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An Efficient *Trans* Complementation System for In Vivo Replication of Defective Poliovirus Mutants

Efficient *trans* Complementation of PV Replication

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Abstract: The picornavirus genome encodes a large single polyprotein that is processed by viral proteases to form an active replication complex. The replication complex is formed with the viral genome, host proteins, and viral proteins that are produced/translated directly from each of the viral genomes (*cis*-provided viral proteins). Efficient complementation *in vivo* of replication complex formation by *trans*-provided viral proteins, thus exogenous or ectopically expressed viral proteins, remains to be demonstrated. Here we report an efficient *trans*-complementation system for the replication of defective poliovirus (PV) mutants by a viral polyprotein precursor in HEK 293 cells. Viral 3AB in the polyprotein, but not 2BC, was processed exclusively in *cis*. Replication of a defective PV replicon mutant, with a disrupted cleavage site for viral 3C^{pro} protease between 3C^{pro} and 3D^{pol} (3C/D[A/G] mutant) could be rescued by a *trans*-provided viral polyprotein. Only a defect of 3D^{pol} activity, of the replicon could be rescued in *trans*; inactivating mutations in 2C$^{ATPase/hel}$, 3B, and 3C^{pro} of the replicon completely abrogated the *trans*-rescued replication. An intact N-terminus of the 3C^{pro} moiety, of the *trans*-provided 3CD^{pro}, was essential for the *trans*-rescue activity. By using this *trans*-complementation system, a high-titer defective PV pseudovirus (> 10^7 infectious units per mL) could be produced with the defective mutants, whose replication was completely dependent on *trans*-complementation. This work reveals potential roles of exogenous viral proteins in PV replication and offers insights into protein/protein and protein/RNA interactions during picornavirus infection.

Keywords: engineered cells; virus; picornavirus; enterovirus; *trans* complementation

Importance

Viral polyprotein processing is an elaborately controlled step by viral proteases encoded in the polyprotein; fully processed proteins and processing intermediates need to be correctly produced for replication, which can be detrimentally affected even by a small modification of the polyprotein. Purified/isolated viral proteins can retain their enzymatic activities required for viral replication, such as protease, helicase, polymerase, etc. However, when these proteins of picornavirus are exogenously provided (also called *trans*-provided) to the viral replication complex with a defective viral genome, replication is generally not rescued/complemented, suggesting the importance of viral proteins endogenously provided (also called *cis*-provided) to the replication complex. In this study, we discovered that only the viral polymerase activity of poliovirus (the typical member of picornavirus family) could be efficiently rescued by exogenously expressed viral proteins. The current study reveals potential roles for exogenous viral proteins in viral replication and offers insights into interactions during picornavirus infection.

1. Introduction

Picornavirus is a small non-enveloped virus with a positive-sense single-stranded RNA genome of about 7,500 nt, including poliovirus (PV) as the typical member of this family (*Enterovirus C* species, the genus *Enterovirus,* in the family *Picornaviridae*) (1). The genome of PV encodes a single large

polyprotein (about 2,200 amino acid[aa] residues) that is subsequently processed into each viral protein, and a small protein derived from an upstream open reading frame (uORF) (2). The polyprotein is initially processed into three precursor proteins, P1 (coding VP4VP2VP3VP1), P2 (coding 2A2B2C), and P3 (coding 3A3B3C3D), by viral proteases (2A^{pro} and 3C^{pro}/3CD^{pro}/3ABC^{pro}) (3-5). Subsequently, P1 is processed into viral capsid proteins (VP4VP2[VP0], VP3, VP1), P2 is processed into proteins that have roles in viral RNA synthesis and also in virion production/release (2A^{pro} protease, 2B viroporin, 2C$^{ATPase/hel}$ ATPase/helicase) (3, 6-10), and P3 is processed into proteins that most directly serve for RNA synthesis (3A [unknown enzymatic function / recruitment of host proteins GBF1/ACBD3/PI4KB], 3B [also known as VPg, the primer for RNA synthesis], 3C^{pro} proteases, 3D^{pol} polymerase) (1, 11-17). The uORF protein might pose a tissue-specific role in virus growth in gut epithelial cells (2), thus is absent from some members of enterovirus (EV) (*i.e.*, *Enterovirus D* and *Rhinovirus*), which prefer infection in the upper respiratory tract rather than in the gut.

