9-2011

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JOURNAL OF VIROLOGY, Sept. 2011, p. 9459–9471 Vol. 85, No. 18
0022-538X/11/$12.00 doi:10.1128/JVI.05179-11

Antagonistic Effects of Cellular Poly(C) Binding Proteins on Vesicular Stomatitis Virus Gene Expression▽

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Received 20 May 2011/Accepted 29 June 2011

Immunoprecipitation and subsequent mass spectrometry analysis of the cellular proteins from cells expressing the vesicular stomatitis virus (VSV) P protein identified the poly(C) binding protein 2 (PCBP2) as one of the P protein-interacting proteins. To investigate the role of PCBP2 in the viral life cycle, we examined the effects of depletion or overexpression of this protein on VSV growth. Small interfering RNA-mediated silencing of PCBP2 promoted VSV replication. Conversely, overexpression of PCBP2 in transfected cells suppressed VSV growth. Further studies revealed that PCBP2 negatively regulates overall viral mRNA accumulation and subsequent genome replication. Coimmunoprecipitation and immunofluorescence microscopic studies showed that PCBP2 interacts and colocalizes with VSV P protein in virus-infected cells. The P-PCBP2 interaction did not result in reduced levels of protein complex formation with the viral N and L proteins, nor did it induce degradation of the P protein. In addition, PCBP1, another member of the poly(C) binding protein family with homology to PCBP2, was also found to interact with the P protein and inhibit the viral mRNA synthesis at the level of primary transcription without affecting secondary transcription or genome replication. The inhibitory effects of PCBP1 on VSV replication were less pronounced than those of PCBP2. Overall, the results presented here suggest that cellular PCBP2 and PCBP1 antagonize VSV growth by affecting viral gene expression and highlight the importance of these two cellular proteins in restricting virus infections.

Vesicular stomatitis virus (VSV) is an enveloped RNA virus in the family *Rhabdoviridae*. The negative-sense RNA genome of VSV is 11,161 nucleotides in length and encodes 5 proteins: the nucleocapsid protein (N), the phosphoprotein (P), the matrix (M), the glycoprotein (G), and the large protein (L). Inside the virion, the viral genome is tightly encapsidated with the N protein to form the ribonucleocapsid complex (RNP or NC) with which the viral RNA-dependent RNA polymerase (RdRp) complex of P-L proteins (41) is associated. The G protein forms spikes on the surface of the VSV virion and mediates virus attachment as well as entry by clathrin-mediated endocytosis. Based on the low-pH environment of the endosome, the VSV G protein mediates fusion of the viral envelope with the endosomal membrane, releasing the viral RNP into the cytoplasm of the infected cell. Once inside the cytosol, the RNP undergoes primary transcription by the associated RdRp activity of the L protein along with its cofactor P protein. Translation of the transcripts by the cellular translation machinery generates viral proteins, which are used for further steps in the infection process, including genome replication, secondary transcription, assembly, and budding (53).

The viral P is a multifunctional protein that modulates the association of viral replicative proteins and is indispensable for viral growth. It is required for viral genome transcription and replication (18) and also plays a role in packaging and assembly of infectious VSV (19). Phosphorylation at different sites within the protein appears to regulate its activity in transcription and replication (18, 35, 49). Based on structural studies of fragments of this protein (22, 23) and many functional studies in the past, three domains have been recently proposed (48). The carboxy-terminal domain (P_{CTD}), spanning residues from approximately 183 to 265, interacts with the N protein and is required for replication (32). The amino-terminal domain (P_{NTD}), spanning residues 1 to 106, likely encodes critical determinants of transcription activity and the L protein binding activity (20). The central domain (P_{CD}) of the molecule, spanning residues 107 to 177, is involved in the oligomerization of the protein (22). Oligomerization of the P protein is crucial for its association with the L protein and its function as a cofactor in enzymatic activities of the viral RdRp.

Because of the multifunctional nature of the P protein, it is likely that it interacts with many cellular proteins in virus-infected cells. Studies have shown that the P protein is phosphorylated by cellular casein kinase II for its activity in viral RNA synthesis and it also associates with c-src (3). The P protein of rabies virus (another member of the family *Rhabdoviridae*) interacts with dynein light chain LC8 (52), and this interaction is suggested to be required for viral genome transcription and replication *in vivo* and *in vitro* (56). Therefore, identification of the cellular proteins that interact with the VSV P protein and understanding how these interactions modulate P protein functions will provide a better understanding of the involvement of the cellular and the viral proteins in the replicative cycle of VSV. Apart from participating in genome replication and transcription, viral replication proteins are also involved in regulating the host cell response to virus infection.

In this study, we have used immunoprecipitation (IP) and mass spectrometry (MS) analysis to identify the cellular proteins that interact with the P protein. Our results reveal that

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▽ Published ahead of print on 13 July 2011.

TABLE 1. Primers used in this study

Primer name	Primer sequence (5′–3′)[a]	Use
NheI-HA-KpnI-PCBP1F	GCCGCTAGCATG*TACCCATACGATGTTCCAG ATTACGCT*GGTACCGATGCCGGTGTGACT GAAAG	Amplification of PCBP1 with HA tag coding sequence at 5′ end
EcoRI-PCBP1R	ATATGAATTCCTAGCTGCACCCCATGCC	
KpnI-PCBP2F	ATATGGTACCGACACCGGTGTGATTGAAGG	Amplification of PCBP2
EcoRI-PCBP2R	ATATGAATTCCTAGCTGCTCCCCATGCC	
N-For	ATGTCTGTTACAGTCAAGAG	Amplification of fragment of VSV N mRNA
N-Rev	CTGGAAGACCGTGTTCTC	
P-For	ATTATGAAGGAGCGCCAG	Amplification of 3′ end of P gene, P-M gene junction, and 5′ end of M gene in VSV antigenome
M-Rev	GTGTAGACTCATACAAGAC	

[a] Underlined portions of sequences indicate restriction enzyme sites; the portion in italics shows the sequence for the HA epitope tag.

among the many proteins identified by this method, the cellular poly(C) binding protein 2 (PCBP2) was consistently detected in several repeat experiments. PCBP2, along with its closely related isoform PCBP1 (which was also detected in our studies), belongs to a family of nucleic acid binding proteins with high affinity for binding to homopolymeric nucleic acids (15). Our studies reported here show that depletion of PCBP2 enhances VSV replication, whereas overexpression of PCBP2 in plasmid-transfected cells inhibits virus replication. PCBP2 was further shown to negatively regulate the levels of viral mRNA and subsequent genome replication without adversely affecting the viral entry and uncoating steps or virus budding. Interestingly, PCBP1 inhibited VSV primary mRNA transcription without affecting secondary transcription or viral genome replication. The P-PCBP2 interaction did not hinder the normal association of P with the N or the L protein or the homo-oligomerization of P molecules. Additionally, we have shown that the P protein is ubiquitinated, and its degradation is dependent on the proteasome pathway. However, the interaction of PCBP2 with the P protein does not lead to the degradation of this viral protein.

MATERIALS AND METHODS

Cell culture and reagents. Monolayer cultures of HeLa and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and the antibiotics penicillin (100 units/ml), kanamycin (20 units/ml), and streptomycin (20 units/ml) (PKS). Baby hamster kidney (BHK-21) cells were maintained as described earlier (16). The NPeGFPL stable cell line (47) derived from 293 cells was maintained as described before (47) in the presence of 1 mg/ml G418. Cycloheximide, MG132, and protease inhibitor cocktail were obtained from Sigma-Aldrich and used at concentrations of 100 μg/ml, 2 to 30 μM, and 1×, respectively.

Viruses, VSV DI particles, and nucleocapsid preparation. Recombinant vaccinia virus vTF7-3 (25) was prepared and titrated in BHK-21 cells as described before (47). Stocks of VSV, VSV-eGFP, and VSV-PeGFP were prepared and titrated as described earlier (17, 18). Defective interfering T particles (DI-T) (39) of wild-type (wt) VSV were prepared and stored at −80°C in small aliquots until use. For generating VSV-PeGFP-ΔG virus, the G protein open reading frame from pVSV-PeGFP (17) was removed by restriction digestion of the full-length viral cDNA-carrying plasmid followed by religation. VSV-PeGFP-ΔG virus was recovered using VSV rescue methods (18) in the presence of G protein expressed from a transfected plasmid under T7 RNA polymerase promoter. The VSV-PeGFP-ΔG virus was amplified by passaging the transfected cell culture supernatants in cells expressing the G protein from a plasmid under the cytomegalovirus (CMV) promoter (pHyg-G, in which the entire coding region of the G protein of VSV is cloned in place of the HygeGFP fusion protein in the pHygeGFP vector from Clontech). The titration of VSV-PeGFP-ΔG was performed on VSV G-expressing BHK-21 cells and by determining the number of fluorescent foci of infection. The viral NC from VSV-PeGFP was prepared with modification to the protocol described before (21). Briefly, purified VSV-PeGFP virus pellet was resuspended in nucleocapsid generation buffer (10 mM Tris-HCl, pH 8.0; 0.4 M NaCl; 1.85% Triton X; 5% glycerol; 0.6 mM dithiothreitol) and incubated on ice for 2 h with intermittent mixing. The sample was then centrifuged at 32,000 rpm for 3 h to pellet the NC. The pelleted nucleocapsid was then resuspended in resuspension buffer (5% glycerol and 10 mM Tris-HCl, pH 8.0), aliquoted, and frozen at −80°C until use.

