are similar levels of viral proteins present to be used for RNA synthesis, allowing for a direct comparison of the levels of negative-strand RNA synthesis.

RNA replication was analyzed by the incorporation of [³²P]CMP into newly synthesized single-stranded RNA or the partially double-stranded replicative intermediate. Labeled RNAs produced *in vitro* were analyzed on an agarose gel, and the ethidium bromide-stained gel was utilized to indicate levels of rRNA as a loading control (Fig. 6A, right panel). Negative-strand RNA synthesis depends on levels of newly synthesized viral proteins, and RNA replication will be less efficient if there are fewer viral proteins synthesized. Since there are equivalent levels of rRNA (Fig. 6A, right panel) and newly synthesized viral proteins (Fig. 5), RNA replication can be directly compared in the presence of wild-type PCBP2 or uncleavable PCBP2 (QS→ID). As seen in Fig. 6A (left panel), poliovirus virion RNA replicates to a high level in mock-sequestered extracts, as indicated by the single-stranded

RNA band and the partially double-stranded replicative intermediate (RI/RF) band (lane 1). In the presence of guanidine hydrochloride (GuHCl), there is no RNA replication (lane 2), as GuHCl inhibits negative-strand RNA synthesis (30, 31). In the presence of poly(rC) RNA (lane 3), levels of RNA replication are significantly reduced, as expected, due to both the decreased levels of translation and the absence of available PCBP2 for ternary complex formation. However, with the addition of wild-type PCBP2 (lanes 7 to 9), there is a rescue of RNA replication that is not observed when uncleavable PCBP2 (QS→ID) is added (lanes 4 to 6). To directly compare levels of RNA synthesis with added wild-type PCBP2 or PCBP2 (QS→ID), the ssRNA band was quantified and relative levels of RNA synthesis were normalized to the level of RNA replication with poly(rC) RNA in the absence of added protein (Fig. 6B). The data shown are representative of results of two independent experiments. Taken together, these data show that there is a pronounced rescue of RNA replication with the addition of wild-type PCBP2 compared to uncleavable PCBP2 (QS→ID). Therefore, the data suggest that the cleavage of PCBP2 is important for efficient poliovirus RNA replication.

DISCUSSION

The picornavirus growth and replication cycle utilizes the genomic RNA as a template for both translation and RNA replication. Translation necessarily precedes RNA replication because several viral proteins are required for initiation and elongation of RNA synthesis. However, it has been shown that a template that is actively being translated cannot function as a template for RNA replication, indicating a switch in template usage from translation to RNA replication (4, 5). Host proteins, including PCBP2, mediate viral translation by binding to stem-loop IV of the IRES (11–14). PCBP2 is also involved in RNA replication and binds to stem-loop I of the 5' NCR to form a ternary complex with 3CD proteinase that is needed for initiation of negative-strand RNA synthesis (13, 15–17). Previously, Perera and coworkers showed that PCBP2 is cleaved by the viral proteinase 3CD during the course of poliovirus infection (26). This cleavage event removes the KH3 domain of the protein, disrupting the interaction with stem-loop IV, thereby inhibiting translation initiation. The cleaved version of PCBP2 can also no longer interact with SRp20, further inhibiting translation by disrupting ribosome recruitment. However, the shortened form of PCBP2 is functional in RNA replication, as it can efficiently bind to stem-loop I to form the ternary complex during negative-strand RNA synthesis initiation (26). Therefore, the cleavage of PCBP2 could, in part, mediate a switch in template usage from translation to RNA replication.

PCBP2 cleavage occurs in HeLa cells during infection by poliovirus or coxsackievirus, two closely related picornaviruses (Fig. 1). *In vitro* cleavage assays further show that poliovirus 3CD, as well as HRV16 3CD proteinase, can cleave PCBP2 (Fig. 2). Knowing that PCBP2 can be cleaved by the 3CD proteinase of several different picornaviruses raises key questions: is the cleavage of PCBP2 important for efficient RNA replication and production of progeny virus, and is it involved in the switch in template usage? To understand how PCBP2 functions in mediating a possible switch in template usage, an uncleavable version of PCBP2 (QS→ID) was utilized that retained the ability to function like wild-type PCBP2 in binding to stem-loop IV or stem-loop I, forming a ternary complex with stem-loop I and 3CD, and also to