As well as the completely processed viral proteins, processing intermediates, derived from P1, P2 or P3 (i.e., 2BC, 3AB, 3CD^{pro}, etc.) are also produced from the polyprotein. Other processing intermediates that span these precursors (*e.g.*, 2C3AB, 2ABC3AB) are produced but only in trace amounts (18, 19). Both processing intermediates and fully processed proteins have critical roles in replication, such as remodeling of the endoplasmic reticulum by 2BC and 3A (20), stimulation of 3D^{pol} activity by 3AB (21-23), efficient cleavage of P1 (4), switching of the viral genome from translation to RNA replication (24, 25), and stimulation of uridylylation of 3B by 3CD^{pro}(26). Disruption of each of the processing intermediate was lethal for infectivity (27), indicating the importance of the processing intermediates in replication.

RNA replication of PV predominantly depends on *cis*-provided viral proteins (28-30). Processing is critically controlled by *cis* cleavage (*i.e.*, cleavage of the polyprotein by 2A^{pro}/3C^{pro}/3CD^{pro}/3ABC^{pro}, which are coded in the target polyprotein itself, thus authentic self-cleavage) and by *trans* cleavage (*i.e.*, cleavage of polyprotein by the 2A^{pro}/3C^{pro}/3CD^{pro}/3ABC^{pro}, which are coded in polyproteins other than the target polyprotein). Disruptions of the polyprotein downstream of the 2B (27) or introduction of mutations in the 2B2CP3 region without affecting the protease activity (31-35) interfered with *cis* cleavage, resulting in aberrant processing and lethality of virus, underscoring the importance of *cis* cleavage in picornavirus replication and of an intact 2B2CP3 precursor. Recently, involvement of a host PI4KB/OSBP family I (OSBP and OSBP2/ORP4) pathway (16, 36) in processing of 3AB was discovered (37-39). The pathway is essential for the development of a viral replication organelle (RO) (40), formation of the replication complex and synthesis of viral plus-strand RNA (41-43), and enhancement of viral growth and infectivity (44). PI4KB and OSBP family I are the target of anti-EV drug candidates (36, 45-50), suggesting that polyprotein processing is a promising target for antiviral development. Enigmatically, processing of 3AB occurs inefficiently especially in the early phase of replication (51); only 4% of 3AB is processed to provide 3A and 3B for RNA synthesis. Interestingly, resistant PV mutants against PI4KB/OSBP inhibitors have mutations in the 3A region, which enhance the processing of 3AB (37, 40), suggesting that cleavage of 3AB or the final products (3A and 3B) could be a target of exogenous intervention to control infection.

Here we have developed an efficient *trans* rescue system for *in vivo* replication of defective PV replicons targeting polyprotein processing. We established cell lines that could conditionally express an entire precursor protein and analyzed *cis* and *trans* roles of viral proteins. We found that cleavage of 3AB occur exclusively in *cis* in the polyprotein. Among the defective PV replicon mutants examined, only a mutant (3C/D[A/G]) that has a disrupted cleavage site between 3C^{pro} and 3D^{pol} showed an efficient *trans*-rescued replication and produced pseudovirus at a high titer, comparable to that of the WT replicon. We identified an intact 3CD^{pro} as the minimal protein required for *trans* rescue.

2. Results

Doxycycline (DOX)-inducible expression of PV non-structural proteins. To analyze the potential role of *trans*-provided PV proteins in replication, we generated a HEK293 cell line (Tet-AG-