Antibodies. Anti-M (23H12) and anti-N (10G4) monoclonal antibodies were kindly provided by D. Lyles (9). Mouse anti-VSV (Indiana serotype) polyclonal antibody and rabbit anti-P polyclonal antibody have been described before (19). Antibodies against actin (sc-47778), PCBP1 (sc-16504), PCBP2 (sc-30725), ubiquitin (sc-9133), and green fluorescent protein (GFP; sc-9996) were purchased from Santa Cruz Biotechnology Inc. Antihemagglutinin (anti-HA) monoclonal antibody HA-7 (H3663) and the polyclonal antibody (H6908), anti-Flag monoclonal antibody M2 (F3165) and the polyclonal antibody (F7425), goat anti-rabbit immunoglobulin (IgG)–horseradish peroxidase (A6154) conjugate, goat anti-mouse IgG–horseradish peroxidase (A4416) conjugate, and rabbit anti-goat IgG–horseradish peroxidase (A4174) conjugate were obtained from Sigma-Aldrich. Alexa Fluor 488 goat anti-rabbit (A11034) and Alexa Fluor 594 goat anti-mouse IgG (A11032) were purchased from Invitrogen.

Plasmid constructs. pTKRL (Promega) and pISG56-luc plasmids have been described before (7). HA-ubiquitin was expressed from the plasmid pMT123-HA-ubiquitin, which was described previously (59). VSV P with a Flag tag at the amino (N) terminus was cloned into the pHygeGFP vector. Coding sequences of PCBP1 (NM_006196.3) and PCBP2 (NM_031989.4) were amplified using total RNA recovered from HEK293 cells by using a LongAmp kit (New England BioLabs). The reverse transcription-PCRs (RT-PCRs) for PCBP1 amplification were performed using the forward primer NheI-HA-KpnI-PCBP1F containing two restriction sites flanking the sequence for the HA epitope tag (shown in italics) and the reverse primer EcoRI-PCBP1R (Table 1). The PCR product was digested with NheI and EcoRI and cloned in the pcDNA3.1(+)Neo vector (Invitrogen) digested with the same enzymes, and the plasmid was named pcDHA-PCBP1. PCBP2 was amplified similarly with primers KpnI-PCBP2F and EcoRI-PCBP2R (Table 1). The PCR product was digested with KpnI and EcoRI. These two enzymes were also used to remove the PCBP1-carrying fragment from the pcDHA-PCBP1 plasmid to generate a linear vector to clone the PCBP2-carrying DNA fragment. This plasmid, pcDHA-PCBP2, carries PCBP2 with an HA tag at its N terminus. The entire sequences of the genes in these plasmids were confirmed by nucleotide sequencing. These HA epitope-tagged proteins could be expressed by both CMV and T7 promoter-driven expression systems.

siRNA-mediated silencing. For depletion of PCBP1 and PCBP2 in HeLa as well as in HEK293 cells, nontargeting (NT) control small interfering RNA (siRNA) or siRNAs (pool of four different siRNAs) targeting PCBP1 or PCBP2 (Dharmacon catalog numbers M-012243-01-0005 and M-012002-01-0005, respectively) were transfected at a final concentration of 10 nM (except where indicated otherwise) following the protocol of reverse transfection with Lipofectamine RNAiMax (Invitrogen) as recommended by the manufacturer. At 24 h posttransfection (hpt), the transfection mix was replaced with DMEM containing 10% FBS and PKS and the cells were incubated further for 40 to 48 h to allow knockdown of the gene.

Plasmid DNA and RNP transfection. Transfection of plasmids was performed using Lipofectamine2000 (Invitrogen) as per the manufacturer's instructions and as described previously (2). At 4 hpt, the transfection mix was replaced with complete growth medium and incubated for 40 to 44 h before being used for

assays. Transfections of viral RNPs were also performed using the same procedure as used for plamsid transfection.

Co-IP and MS. Cell monolayers were lysed in radioimmunoprecipitation assay (RIPA) buffer without SDS (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 1× protease inhibitor cocktail) and subjected to coimmunoprecipitation (co-IP) following the procedure described previously (16). For MS analysis, the immunoprecipitated proteins were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels, the proteins were stained using the silver staining method following a published protocol (11), and the selected bands were excised from the stained gel for liquid chromatography-MS analysis.

WB. Protein samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were soaked in blocking buffer (phosphate-buffered saline [PBS] containing 5% nonfat dry milk and 0.05% Tween 20) for 1 h and then probed with specific antibodies as indicated below in each figure. Proteins were revealed by horseradish peroxidase-labeled secondary antibody and visualized with an enhanced chemiluminescence detection system (Pierce) following the manufacturer's recommendations. Quantitative levels of protein expression were determined by scanning of Western blotting (WB) data using Quantity-One software (Bio-Rad).

Semiquantitative RT-PCR. Total RNA from infected cells was extracted with TRIzol (Invitrogen). For quantitation of viral mRNAs, first-strand cDNA was synthesized using total RNA and oligo(dT) primer (Roche) followed by PCR amplification of VSV N mRNA with specific primers (Table 1). For an internal control, oligo(dT)-derived cDNA was used to amplify ribosomal protein L32 mRNA by using specific primers (Table 1) as described before (7). For quantitation of full-length antigenome (replication product), total RNA was used to synthesize cDNA with a reverse primer (Table 1) which binds to the antigenome of VSV at sequences corresponding to the N terminus of M protein. The cDNA was then used in PCR using the same reverse primer and a forward primer (Table 1) that bound to the sequences corresponding to the C terminus of P protein. The total RNA was also used to generate L32 PCR product (described above) as an internal loading control for the amount of RNA used in the assays. PCR cycling conditions used were 30 s at 94°C, 30 s at 54°C, and 40 s at 72°C for 25 cycles (protein-depleted cells) or 35 cycles (protein-overexpressing cells).

Virus infection and titration. Virus infection in each experiment was performed at a multiplicity of infection (MOI) of 1 or 0.01 PFU per cell, except where indicated otherwise, and the titers were determined in BHK-21 or HeLa cells by plaque assay.

Immunofluorescence. HeLa cells grown on glass coverslips were transfected with a vector encoding HA sequences alone (EV) or HA-PCBP2 or HA-PCBP1 protein-expressing plasmids. At 48 hpt, cells were infected with wt VSV at an MOI of 1 for 3 h, followed by fixation with methanol-acetone (1:1) for 15 min at −30°C. After washing the cells with PBS twice, cells were stained with mouse anti-HA monoclonal antibody and rabbit anti-P polyclonal antibody. Cells were subsequently stained with Alexa Flour 594 goat anti-mouse IgG and Alexa Flour 488 goat anti-rabbit IgG. Images were obtained with an Olympus FV500/IX81 inverted laser scanning confocal microscope.

Statistical analyses. Statistical analyses were performed using the MIXED procedure (SAS Institute Inc., Cary, NC). The statistical modeling included treatment as a fixed effect. Data are presented as means ± standard errors of means. A P value of <0.05 was considered statistically significant.

RESULTS

Identification of cellular proteins that interact with VSV P protein by IP and MS analysis. In an attempt to identify and characterize cellular factors that interact with VSV P protein and are involved in the VSV life cycle, we employed an IP assay with lysates of cells expressing the VSV P protein, followed by identification of the cellular proteins using MS analysis. The plasmid carrying the amino-terminal Flag-tagged P protein (Flag-P), which is functional in viral genome transcription and replication (unpublished data), or a vector encoding only the Flag sequences (EV) was transfected into 293T cells. IP of cell extracts was performed with anti-Flag antibody. The immunoprecipitated proteins were separated by SDS-PAGE, and the protein bands were detected by silver staining. Several protein bands (identified by asterisks in Fig. 1) based on increased or decreased intensity were observed in the Flag-P-overexpressing lane compared to the EV lane. MS analysis of some of these bands identified many cellular proteins, and only those protein candidates that had at least two peptides (with individual MASCOT scores of >35) and that represented at least 4% coverage of the corresponding full-length proteins are shown on the right side of Fig. 1. For the purpose of confirming the identity of the P protein, when we analyzed the presumptive P protein band by MS, we detected other cellular proteins, including PCBP1 (also known as hnRNP E1) and PCBP2 (hnRNP E2), in addition to the VSV P protein. Besides PCBP1 and PCBP2, other cellular proteins were also identified (Fig. 1), and several of these proteins have been reconfirmed to interact with the P protein. Many of these proteins are generally involved in cellular RNA metabolism and protein modification processes (34, 36, 42). In this study, we focused on examining the role of PCBP2 and PCBP1 in the VSV life cycle.