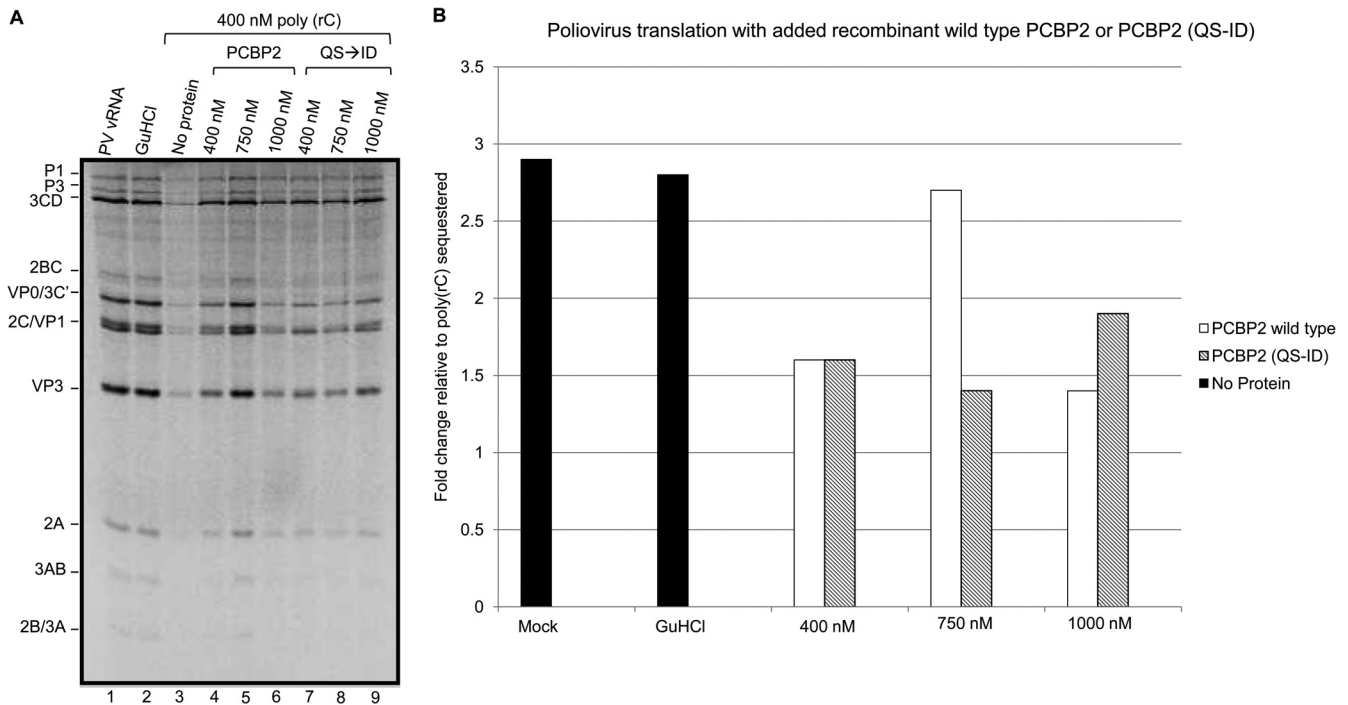


FIG 5 *In vitro* translation of poliovirus RNA in the presence of wild-type or uncleavable PCBP2. HeLa cytoplasmic extract was preincubated with poly(rC) RNA to bind and sequester PCBP2. Poliovirus virion RNA was then added with or without increasing amounts of recombinant wild-type PCBP2 or uncleavable PCBP2 (QS→ID). (A) Translation of [³⁵S]methionine-labeled viral proteins was analyzed by SDS-PAGE. Viral proteins are indicated to the left of the gel image. Lane 1 is the positive control with cytoplasmic extract mock depleted and incubated with poliovirus virion RNA (vRNA). The addition of guanidine hydrochloride (GuHCl) in lane 2 has no effect on translation. Poly(rC) is added in lanes 3 through 9, with increasing concentrations of added PCBP2 wild type (lanes 4 to 6) or uncleavable PCBP2 (QS→ID) (lanes 7 to 9). (B) Quantitation of viral translation was performed using Quantity One software to determine the concentration of the VP3 band (optical density per mm²). A ratio of the determined optical density with the addition of wild-type PCBP2 (white) or PCBP2 (QS→ID) (hatched) to the optical density for poly(rC) was calculated to determine the fold change. The positive controls indicating levels of translation in the absence of poly(rC) RNA or recombinant protein (no protein) are depicted as black columns. Results shown are representative of two independent experiments.