PV-2B2CP3[WT]) that could express a polyprotein of PV non-structural proteins (2BC3ABCD) in the presence of DOX (1 mg/L) as a form of an N-terminally Azami green (AG)-fused protein, which allowed a high expression level of protein (52) (Figure 1). Expression of the AG-fused PV polyprotein caused rounded morphology of the cells, similar to the cytopathic effect observed in PV-infected cells. Localization of AG, which is cleaved from the polyprotein by 3C^{pro}, was in the nucleus and cytoplasm (Figure 1A). In the presence of a reversible 3C^{pro} inhibitor GC376 (53), a normal morphology of the cells was retained even after protein expression. In the presence of GC376, AG, which remained attached to the polyprotein, showed a dot-like localization in the cytoplasm, in contrast to that of free AG. Processing of the polyprotein was completely inhibited in the presence of GC376 or rupintrivir (also known as AG7088, an irreversible 3C^{pro} inhibitor) (54), and the polyprotein (161 kDa) remained as an intact precursor (Figure 1A). In the absence of, or at lower concentration of the 3C^{pro} inhibitors, processing intermediates (P3/3ABCD, 3CD^{pro}) and a fully processed viral protein (3D^{pol}) was detected by anti-3D^{pol} antibody similar to those present in PV-infected cells (Figure 1B). These results suggested that Tet-AG-PV-2B2CP3(WT) cells could express a PV polyprotein precursor, which was subsequently processed in the absence of 3C^{pro} inhibitors.
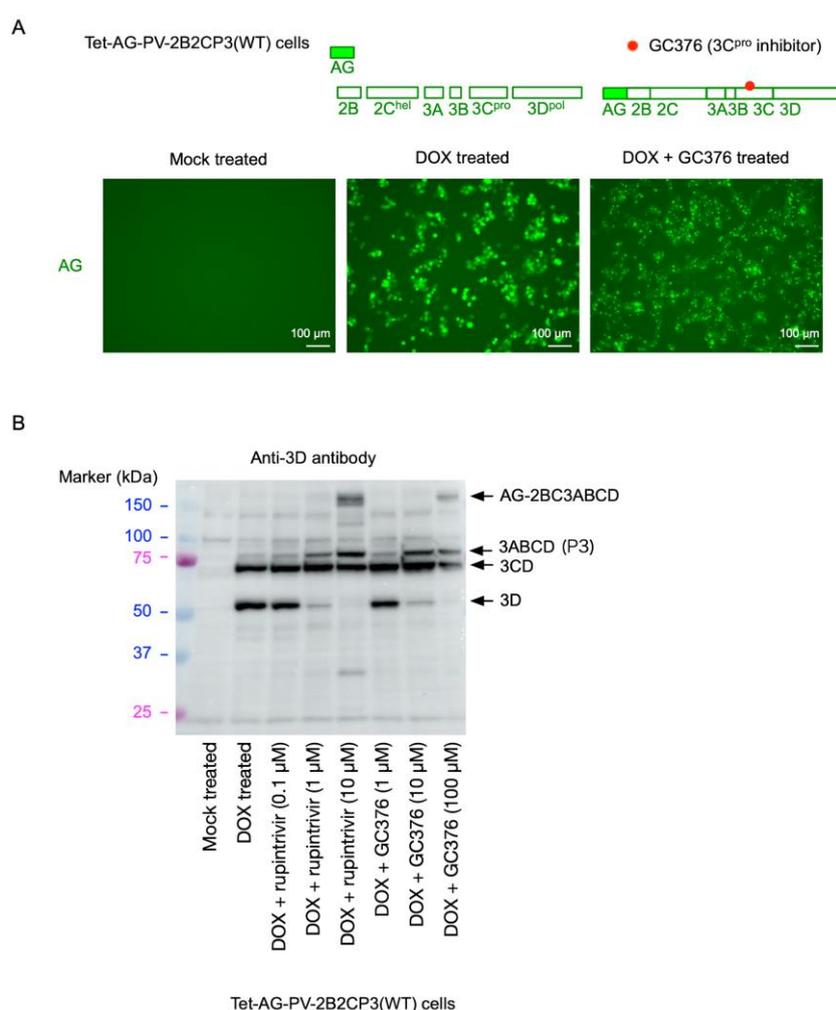

Figure 1. Doxycycline (DOX)-inducible expression of PV non-structural proteins in Tet-AG-PV-2B2CP3(WT) cells. (A) Expression of PV non-structural proteins in Tet-AG-PV-2B2CP3(WT) cells. PV non-structural proteins were expressed as a form of an N-terminally Azami green (AG)-fused single polyprotein in the presence of doxycycline (DOX). The cells were treated with DOX (1 mg/L) for 17 h in the presence of absence of a 3C protease inhibitor GC376 (100 μM). Fluorescent microscope images of the cells with or without DOX treatment are shown. (B) Western blot analysis of the expressed proteins in the cells. Tet-AG-PV-2B2CP3(WT) cells were treated with or without DOX for 17 h in the

presence of absence of 3C protease inhibitors GC376 (1, 10, or 100 μM) or rupintrivir (0.1, 1, or 10 μM). Processing intermediates of the polyprotein were detected with an anti-3D antibody.