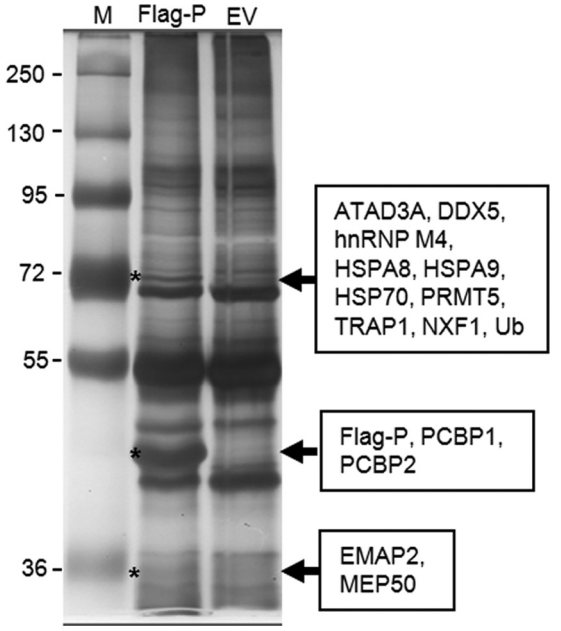

FIG. 1. Identification of interacting partners of VSV P protein. Flag-P carrying plasmid (5 μg) or empty vector (EV; 5 μg) were transfected into 293T cells. At 48 hpt, cells were harvested for IP using anti-Flag monoclonal antibody-conjugated beads for 12 h, followed by separation of the proteins by 10% SDS-PAGE and silver staining. Molecular mass markers (in kDa) are separated in lane M. The protein bands identified by asterisks to the right of lanes were excised from the gel and subjected to MS analysis. Proteins identified from MS analysis of the bands are shown. ATAD3A, ATPase family AAA domain-containing protein 3A; DDX5, RNA helicase p68; EMAP2, endothelial-monocyte activating polypeptide II; hnRNP M4, heterogeneous nuclear ribonucleoprotein M4; HSPA8, heat shock 70-kDa protein 8; HSPA9, heat shock 70-kDa protein 9; HSP70, heat shock protein 70; MEP50, methylosome protein 50; PRMT5, protein arginine N-methyltransferase 5; NXF1, nuclear RNA export factor 1 (also known as TAP); TRAP1, tumor necrosis factor type 1 receptor-associated protein.

Both PCBP2 and PCBP1 interact and colocalize with VSV P protein. To ascertain whether detection of PCBP2 in the IP-MS analysis was due to specific interaction with the viral P protein or was simply nonspecific binding with the antibody, we examined interaction between PCBP2 and P proteins in trans-

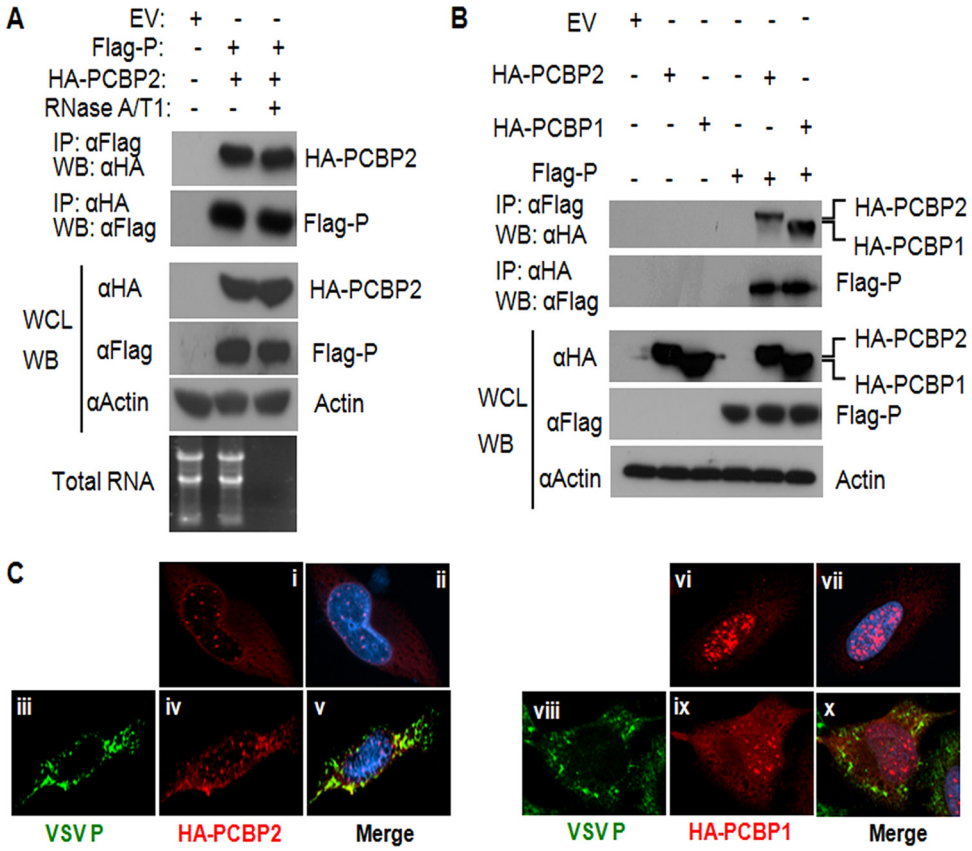

FIG. 2. VSV P protein interacts and colocalizes with PCBP2 and PCBP1. (A) P-PCBP2 interaction. Two micrograms of each of the plasmids carrying Flag-P and HA-PCBP2 was cotransfected into 293T cells. At 48 hpt, cells were lysed in RIPA buffer without SDS. An aliquot (40%) of the clarified cell lysate was treated with RNase A (2.5 U/100 μl lysate) and RNase T_1 (10 U/100 μl lysate) for 1 h at room temperature. Equal amounts of lysates with or without RNase treatment were subjected to co-IP with anti-Flag or anti-HA antibody, and WB was performed with anti-HA or anti-Flag antibody, respectively. Approximately 3% of whole-cell lysates (WCL) was also examined by WB with the antibodies shown. Actin served as a loading control. The panel (total RNA) shows the removal of RNA (lane 3) following RNase A/T_1 treatment. (B) WB to demonstrate the interaction of PCBP1 with P. The experiment was conducted as described for panel A. The interaction with PCBP2 was used as a positive control in this experiment. (C) Colocalization of PCBP2 and PCBP1 with VSV P protein in VSV-infected HeLa cells. HeLa cells were transfected with plasmids carrying HA-PCBP2 (panels i to v) or HA-PCBP1 (panels vi to x), and at 48 hpt cells were infected with VSV (panels iii to v and viii to x) or mock infected (panels i, ii, vi, and vii) for 3 h. The cells were immunostained with anti-HA monoclonal and anti-P polyclonal antibody followed by anti-mouse Alexa Fluor 594 (red), anti-rabbit Alexa Fluor 488 (green), and 4′,6-diamidino-2-phenylindole (blue) staining. Merged images are also shown.

fected cells. Plasmids carrying HA-tagged PCBP2 (HA-PCBP2) and Flag-tagged P (Flag-P) proteins were cotransfected into 293T cells, immunoprecipitated with anti-Flag or anti-HA antibody, resolved by SDS-PAGE, and then immunoblotted with anti-HA or anti-Flag antibody. The results (Fig. 2A) demonstrated a specific interaction when these two proteins were coexpressed. Since the functions of PCBP2 are known to be modulated by its interaction with RNA (12, 46), we examined if the PCBP2-P interaction is mediated through RNA. When the cell extracts were treated with ribonucleases prior to IP, the PCBP2-P interaction was still detected at levels similar to those without RNase treatment. The results showed that PCBP2 interacts with the P protein and that this interaction is independent of RNA.

In the family of PCBPs that contain 3 KH domains, the human PCBP1 protein shares 89% amino acid sequence identity with PCBP2 protein and is slightly smaller (10 amino acids) (58). This protein was also identified in the IP-MS analysis as another P-interacting protein. Therefore, we investigated if P protein also interacts with PCBP1 in transfected cells. To this end, we coexpressed HA epitope-tagged PCBP1 (HA-PCBP1) together with Flag-P in 293T cells. Interaction of P with these proteins was examined as described above. The results (Fig. 2B) showed that the P protein also interacts with PCBP1 when ectopically expressed in transfected cells.