retain the ability to rescue translation similarly to wild-type PCBP2 (as shown in Fig. 3 and 4). Therefore, the importance of PCBP2 cleavage on RNA replication could be analyzed utilizing this uncleavable form of PCBP2. An *in vitro* translation and RNA replication assay showed that when poly(rC) RNA sequestered PCBP2 present in the cytoplasmic extract, translation, and therefore RNA replication, was decreased, as expected. When recombinant wild-type PCBP2 or PCBP2 (QS→ID) was added following PCBP2 sequestration with poly(rC) RNA, translation levels were increased relative to translation with just poly(rC) RNA (Fig. 5). Importantly, levels of translation were similar with added wild-type PCBP2 or PCBP2 (QS→ID), allowing for a direct comparison of levels of negative-strand RNA synthesis. In addition, recombinant PCBP2 (QS→ID) bound stem-loop I RNA with an affinity similar to that of wild-type PCBP2, further indicating that the two proteins could potentially function in RNA replication with a similar efficiency (Fig. 3B). Therefore, we concluded that the significantly lower level of viral RNA synthesis observed with added PCBP2 (QS→ID) than with wild-type PCBP2 was evidence that RNA replication could not be rescued by the addition of uncleavable PCBP2 (Fig. 6).

Our results suggest that PCBP2 cleavage is important for efficient poliovirus RNA replication. Although we have not provided direct evidence, our data support a role for this cleavage in facilitating a switch in template usage from translation to RNA replication. We propose an updated model in which full-length PCBP2

binds to stem-loop IV to form an RNP complex necessary for initiation of viral translation (Fig. 7). PCBP2 also binds to cellular protein SRp20 to form a complex that has been proposed to help recruit ribosomes for translation (22). In addition, host protein PTB is known to bind to stem-loop IV and stimulate poliovirus translation (7–10). As translation of the polyprotein proceeds, the concentration of 3CD proteinase, along with that of other viral proteins, increases and the concentration of cleaved PCBP2 accumulates. Also, PTB is cleaved by 3C proteinase, which has also been proposed to play a role in mediating a switch in template usage from translation to RNA replication (9, 36). Although not shown as a cleaved polypeptide in Fig. 7, host cell poly(A) binding protein (PABP) has also been shown to be cleaved by poliovirus 3C or 3CD proteinase, contributing to the shutdown of cap-independent viral translation (37). With the increased concentration of viral proteins, RNA replication complexes are formed, leading to cytoplasmic foci with higher concentrations of cleaved PCBP2 than of full-length PCBP2. Under these conditions, cleaved PCBP2 no longer binds to stem-loop IV or SRp20 and translation initiation is inhibited, allowing the template to be cleared of translating ribosomes. The cleaved PCBP2 is instead bound to stem-loop I, along with 3CD, to form the ternary complex necessary for initiation of negative-strand RNA synthesis.

The switch from translation to RNA replication is a necessary part of the poliovirus replication cycle. As shown in Fig. 1, PCBP2 is also cleaved during the course of coxsackievirus infection. In