Trans **rescue of replication of defective PV replicons.** Next, we attempted to *trans* rescue defective PV replicon mutants in Tet-AG-PV-2B2CP3(WT) cells (Figure 2A). Tet-AG-PV-2B2CP3(WT) cells were first treated with DOX and GC376 to induce expression of the unprocessed polyprotein. The cells were then washed to remove DOX and GC376, and then were transfected with RNA transcripts of the defective PV replicon mutants. Replication was monitored by firefly luciferase or mCherry reporters encoded in the replicons. PV replicon mutants that have disrupted cleavage sites (aa substitution of the conserved Q/G to A/G in the cleavage site) for 3C^{pro} between the viral proteins (37, 55, 56), to inhibit production of fully processed viral proteins (*i.e.*, 2A^{pro}, 2B, 2C$^{ATPas/hel}$, 3A, 3B, 3C^{pro}, and 3D^{pol}) or lack each of the viral genes were examined (Figure 2B).
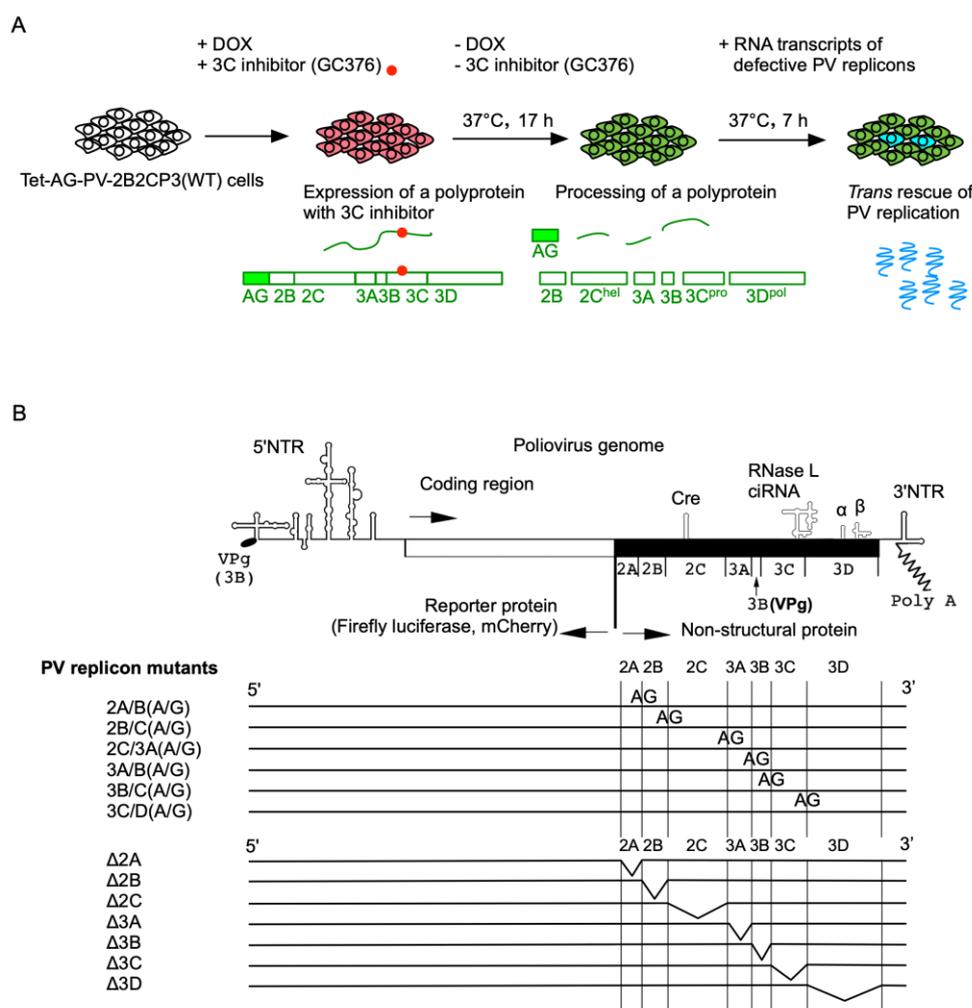

Figure 2. Experimental design of *trans* rescue of replication of defective PV replicons. (A) Schematic view of the *trans*-rescue experiment. PV non-structural proteins were expressed in the presence of DOX (1 mg/mL) and GC376 (100 μM) at 37°C for 17 h. Then, RNA transcripts of each PV replicon mutants that have firefly luciferase or mCherry reporters were transfected into cells in the absence of DOX and GC376. The luciferase signals or fluorescence of mCherry in the cells were analyzed at 7 h p.t. of RNA transcripts. (B) Schematic view of PV replicon mutants. Firefly luciferase or mCHerry was used as reporters for replication. Introduced amino acid substitutions in the cleavage sites by 3C^{pro} or deletions of each viral gene are shown.