Since VSV infection results in redistribution of hnRNP A1, K, and C1/C2 proteins in the cytoplasm in the infected cells (51), we investigated if the interaction of P with PCBP2 or PCBP1 affects their intracellular distribution. Consistent with a previous study (15), we found that in cells expressing HA-PCBP2 or HA-PCBP1, these proteins were found to be distributed throughout the cells. The PCBP1 was predominantly localized in the nucleus (Fig. 2C, panel vi), whereas PCBP2 was localized in both the nucleus and the cytoplasm (Fig. 2C, panel ii), consistent with their association with RNA-processing centers (12). In cells infected with VSV, the intracellular

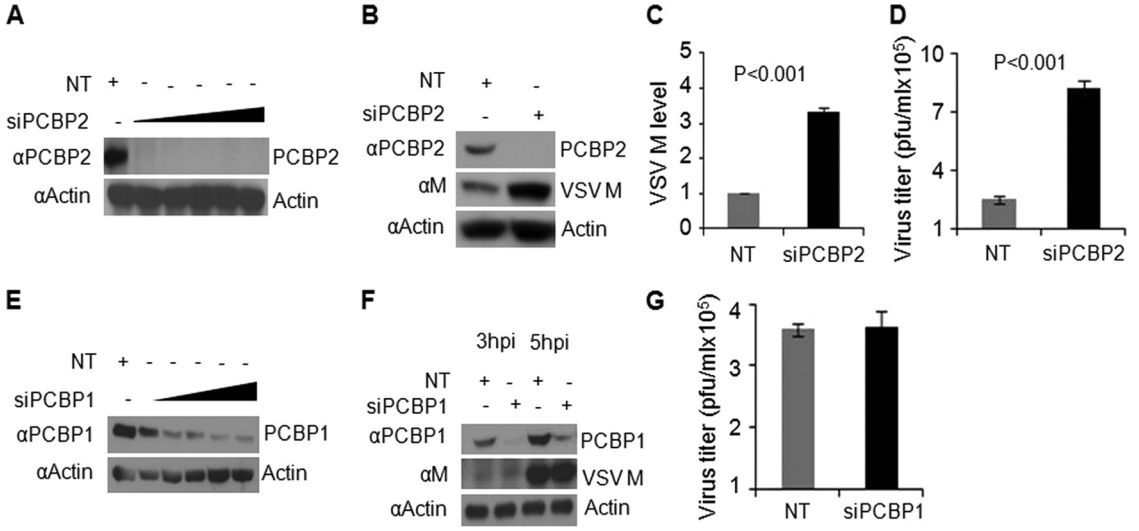

FIG. 3. Knockdown of PCBP2 but not PCBP1 enhances VSV growth. (A) Knockdown efficiency of siRNA against PCBP2. HeLa cells were treated with siRNA targeting PCBP2 at final concentrations of 0.1, 1, 5, 10, and 20 nM or with 20 nM nontargeting (NT) siRNA. PCBP2 expression was determined by WB at 72 hpt with anti-PCBP2 polyclonal antibody. (B) VSV infection is enhanced in PCBP2-depleted cells. HeLa cells treated with NT siRNA or PCBP2 siRNA at 10 nM for 68 to 72 h were infected with VSV-eGFP at an MOI of 0.01 for 8 h. VSV M protein and endogenous PCBP2 were detected by WB with anti-M monoclonal antibody and anti-PCBP2 antibody. (C) Quantitation of M protein levels (arbitrary units) from three independent experiments, as described for panel B. The level of M expression in the NT siRNA-treated sample was set at 1. (D) VSV titer in PCBP2-depleted cells. siRNA treatment and virus infections were performed similarly, as for panel B, and virus titers in supernatants collected at 12 hpi were determined by plaque assay and expressed as PFU/ml. (E) Knockdown efficiency of siRNA against PCBP1. siRNA concentrations used were similar to those for panel A. PCBP1 expression was determined by WB at 72 hpt with anti-PCBP1 polyclonal antibody. (F) VSV infection in PCBP1-depleted cells. Cells, silencing conditions, and the MOI used were similar to those for panel B, but cells were harvested at 3 or 5 hpi. WB was done with anti-M and anti-PCBP1 antibody. Actin served as a loading control. (G) VSV growth in PCBP1-depleted cells. Cells, PCBP1 siRNA treatment, infection with VSV, and virus titration were similar to those described for panels B and D. Error bars represent the standard errors of means from at least 3 independent experiments.

distribution of both PCBP2 and PCBP1 (panels iv and ix) was not significantly altered. However, we could detect the colocalization of VSV P with PCBP2 throughout the cytosol (panel v), whereas the colocalization of VSV P with PCBP1 was observed in some regions of the cytoplasm (panel x). These data indicate that VSV infection does not significantly alter the normal localization of PCBP2 and PCBP1 in infected cells but that VSV P protein colocalizes with PCBP2 more so than with PCBP1 protein.

Depletion of PCBP2 but not PCBP1 by siRNA enhances VSV growth. The observation that PCBP2 and PCPB1 interact with VSV P prompted us to examine the relevance of this interaction for the VSV life cycle. To this end, we used specific siRNAs to deplete PCBP2 or PCBP1 from HeLa cells. Treatment of cells with siRNA for PCBP2 led to efficient depletion of the protein in these cells, even at the lowest concentration (0.1 nM) of siRNA used (Fig. 3A). In cells depleted of PCBP2, VSV infection resulted in a significant increase in viral proteins (as a function of viral gene expression) compared to the cells treated with NT siRNA (Fig. 3B and C). PCBP2-silenced cells exhibited more than a 3-fold increase of viral M protein, which also correlated well with a similar increase in infectious viral titer in the supernatant (Fig. 3D). Although we had observed similar effects of PCBP2 depletion or overexpression on the levels of the VSV N, P, M, and G proteins (data not shown), only the data for the M protein have been presented here. For PCBP1, the best depletion was achieved at a 10 to 20 nM concentration of siRNA (Fig. 3E). Under these silencing conditions, we did not observe any significant differences in viral protein expression (Fig. 3F) or infectious virus production in the culture supernatants (Fig. 3G). Additionally, no differences in viral protein production or infectious virus titers were observed at early (6 hpi) or late (15 hpi) times of infection (data not shown). Overall, the results show that depletion of cellular PCBP2 has a negative consequence on virus gene expression and growth, whereas depletion of PCBP1 does not adversely affect these virus-specific steps.

Overexpression of PCBP2 but not PCBP1 suppresses VSV growth. The observation that depletion of PCBP2 resulted in enhanced gene expression and viral growth prompted us to examine the effects of overexpression of this protein on VSV. HeLa cells were transiently transfected with the plasmid carrying HA-PCBP2 and subsequently infected with VSV. In these cells, HA-PCBP2 could be readily detected (Fig. 4A). When the cells were infected with VSV, viral gene expression as assayed by detection of M protein was reduced by approximately 3-fold (Fig. 4A and B). A similar reduction in viral titer was also observed in the supernatant of these cells (Fig. 4C). On the other hand, overexpression of HA-PCBP1 did not result in any significant difference in viral gene expression or infectious virus production (Fig. 4D and E). Thus, these results together with those from knockdown experiments highlight the antagonistic properties of cellular PCBP2 on VSV infection.

The interaction of PCBP2 with P does not disrupt interactions among viral proteins. In VSV-infected cells, the P protein interacts with the other viral proteins, namely, the N and L proteins, as well as with itself to form complexes that are required for viral RNA synthesis (20, 32, 41). The observed

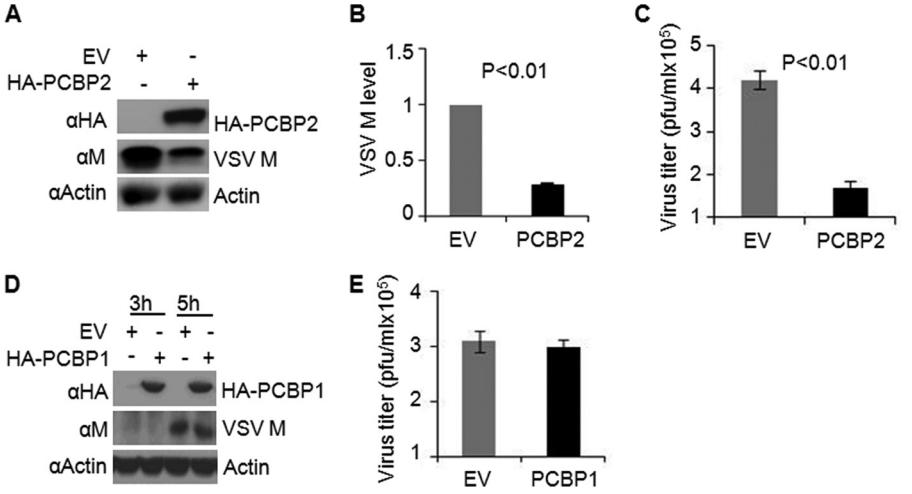

FIG. 4. Overexpression of PCBP2 but not PCBP1 suppresses VSV growth. (A) VSV infection is suppressed in PCBP2-overexpressing cells. HeLa cells transfected with 1.5 μg of EV- or HA-PCBP2-carrying plasmid for 48 h were infected with VSV at an MOI of 0.01 for 8 h, and cell lysates were analyzed by WB using anti-M or anti-HA antibodies. Actin served as a loading control. (B) Quantitative determination of levels of M (arbitrary units) from three independent experiments as described for panel A. The level of M expression in EV-transfected cells was set at 1. (C) Reduced VSV growth in PCBP2-overexpressing cells. Transfection and infection conditions were as described for panel A, but the titer of virus in the supernatant collected at 12 hpi was determined. (D) VSV infection in PCBP1-overexpressing cells. HeLa cells transfected with EV or HA-PCBP1 plasmid were infected with VSV at an MOI of 0.01 for 3 or 5 h, and the cell lysates were analyzed by WB using anti-M or anti-HA antibodies. Actin served as a loading control. (E) VSV growth in PCBP1-overexpressing cells. Transfection and infection conditions were as described for panel C. Virus titers in the supernatants collected at 12 hpi were determined. Error bars represent the standard errors of means from 3 independent experiments.