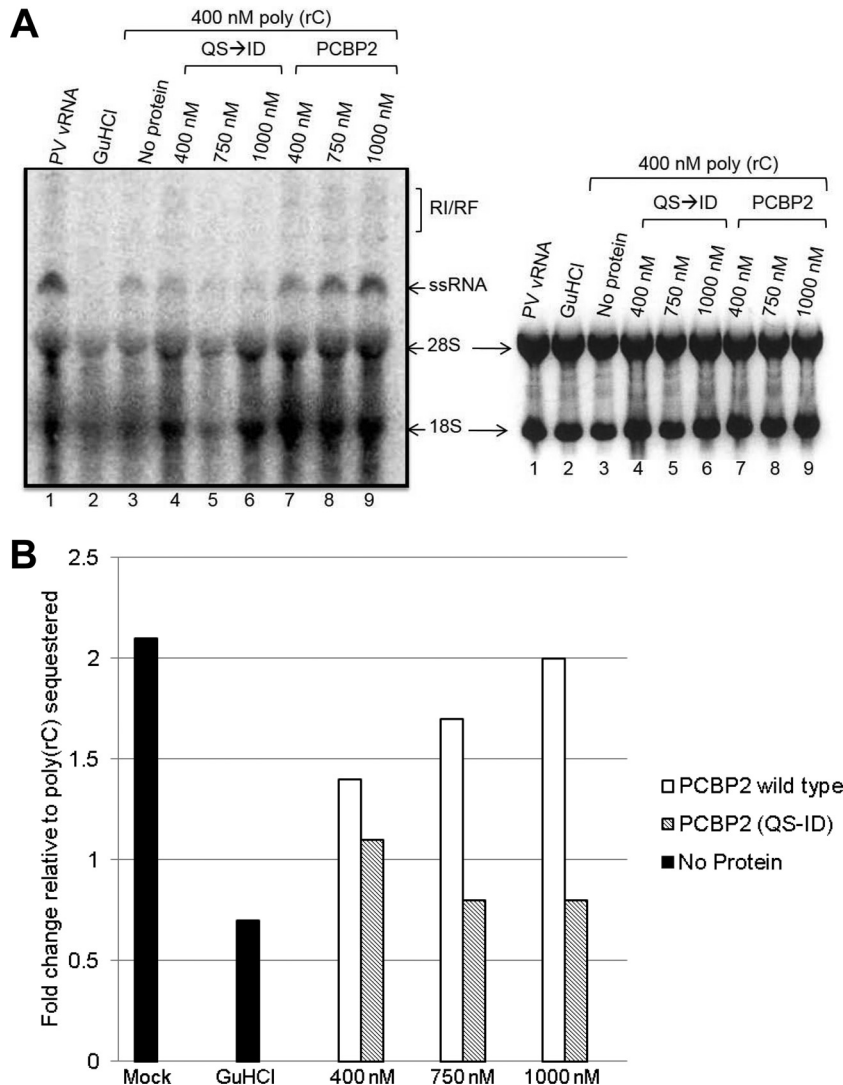


FIG 6 *In vitro* poliovirus RNA replication in the presence of added wild-type or uncleavable PCBP2. As in Fig. 5, HeLa cytoplasmic extract was preincubated with poly(rC) RNA to bind and sequester PCBP2. Poliovirus virion RNA was then added with or without increasing amounts of recombinant PCBP2 or uncleavable PCBP2 (QS→ID). (A) RNA replication was analyzed by incorporation of [³²P]CMP and the presence of labeled ssRNA and the partially double-stranded RI/RF intermediate, as indicated to the right of the gel image, following agarose gel electrophoresis. Lane 1 is the positive control for RNA synthesis with poliovirus virion RNA (vRNA) and no poly(rC) RNA. The addition of guanidine hydrochloride (GuHCl) in lane 2 is a negative control for RNA synthesis. Poly(rC) is added in lanes 3 through 9, with increasing concentrations of added PCBP2 wild type (lanes 7 to 9) or uncleavable PCBP2 (QS→ID) (lanes 4 to 6). The left panel analyzes ssRNA and the RI/RF intermediate based on scans of phosphor screens using Personal Molecular Imager FC. The right panel depicts the ethidium bromide stained agarose gel to analyze levels of rRNA. (B) Quantitation of viral RNA synthesis was analyzed using Quantity One software to determine the concentration of the ssRNA band (optical density per mm²). The fold change with added recombinant protein was then determined as a ratio of the determined optical density when wild-type PCBP2 (white) or PCBP2 (QS→ID) (hatched) was added to the optical density for poly(rC). Positive and negative controls in the absence of poly(rC) RNA or recombinant protein (no protein) are depicted in black. Results shown in the figure are representative of those of two independent experiments.