In Tet-AG-PV-2B2CP3(WT) cells, without DOX treatment, only the WT and Δ2A mutant could replicate (Figure 3A), consistent with a previous report of a viable 2A^{pro} deletion mutant (57). Other mutants produced only basal levels of signals in the cells, which are derived from initial protein

synthesis from transfected RNA transcripts and could not be suppressed in the presence of a PV replication inhibitor GuHCl (a $2C^{ATPas/hel}$ inhibitor), suggesting no replication occurred. In Tet-AG-PV-2B2CP3(WT) cells treated with DOX and GC376 before RNA transfection, interestingly, a mutant (3C/D[A/G]) could replicate as well as the WT and Δ2A mutant. Replication of 2B/C(A/G) and Δ2B mutants could be detected, albeit at low levels (2- to 4-fold increment compared to that in GuHCl-treated cells) (Figure 3B). This suggested that each viral protein (*i.e.*, 2B, $2C^{ATPas/hel}$, 3A, 3B, $3C^{pro}$, and $3D^{pol}$) is essential for replication and can not be *trans* complemented. In addition, replication of a defective PV replicon, which could express $3CD^{pro}$ but not $3C^{pro}$ and $3D^{pol}$, could be efficiently *trans* rescued by a viral polyprotein.
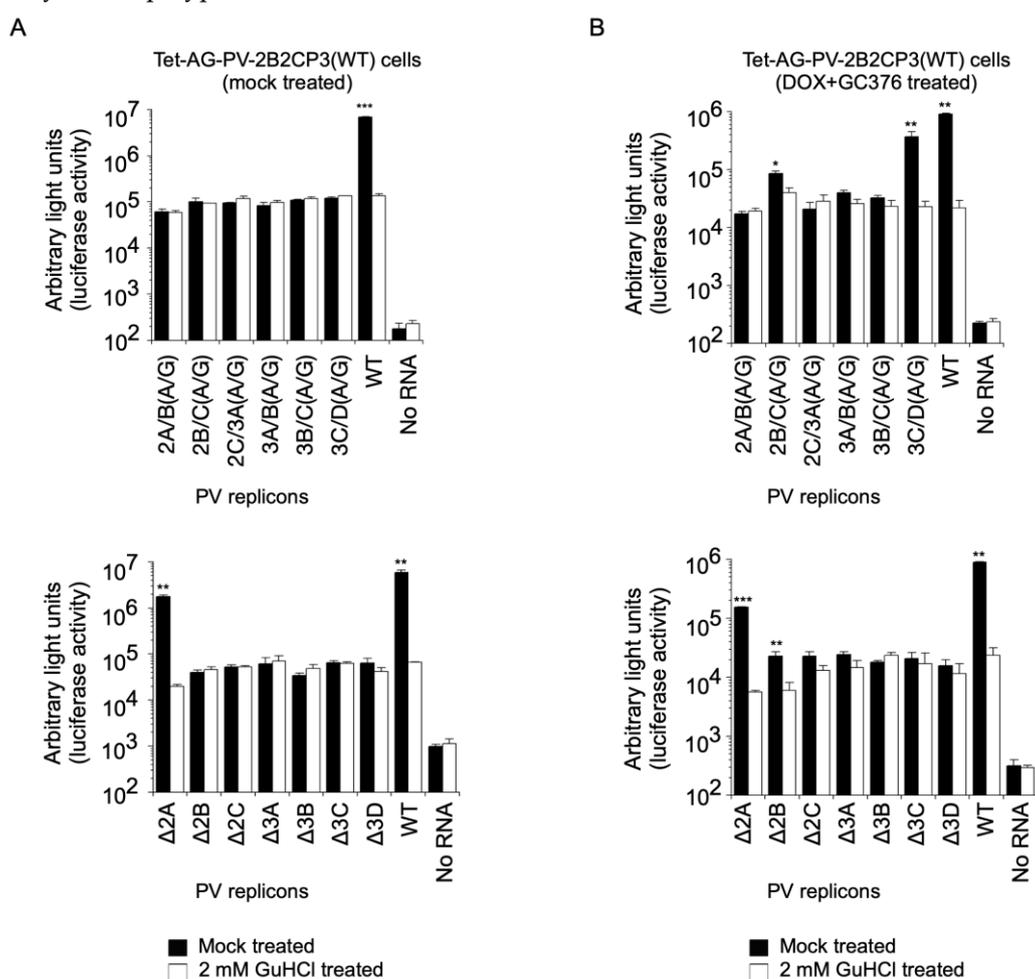

Figure 3. *Trans* rescue of replication of defective PV replicons in Tet-AG-PV-2B2CP3(WT) cells. Without DOX treatment (A) or after DOX and GC376 treatment (B), the cells were transfected with RNA transcripts of each replicon that have firefly luciferase reporter, in the presence of absence of GuHCl (a 2C inhibitor). The luciferase signals measured at 7 h p.t. of the RNA transcripts are shown.