decrease of virus replication in cells when PCBP2 was ectopically expressed raised the possibility that the interaction of PCBP2 with P protein may interfere with the interactions of the viral proteins with the P protein. To explore this possibility, we used the stable cell line 293-NPeGFPL, which constitutively expresses the VSV replication proteins, N, PeGFP (a fusion protein with enhanced GFP [eGFP] inserted in frame after amino acid 196 of VSV P protein), and the L protein and supports efficient VSV defective interfering particle genome replication (47). These cells were cotransfected with the plasmid carrying Flag-P together with increasing amounts of the plasmid carrying HA-PCBP2. Cell extracts from these cells were subjected to co-IP with anti-Flag antibody, and the protein species were detected by immunoblotting with various antibodies. If the P-PCBP2 interaction abrogated or affected the interaction of P with PeGFP or N, the amount of PeGFP or N proteins bound and precipitated by Flag-P would be expected to be reduced during co-IP with anti-Flag antibody. Results showed that similar amounts of PeGFP and N protein were immunoprecipitated by anti-Flag antibody, irrespective of the level of HA-PCBP2 expressed in these cells (Fig. 5A). To examine if the P-L interaction was affected by the presence of overexpressed PCBP2, the 293-NPeGFPL cells were transfected with increasing amounts of the plasmid carrying HA-PCBP2, and the cell extracts were used for immunoprecipitation with anti-L antibody and immunoblotted with the antibodies shown. The amount of PeGFP pulled down by the antibody was not affected by the presence of overexpressed PCBP2 (Fig. 5B). These results indicate that overexpression of PCBP2 does not affect the interaction of PeGFP with L protein. It is worth mentioning here that the PeGFP fusion protein interacts with PCBP2 to a similar extent as the Flag-P protein (data not shown). Taken together, these results demonstrate that the presence of HA-PCBP2 and its interaction with VSV P does not interfere with the interactions among the viral proteins to form functional complexes for viral gene expression.

PCBP2 does not induce degradation of P protein. PCBP2 has been shown to interact with MAVS and induce its degradation via ubiquitination by E3 ubiquitin ligase AIP4 through proteasome pathway (67). It is then possible that inhibition of virus growth by PCBP2 could be in part due to degradation of the P in infected cells. Interestingly, by MS analysis, we detected ubiquitin in the Flag-P pull-down sample (Fig. 1), indicating that P may be ubiquitinated in cells. Examination of ubiquitination of P protein in cells transfected with plasmids carrying HA-tagged ubiquitin (HA-Ub) and P proteins revealed that the P protein was indeed ubiquitinated (Fig. 6A). In cells infected with VSV, the P protein was also found to be ubiquitinated (Fig. 6B). Since protein degradation by the proteasome pathway is regulated by ubiquitination of protein, we examined whether the P protein is degraded by the proteasome pathway. When P protein-expressing cells were treated with the proteasomal inhibitor MG132, the level of P protein increased significantly compared to the cells not treated with the inhibitor (Fig. 6C). These observations indicate that VSV P protein is ubiquitinated and its degradation is dependent on the proteasome pathway.

To examine if VSV P protein degradation is regulated by PCBP2, we determined the levels of P protein in cells cotransfected with a fixed amount of the plasmid carrying the P protein and increasing amounts of the plasmid carying HA-PCBP2. Results showed no observable differences in the level of P protein in cells expressing increasing amounts of HA-

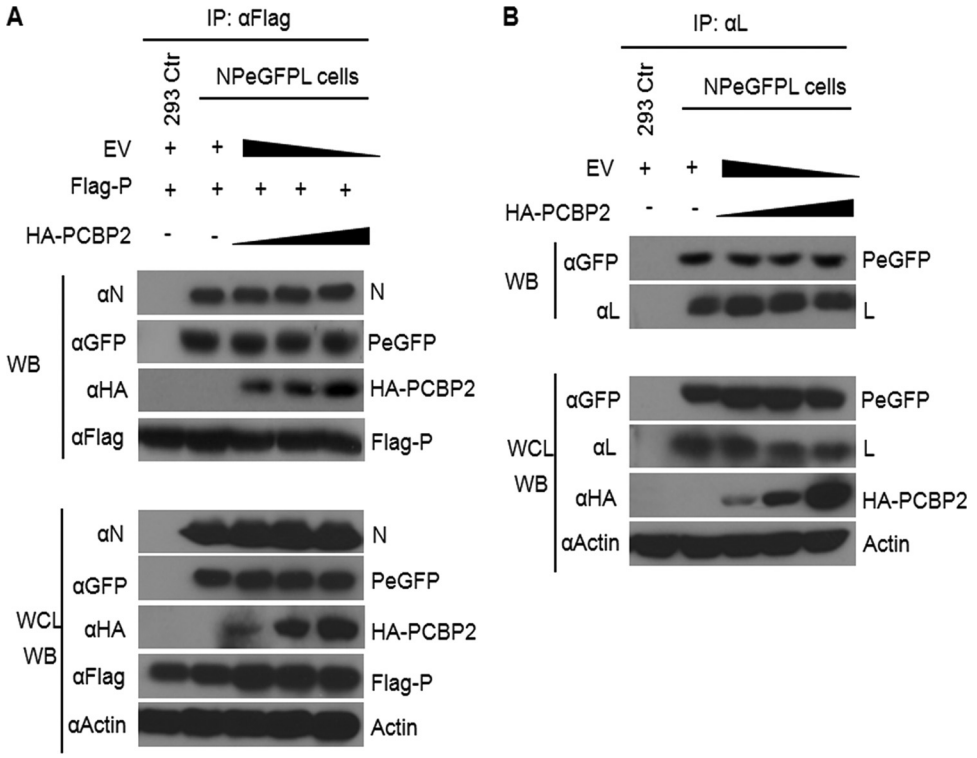

FIG. 5. The P-PCBP2 interaction does not disrupt the formation of viral protein complexes. (A) Formation of N-P and P-P complexes in the presence of PCBP2. The NPeGFPL stable cell line (clone 206) along with a stable 293 cell control (293 Ctr) were transfected with 2 μg Flag-P-carrying plasmid along with EV or plasmid carrying HA-PCBP2 at increasing concentration of 0.5 μg, 1 μg, and 2 μg (wedge). At 48 hpt, cell lysates were used for co-IP with anti-Flag antibody followed by WB with the indicated antibodies (on the left). The detected proteins are shown on the right. WB results with whole-cell lysates (WCL) corresponding to 3% of total extract with the indicated antibodies (bottom panels) show the amount of various proteins. (B) Formation of the P-L complex in the presence of HA-PCBP2. NPeGFPL stable cells were transfected with increasing amounts of HA-PCBP2 carrying plasmid at 2 μg, 5 μg, and 10 μg (wedge). At 48 hpt, anti-L polyclonal antibody was used in co-IP of cell lysates, and subsequently the samples were analyzed by WB with the indicated antibodies. Actin served as a loading control in these experiments. WB results with whole-cell lysates corresponding to 3% of total extract with the indicated antibodies (bottom panels) show the amounts of various proteins.

PCBP2 (Fig. 6D), indicating that PCBP2 has no effect on P protein degradation. Since PCBP2 is known to induce MAVS degradation to prevent uncontrolled interferon (IFN) production (67), we then asked if in the context of activated IFN production, PCBP2 might function to reduce the P protein level by inducing its degradation, which otherwise might not be observed in the absence of IFN induction. To address this possibility, we expressed HA-PCBP2 in the 293-NPeGFPL cell line and then mock infected cells or infected cells with DI particles of VSV. Infection of these cells with DI particles resulted in stimulation of IFN production by up to 90-fold (Fig. 6E), consistent with our previous report (47). In the presence of PCBP2, the activation of the IFN pathway is partially repressed (Fig. 6E), as has been documented before (67). Nevertheless, the level of PeGFP protein was similar in cells with or without HA-PCBP2 expression (Fig. 6F). Taken together, the results from these experiments suggest that interaction of PCBP2 with VSV P protein does not lead to degradation of this viral protein.

Although our previous experiments indicated that VSV P protein is not a direct target of the PCBP2 degradation pathway, it is possible that PCBP2's restrictive effect on viral growth is mediated through proteasomal degradation of other cellular proteins. To investigate if a functional proteasome is required for PCBP2-mediated suppression of viral growth, we used MG132 to block the proteasomal pathway in cells treated with NT siRNA or PCBP2 siRNA (dimethyl sulfoxide [DMSO] treatment was used as the vehicle control). Under these conditions, we still observed a 2.5-fold increase of virus titer in PCBP2-silenced cells compared to NT siRNA-transfected cells in MG132-treated cells (Fig. 6G, right panel). It should be noted that treatment of cells with MG132 resulted in significant inhibition of VSV growth (Fig. 6G, right panel) compared to DMSO treatment (Fig. 6G, left panel), consistent with a previously reported inhibitory effect of MG132 on VSV growth (33). A similar increase in titers was also observed with DMSO treatment (Fig. 6G, left panel). This suggests that the inhibitory effect of PCBP2 on VSV growth is independent of the proteasome pathway.