addition, PCBP2 has been shown to be cleaved during human rhinovirus infection of HeLa cells (41), suggesting that PCBP2 cleavage could be a mechanism for template switching utilized by multiple picornaviruses. However, our recent data suggest that a switch in template usage during human rhinovirus infection is mediated by a different mechanism (Chase and Semler, submitted). Thus, there may be redundant functions involved in mediating the switch in template usage for picornaviruses. PCBP2 is a known IRES *trans*-acting factor (ITAF) involved in both RNA replication and translation, but other ITAFs involved only in enhancing translation could also be important in mediating the

switch. PTB is an ITAF that is known to enhance translation and has also been shown to be cleaved by poliovirus 3C proteinase during infection, suggesting that this protein could also be involved in mediating a switch in template usage (36, 38). Interestingly, the La autoantigen (the first ITAF discovered for poliovirus [39]) is cleaved during poliovirus infection; however, the truncated form is still able to stimulate poliovirus translation in rabbit reticulocyte lysates (40). Further work is necessary to elucidate which other mechanisms, if any, could be involved in mediating the switch in template usage and to determine if those mechanisms act synergistically or redundantly. Additional studies also

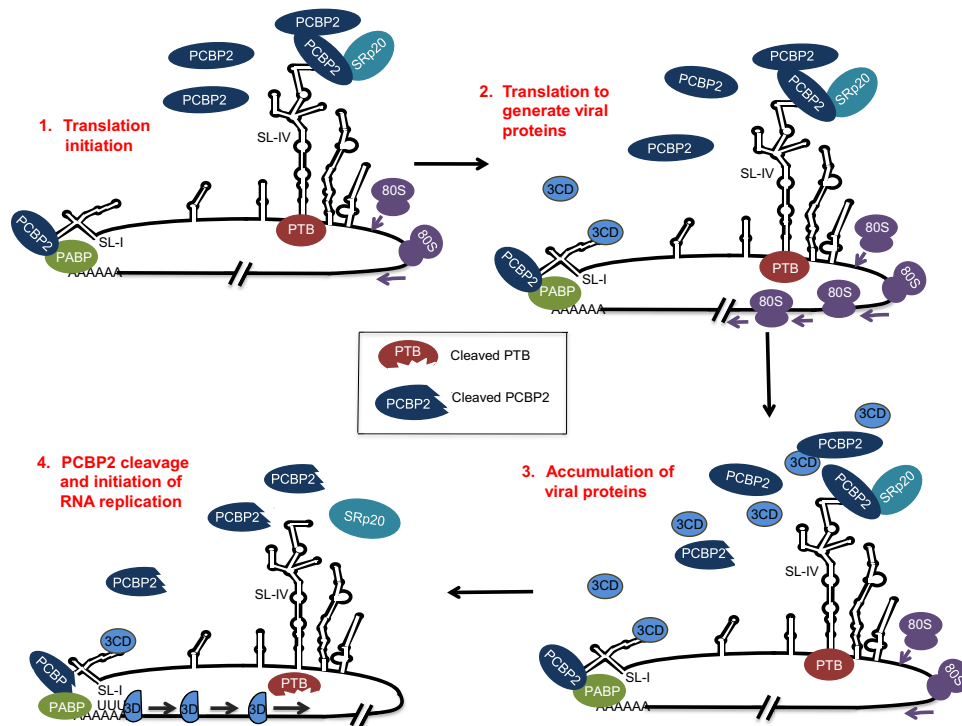


FIG 7 Template switching model for poliovirus replication. The switch in template usage based on the cleavage of PCBP2 is described (1). During translation, full-length PCBP2 is bound to stem-loop IV, forming an RNP complex to allow translation. PTB binds to the base of stem-loop IV to stimulate viral translation, and SRp20 forms a complex with PCBP2 on stem-loop IV that is also necessary for translation (2). Translation and processing of the polyprotein produces viral proteins, such as 3CD proteinase (3). As translation proceeds, the concentration of 3CD increases, leading to cleavage of PCBP2 (4). As the concentration of cleaved PCBP2 increases, PCBP2 no longer binds to stem-loop IV and no longer forms a complex with SRp20. Therefore, translation is inhibited and the template is cleared of translating ribosomes. Cleaved PCBP2 can still bind to stem-loop I and forms a functional ternary complex with 3CD viral proteinase, allowing initiation of RNA synthesis and elongation by the viral 3D polymerase. Poly(A) binding protein (PABP) is also depicted in the model. Although not shown as a cleaved polypeptide in the figure, PABP has been shown to be cleaved by poliovirus 3C/3CD proteinase, contributing to the shutdown of cap-independent viral translation (37).

need to be carried out to elucidate whether the cleavage of PCBP2 by 3CD proteinase to mediate a switch in template usage is a mechanism unique to poliovirus or is a mechanism utilized by other picornaviruses.

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