Cis role of viral proteins in the replication of a PV 3C/D(A/G) mutant. Next, to determine the roles of *cis*-provided viral proteins in PV replication, mutations that inactivate activities of viral proteins were introduced into the 3C/D(A/G) mutant (Figure 4A): 2C-K153A aa substitution that disrupts ATPase activity of $2C^{ATPas/hel}$) (6), 3B-Y3F aa substitution that inhibits uridylylation of the 3B protein (58), 3C-C147A aa substitution that inactivates the protease activity of $3C^{pro}/3CD^{pro}$ by disruption of the catalytic triad (59), and 3D-D328N/D329N, aa substitutions that inactivate the polymerase activity of $3D^{pol}$ (60). In Tet-AG-PV-2B2CP3(WT) cells treated with DOX and GC376 before RNA transfection, no replication was observed for mutants with inactivated $2C^{ATPas/hel}$, 3B, or $3C^{pro}/3CD^{pro}$ (Figure 4B). In contrast, a mutant with inactivated $3D^{pol}$ (3C/D[A/G]-3D-D328N/D329N)

replicated as well as the WT and parental 3C/D(A/G) mutant. This suggested that the polymerase activity of the 3C/D(A/G) mutant can be *trans* rescued and that *cis* activities of viral proteins (2C$^{ATPas/hel}$, 3B, and 3C^{Pro}/3CD^{Pro}) are essential for replication.
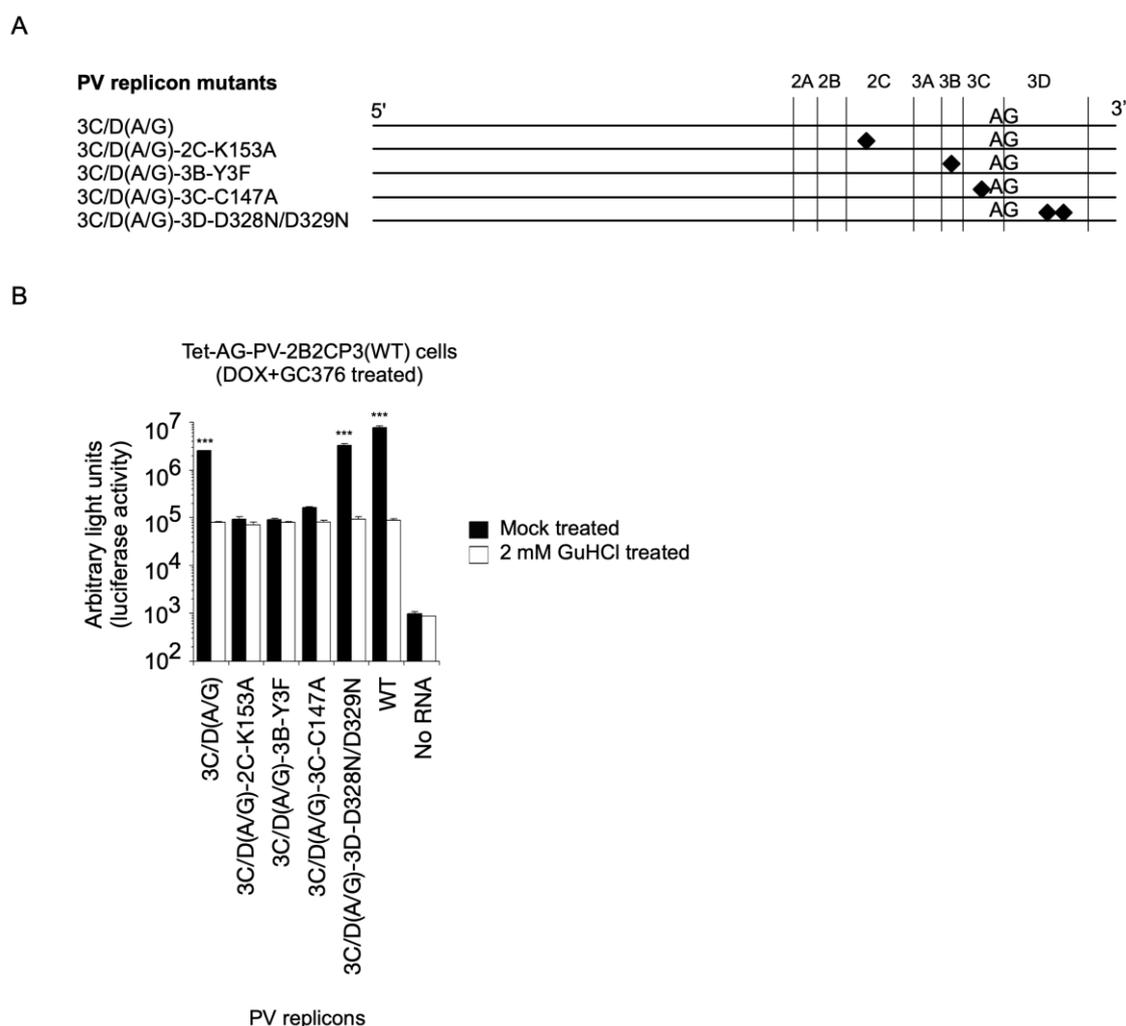