PCBP2 exerts its inhibitory effect on VSV growth at the level of viral gene expression. The observed increases in viral protein expression and virus titer following PCBP2 silencing do not indicate whether this cellular protein exerts its inhibitory effect on VSV replication at the viral entry and uncoating step or at the gene expression step. To address this, we prepared viral RNPs from purified virions and transfected them directly

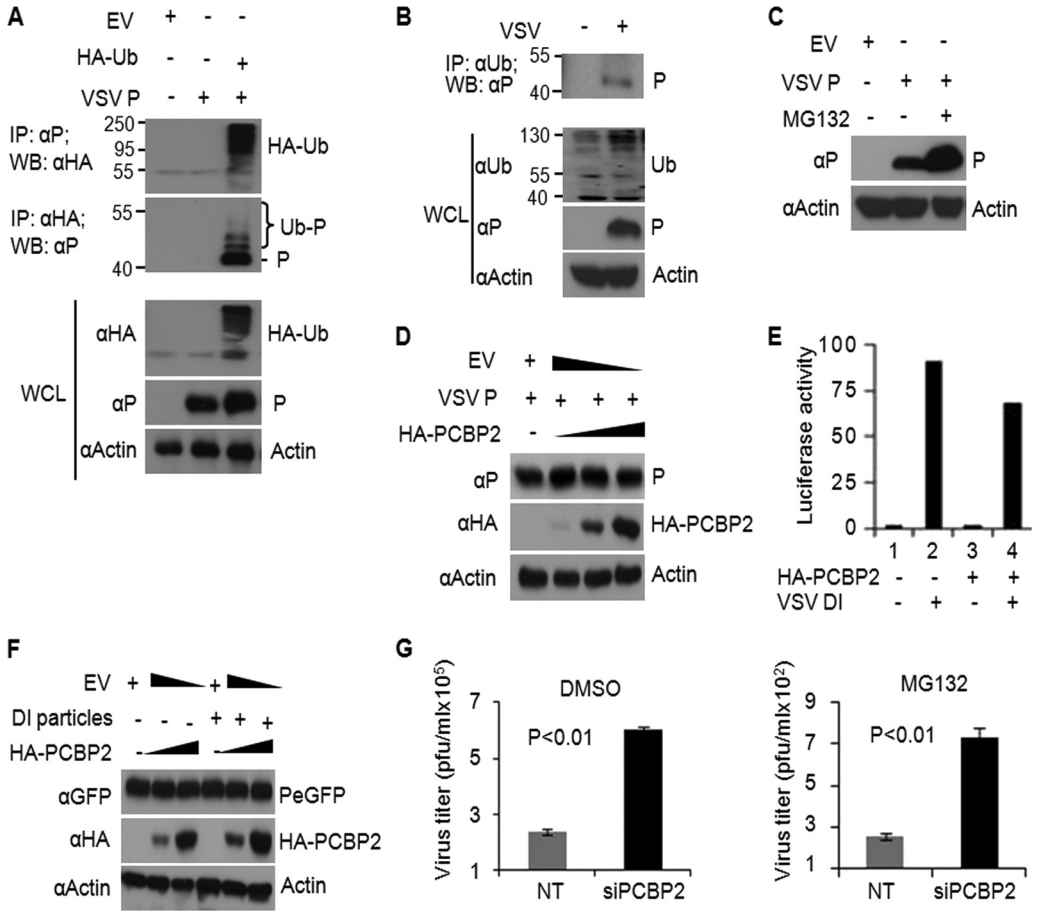

FIG. 6. PCBP2 does not induce the degradation of viral proteins. (A) Ubiquitination of VSV P protein. Cell lysates at 48 hpt from 293T cells transfected with the indicated combination of 2 μg each of EV, VSV P, or HA-ubiquitin-expressing plasmid was used for co-IP with anti-P or anti-HA antibodies, followed by WB with anti-HA or anti-P antibody, respectively. WB results with WCL corresponding to 3% of total extract with various antibodies are also shown at the bottom. Actin served as a loading control in these experiments. Molecular mass markers in kilodaltons are shown on the left. (B) Ubiquitination of P protein in VSV-infected 293T cells. Cells were infected with VSVeGFP at an MOI of 5 for 5 h. Whole-cell lysates were used for IP with antiubiquitin antibody followed by WB with anti-P antibody. Molecular mass markers in kilodaltons are shown on the left. (C) Proteasome-mediated degradation of VSV P protein. VSV P-carrying plasmid (2 μg) was transfected into 293T cells, and at 24 hpt the transfected cells were treated with DMSO or MG132 at 30 μM for 12 h. The amount of P protein was determined by WB with anti-P polyclonal antibody. (D) P protein level when coexpressed with PCBP2. Cells were transfected with 0.5 μg VSV P-carrying plasmid along with increasing amounts (0.1 μg, 0.25 μg, and 0.5 μg) of HA-PCBP2 plasmid. At 48 hpt, cell lysates were prepared for WB with anti-P and anti-HA antibodies. (E) Relative luciferase activity in a stable cell line supporting DI particle RNA replication and overexpressing PCBP2. NPeGFPL stable cells were transfected with 1 μg EV or plasmid carrying HA-PCBP2 along with 10 ng pTKRL and 0.4 μg pISG56-luc. At 28 hpt, the cells were mock infected (lanes 1 and 3) or infected with DI particles (lanes 2 and 4). At 16 hpi, cells were lysed and assayed using the Dual-Luciferase assay kit (Promega). Luciferase activity (lanes 2 and 4) was expressed as the relative fold induction over the level in uninfected control cells (lanes 1 and 3) after normalization with respect to *Renilla* luciferase activity. (F) PeGFP protein levels in a stable cell line supporting DI replication and expressing PCBP2. NPeGFPL stable cells transfected with EV or plasmid carrying HA-PCBP2 were mock infected or infected with DI particles at 28 hpt and harvested at 16 hpi for analysis by SDS-PAGE and WB with anti-GFP or anti-HA antibodies. Actin was the loading control for WCL. (G) VSV titers in cells with PCBP2 depleted and the proteasome pathway inhibited. HeLa cells were treated with siRNA as for Fig. 3B and infected with VSV at an MOI of 0.01. One hour after infection, cells were incubated in complete medium containing DMSO (left panel) or 2 μM proteasome inhibitor MG132 (right panel). The supernatants were collected at 16 h postinfection for a plaque assay. Error bars represent the standard errors of means from 2 independent experiments.

into cells depleted of PCBP2 (by siRNA) or overexpressing PCBP2 (by prior transfection with the HA-PCBP2-carrying plasmid). The viral RNP from purified virions can initiate viral gene expression upon introduction into naïve cells by transfection (unpublished data). If the inhibitory effect of PCBP2 were at the virus entry and uncoating step, bypassing this step through transfection of RNP into the cell would result in viral gene expression that is independent of the presence or absence of PCBP2. Results from such experiments showed that the viral M protein level (as a function of viral gene expression) increased significantly following siRNA-mediated depletion of PCBP2 (Fig. 7A, left panels) and was reduced significantly when PCBP2 was overexpressed (Fig. 7A, right panels). Furthermore, the level of enhancement (in PCBP2-depleted cells) or reduction (in PCBP2-overexpressing cells) of viral gene expression was similar whether RNP transfection or virus infection was used (Fig. 3 and 4). Therefore, the results indicate that PCBP2 inhibits VSV infection at a step after virus entry and uncoating.

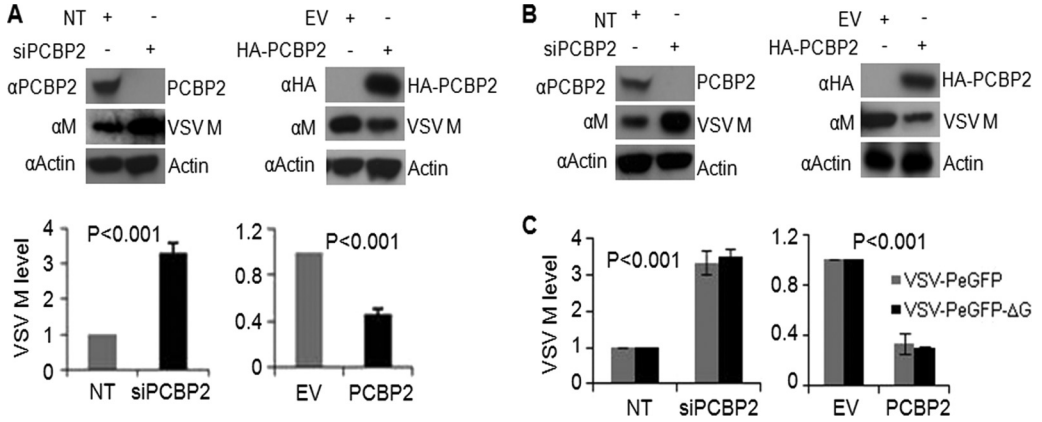

FIG. 7. The antagonistic effect of PCBP2 on VSV infection is not dependent on virus entry and uncoating or virus budding. (A and B) HeLa cells were transfected with 10 nM siRNA for NT or PCBP2 for 72 h (A and B, left panels) or transfected with 1.5 μg of EV or HA-PCBP2-carrying plasmids for 48 h (A and B, right panels). Cells were then supertransfected for 12 h with nucleocapsids prepared from VSV-PeGFP (A) or infected with VSV-PeGFP-ΔG at an MOI of 0.2 for 8 h (B). Equal amounts of cell lysates from both experiments were analyzed by SDS-PAGE and WB with anti-PCBP2, anti-M, or anti-HA antibodies as indicated. Actin was the loading control. M proteins levels were determined by densitometric quantitation. M protein levels (histograms) were set at 1 in NT siRNA-treated cells as well as in EV-transfected cells. (C) Levels of M protein in cells treated with siRNA or transfected with the indicated plasmids and then infected with VSV-PeGFP or VSV-PeGFP-ΔG virus. Error bars represent the standard errors of means from 2 independent experiments.

To ascertain if PCBP2 affects VSV growth at the level of virus budding, we utilized VSV-PeGFP-ΔG virus, which lacks the G coding sequence in the viral genome. Infectious ΔG viruses can be generated from transfected cells by complementing with G protein (65). Since VSV G protein is required for virus budding and infectivity (8, 43), the ΔG virus is defective in producing infectious progeny virions and undergoes only a single round of infection. Using VSV-PeGFP-ΔG, we observed that the level of viral gene expression was increased by approximately 3-fold when the cells were depleted of PCBP2 and decreased similarly when PCBP2 was overexpressed (Fig. 7B). More importantly, the level of increase or decrease in viral gene expression was similar whether the budding-competent VSV-PeGFP or budding-incompetent VSV-PeGFP-ΔG virus was used in these studies (Fig. 7C). Taken together, these studies suggest that PCBP2 primarily inhibits VSV gene expression without adversely affecting the viral entry and uncoating or budding steps.

To further determine if PCBP2 affects VSV RNA synthesis at the level of transcription, replication, or both, we examined the effects of depletion or overexpression of PCBP2 on viral primary transcription. Immediately after uncoating in the cytoplasm, the input RNP undergoes primary transcription to produce viral mRNAs, which are then translated to produce viral proteins that are required for subsequent replication and secondary transcription. In the presence of inhibitors of protein synthesis, such as cycloheximide, viral RNA synthesis will be limited to primary transcription. We therefore conducted an experiment in which the cells were treated with PCBP2 siRNA and then infected with VSV in the presence of cycloheximide. VSV mRNAs resulting from primary transcription alone were quantitated by semiquantitative RT-PCR. Results showed that synthesis of VSV N mRNA was increased greater than 10-fold when PCBP2 was depleted, whereas in cells overexpressing PCBP2, N mRNA synthesis was reduced by approximately 3-fold (Fig. 8A). Examination of VSV P mRNA by semiquantitative RT-PCR showed that the levels of this mRNA were also affected similarly (data not shown), indicating that the effect of PCBP2 is not specific to viral N mRNA transcription. A similar effect on the overall level of transcription in the absence of cycloheximide was observed (data not shown), indicating that PCBP2 inhibits viral mRNA transcription. Examination of viral antigenome (replication product) levels in the absence of cycloheximide revealed that depletion of PCBP2 led to increased genome replication, whereas its overexpression resulted in inhibition of replication (Fig. 8B). These results suggest that PCBP2 negatively affects VSV mRNA synthesis and genome replication.

Although overall growth and gene expression were not influenced by PCBP1 (Fig. 3 and 4), considering its overlapping functions with PCBP2, we examined if PCBP1 might affect certain steps in VSV gene expression. Interestingly, using the same experimental settings as above, we found that cells depleted of PCBP1 could support more viral primary transcription (~8-fold) and, conversely, that overexpression of PCBP1 resulted in a greater-than-2-fold reduction of primary transcription (Fig. 8C). However, the inhibitory effect of PCBP1 on viral genome replication was not observed (Fig. 8D). Taken together, the results indicate that PCBP1 exerts its inhibitory effect at the primary transcription step.

DISCUSSION

The P protein of VSV is the noncatalytic subunit of the viral RdRp. Its phosphorylation status and interactions with the other viral proteins involved in viral genome transcription and replication regulate its activity in these processes (20, 35). Furthermore, P has been shown to play a role in assembly of infectious virus particles (19). Since the P protein regulates multiple functions in the life cycle of VSV, we reasoned that it must interact with many host cell proteins. Currently, little is known about the host cell proteins that interact and modulate

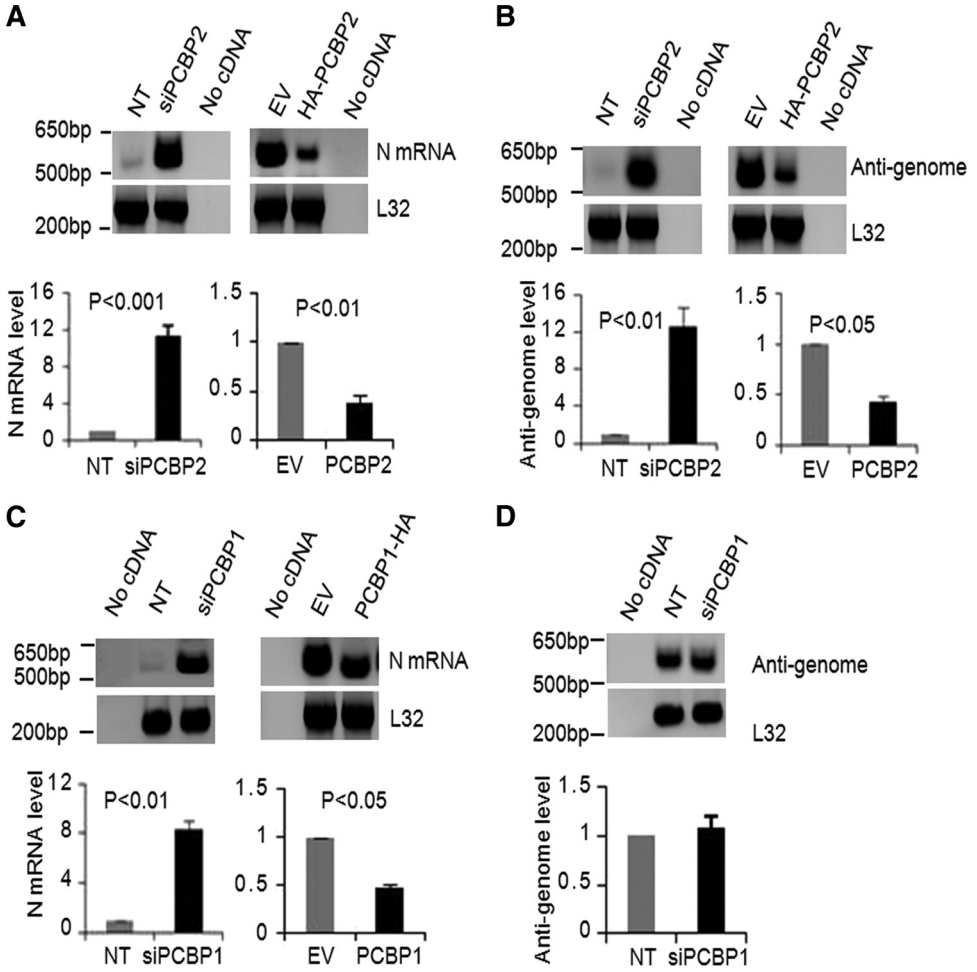

FIG. 8. PCBP2 and PCBP1 restrict VSV at the primary transcription step. HEK293 cells were treated with 10 nM siRNA for NT, PCBP2, or PCBP1 for 68 to 72 h or transfected with 2 μg of EV-, HA-PCBP2-, or HA-PCBP1-carrying plasmids for 48 h. (A and C) VSV primary transcription assays. Cells were treated with cycloheximide for 1 h prior to infection with VSV-eGFP at an MOI of 50 for 2.5 h. Total RNA was extracted from cells by using TRIzol. Subsequently, cDNA was synthesized with oligo(dT) primer followed by PCR with specific primers binding to VSV N mRNA or primers for the mRNA of cellular ribosomal protein L32 as an internal control. The level of N mRNA was normalized to 1 in NT siRNA-treated cells or in EV-transfected cells. "No cDNA," PCR amplification performed without cDNA in the reaction mixture. (B and D) VSV replication (antigenome) assays. Cells were infected with VSV-eGFP at an MOI of 0.2 for 3.5 h, followed by total RNA extraction and semiquantitative RT-PCR using specific primers complementary to P and M coding regions to detect VSV antigenomic RNA. Ribosomal protein L32 was used as an internal control. Error bars represent the standard errors of means from 3 independent experiments. RT-PCR was performed for 25 cycles with samples from PCBP depletion experiments or 35 cycles with samples from protein-overexpressing cells.