Figure 4. *Cis* roles of viral proteins in *trans*-rescued replication of defective PV replicons. (A) Schematic view of defective PV replicon mutants with a disrupted cleavage site between the 3C and 3D regions. Firefly luciferase was used as reporter of the replication. (B) *Trans* rescue of replication of defective PV replicon mutants in Tet-AG-PV-2B2CP3(WT) cells. After DOX and GC376 treatment for 17 h, the cells were transfected with each RNA transcript of the replicon that has firefly luciferase reporter, in the presence of absence of GuHCl. The luciferase signals measured at 7 h p.t. of the RNA transcripts are shown.

Identification of minimal viral proteins required for *trans* rescue of the replication of defective PV replicons. Because the polymerase activity seemed to be the target of the *trans* rescue of the 3C/D(A/G) mutant, we generated a HEK293 cell line (Tet-AG-PV-3CD[WT]) that expresses PV 3CD^{Pro} as a form of an N-terminally AG-fused protein in the presence of DOX, which could be self-processed into 3D^{Pol} with an intact N-terminus, which is essential for the polymerase activity (61) (Figure 5A). Fortuitously, a cell line (Tet-AG-PV-3CD[Δ4-5 aa]) that   expresses a 3CD^{Pro} variant (deletion of aa 4 and 5 of 3C^{Pro}, possibly derived from mutations in the oligo DNAs used for the cloning) was also produced. Both cell lines expressed AG-3CD^{Pro} and a processing intermediate 3CD^{Pro} and 3D^{Pol} in the presence of DOX (Figure 5B). Surprisingly, replication of the 3C/D(A/G) mutant was rescued only in Tet-AG-PV-3CD(WT) cells, but not in Tet-AG-PV-3CD(Δ4-5 aa) cells (Figure 5C), suggesting that an intact 3CD^{Pro} is essential and sufficient for the *trans* rescue. Expression