functions of the P proteins of VSV. One early report suggested that the P protein associates with c-src (6); however, the functional significance of this association is unclear. Interaction of the P protein of rabies virus with dynein light chain LC8 has been shown to be required for its activity in viral genome transcription and replication (52, 56). The P proteins of rabies virus and Borna disease virus have also been shown to antagonize the IFN response (13, 50, 60). The Borna disease virus P protein interacts with GABARAP, and this interaction results in relocalization of GABARAP from the cytoplasm to nucleus (50). The nucleocapsid proteins of negative-strand RNA viruses also interact with host proteins and regulate virus RNA synthesis. Cyclophilin A binds to the N protein and is a required factor for replication of VSV_{NJ} but not VSV_{I} (10). Recently, peroxiredoxin 1 was identified as a binding partner of the nucleocapsid protein of measles virus and was found to be required for its replication and transcription (63). Hantavirus nucleocapsid protein binds to a ribosomal protein, RPS19, and mediates preferential translation of viral mRNA (14). Thus, several viral replication proteins appear to interact with cellular proteins to modulate a variety of functions required for successful replication of the virus in the host.

In an attempt to identify the cellular proteins that interact with VSV P protein and understand the functional importance of these interactions, we employed an IP assay to pull down host factors interacting with the VSV P protein and subsequently used MS analysis to identify these proteins. Among the many cellular proteins identified, we focused our studies on PCBP2 and PCBP1, two members of a family of hnRNPs, which are known to regulate transcription, translation, and mRNA stability in cells (46). In this study, we demonstrated that PCBP2 and PCBP1 function as negative regulators of

VSV infection in human cell types, including HeLa and HEK293. Further examination revealed that these two proteins specifically inhibit viral gene expression.

Although PCBP2 and PCBP1 are two isoforms of the same family of proteins with nearly 90% sequence identity (58), the inhibitory effects of PCBP2 on VSV growth and viral gene expression were significantly more pronounced than those of PCBP1 (Fig. 3 and 4). The reasons for this are unknown at this time but could be in part due to the fact that the amount of PCBP1 is lower in the cytoplasm, where VSV replicates exclusively. It is also possible that the inherent differences in their inhibitory activities on VSV could be due to their associations with other cellular interacting partners. Members of the PCBP family are known to form functional multimers with other cellular proteins for their shuttling between the nucleus and the cytoplasm as well as for their functions. PCBP2 is known to interact with at least hnRNP K, I, and L (37). Furthermore, PCBP2 interacts with myotonic dystrophy type 2 protein ZNF9 in regulating ornithine decarboxylase expression (30) and associates with the nucleo-cytoplasmic SR protein (SRp20) to modulate poliovirus genome translation (5), whereas PCBP1 acts together with hnRNP A2 in negatively controlling the expression of mRNA containing A2 response elements (38). Whether the associations of different cellular proteins with PCBP2 and PCBP1 are responsible for the observed differences in inhibition of VSV growth and gene expression is unknown, and further studies along these lines may help us understand more about the inhibitory effects of these two proteins on VSV. Additionally, PCBP1 and PCBP2 form functional homo- and heterodimers in supporting translation and replication of poliovirus genome RNA. It has been shown that PCBP1 can rescue poliovirus replication when PCBP2 is depleted in the cell (29, 62), demonstrating a functional cooperation between these two proteins in poliovirus replication. Such cooperation does not seem to exist in inhibiting or enhancing VSV replication, as overexpression or depletion of both proteins simultaneously did not inhibit or enhance VSV growth significantly (data not shown) compared to the individual proteins.

To understand how PCBP2 is involved in inhibiting VSV growth and viral gene expression, we examined whether PCBP2 interfered with the formation of viral replication protein complexes, which are required for transcription and replication of the VSV genome. However, our studies showed that the interaction of PCBP2 with VSV P protein did not interfere with the formation of complexes of N-P or L-P or the homo-oligomerization of P-P protein. A recent study showed that PCBP2 interacts with MAVS and mediates its degradation through the proteasome pathway via ubiquitination by the E3 ubiquitin ligase AIP4 (67). Since the proteasome pathway is also involved in inhibition of VSV (33), we examined whether degradation of P through the proteasome pathway by ubiquitination might account for the inhibition of VSV by PCBP2. Although the studies reported here successfully demonstrated for the first time ubiquitination of the VSV P protein and its degradation through the proteasome pathway, the results also sugges that PCBP2 does not directly target the P protein for degradation. So, the inhibition mediated by PCBP2 is independent of the proteasome pathway. Therefore, the exact mechanism by which PCBP2 exerts its inhibitory effect remains to be elucidated. Further studies on PCBP2-interacting proteins may reveal how PCBP2 inhibits VSV growth and gene expression.

The effects of PCBP2 and PCBP1 on replication of other viral pathogens have been found to be variable. For example, PCBP2 binds the 5′-untranslated region (UTR) and enhances translation of the internal ribosome entry site (IRES) containing mRNA of several positive-strand RNA viruses, such as poliovirus, enterovirus, hepatitis A virus, and coxsakievirus (24, 31, 45, 55). On the other hand, its binding to the 5′-UTR of HCV has no effect on translation of HCV IRES-containing genomic RNA (28). Additionally, PCBP1 but not PCBP2 mediated a strong inhibition of gene expression steps in HIV-1 infection (66). While a role for PCBP2 and PCBP1 has not been reported for replication of any of the negative-strand RNA viruses yet, our findings that PCBP2 and PCBP1 are antagonists for VSV infection provide the first example of the involvement of these proteins in gene expression of this class of RNA viruses. The results underscore the complexity of PCBP regulatory functions in virus-infected cells. PCBP2 is induced following virus-mediated IFN induction. Although it downregulates the IFN response stimulated by viral pathogens (67), in this study we showed that it suppresses VSV growth by targeting viral gene expression. Other interferon-induced proteins, such as IFITM3, tetherin, and Ars2, have been shown recently to inhibit VSV replication at different stages of infection. While Ars2 (54) restricts VSV replication at the level of viral RNA synthesis, IFITM3 targets an early event after endocytosis and before primary transcription, and tetherin blocks virion budding from infected cells (64). Thus, the host cells employ multiple and redundant mechanisms to restrict replication of an invading pathogen. In concordance with a previous report (67), we also observed an increase of PCBP2 protein level in VSV-infected cells (data not shown). Thus, PCBP2 seems to restrict VSV replication at the level of primary and secondary transcription as well as genome replication, whereas the role of PCBP1 in VSV replication appears to be more restricted. While the viral genome replication was not adversely affected by PCBP1, only the primary transcription was affected negatively by this protein. Additional studies will be needed to understand further the role of PCBP1 in inhibiting VSV primary transcription. Moreover, oligoadenylate synthetase (OAS) and RNase L, which are also induced by IFN (57), are involved in blocking VSV protein synthesis and thus virus replication (1, 4). Since PCBP2 mRNA is the activator for OAS (44), it would be interesting to examine the relationship between PCBP2, OAS, and RNase L in restricting replication of VSV and other viruses.

The findings reported here add to the ever-increasing list of functions reported for PCBP2 and PCBP1. We have demonstrated the effects of PCBP2 and PCBP1 at the level of viral gene expression during VSV infection. It is possible that PCBP2 and PCBP1 affect the viral mRNA transcription process itself, or mRNA stability, or both. PCBP2 and PCBP1 have been implicated in negatively regulating the stability of p21 mRNA (61). Furthermore, both PCBP1 and PCBP2 have been shown to be components of stress granules (SG) and processing bodies (P-bodies), and thus they have been proposed to participate in targeting newly synthesized mRNA to P-bodies for storage and/or degradation (26, 27). It has been reported that siRNA-mediated depletion of TIA-1, a compo-

nent of SG, results in increased replication of VSV (40). Our preliminary data suggest that VSV induces SG formation in infected cells (P.X.D. and A.K.P., unpublished data). It is possible that PCBP2 and PCBP1 directly interact with viral RNA species (like the genome, antigenome, mRNA, and leader and trailer RNAs) to sequester them in SG, thereby inhibiting viral gene expression, or that their binding impedes the accessibility of the P-L complex to viral RNAs and thus reduces viral gene expression.

In summary, our results demonstrate the antagonistic effects of PCBP2 and PCBP1 on VSV infection in cell culture systems. The inhibitory effects were shown to be at the level of viral gene expression. The molecular mechanism(s) by which PCBP1 and PCBP2 exert their inhibitory effects on VSV RNA synthesis are under investigation.

ACKNOWLEDGMENTS

We thank Y. Zhou and Terry Fangman (UNL Microscopy Core Facility) for help in fluorescence microscopy and Ron Cerny (UNL Mass Spec Core Facility) for performing and analyzing the MS experiment data.

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