of N-terminally AG-fused 3D^{pol}, which could be processed by 3CD^{pro} of the 3C/D(A/G) mutant to give intact 3D^{pol}, could only partially *trans*-rescue the replication of this mutant (Figure S1). The WT replicon replicated to similar levels in both cell lines, suggesting that the effect was specific to the *trans*-rescued replication. To further analyze the specificity of *trans*-provided PV 3CD^{pro}, we attempted to rescue the replication of enterovirus 71 (EV-A71), which belongs to the *Enterovirus A* species, thus another species in EV. A mutation that disrupts the 3C^{pro} cleavage site between 3C^{pro} and 3D^{pol} was introduced in an EV-A71 replicon (EV-A71-3C/D[A/G] mutant), and then replication in cells was analyzed (Figure S2). In contrast to the PV replicon mutant, replication of the EV-A71-3C/D(A/G) mutant was not *trans* rescued by a PV polyprotein or 3CD^{pro}. These results suggested that the intact 3C^{pro} region of 3CD^{pro} and viral species-specific interaction of 3CD^{pro} is essential for the *trans*-rescue activity.
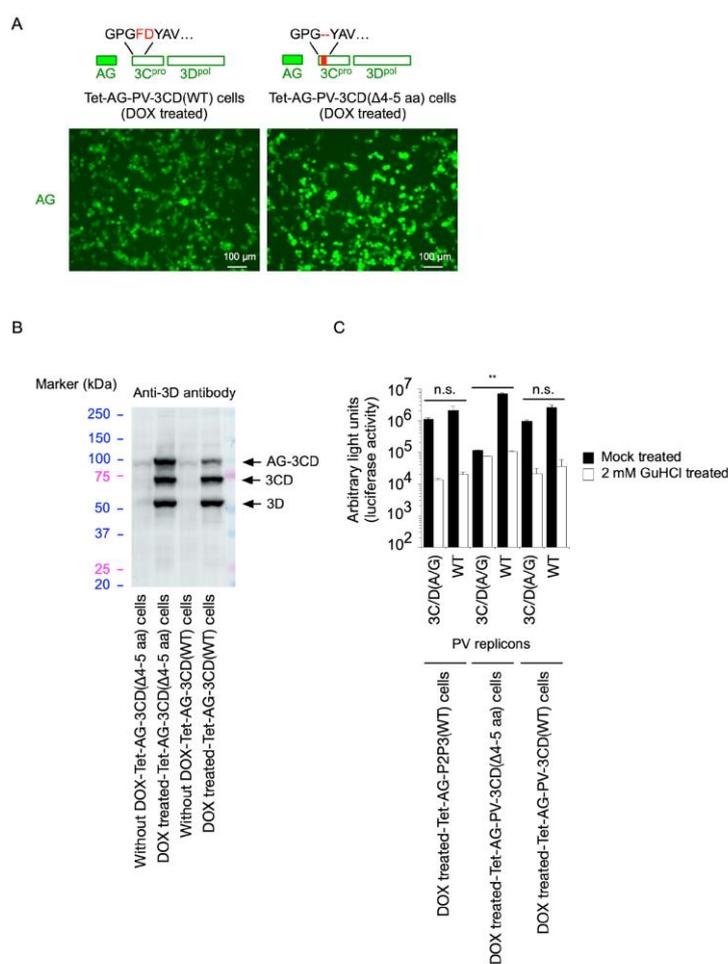

Figure 5. *Trans* rescue of replication of defective PV replicons by 3CD^{pro}. (A) Generation of HEK293 cell lines (Tet-AG-3CD(WT) and Tet-AG-3CD(Δ4-5 aa) that expresses PV 3CD proteins (WT or a Δ4-5 aa variant) in the presence of DOX. Fluorescent microscope images of the cells for AG after DOX treatment for 17 h are shown. (B) Western blot analysis of the expressed proteins in the cells. The cells were treated with or without DOX for 17 h. Processing intermediates of the 3CD proteins were detected by anti-3D antibody. (C) *Trans* rescue of replication of a defective PV replicon in Tet-AG-3CD(WT) and Tet-AG-3CD(Δ4-5 aa) cells. After the DOX and GC376 treatment for 17 h, the cells were transfected with each RNA transcript of the replicon that has firefly luciferase reporter, in the presence or absence of GuHCl. The luciferase signals measured at 7 h p.t. of the RNA transcripts are shown.

Trans rescue of replication of defective PV replicons by 3CD^{pro}. To clarify the role of 3C^{pro} for the *trans* rescue activity of 3CD^{pro}, we introduced aa substitutions into the 3C^{pro} moiety of 3CD^{pro},

focusing on those involved in the binding to viral RNA and phospholipids (4, 24, 26, 62). We introduced 3C-R13N, 3C-K82N, and 3C-R84S aa substitutions into 3CDpro, which abolish the binding to viral RNA and phospholipids (24, 26, 62-64). We also introduced a mutation to disrupt a 3Cpro cleavage site between AG and the 3Cpro region to analyze the effect of potential steric hinderance for the interaction with target molecules around the N-terminus of 3CDpro on the *trans* rescue.

We generated HEK293 cell lines that could express these 3CDpro variants, and analyzed the *trans*-rescue activity for PV1(Fluc)pv(3C/D[A/G]) infection (Figure 6A). Unexpectedly, the 3CDpro variants (3C-R13N, 3C-K82N, and 3C-R84S) efficiently rescued the infection, higher than that by the 3CD(Δ4-5 aa) variant. In contrast, the 3CDpro variants with uncleavable AG could not substantially rescue the infection irrespective of the deletion in the N-terminal region of 3Cpro. This suggested that the integrity of N-terminus of 3Cpro, but not the binding activity to viral RNA or phospholipids, is essential for the *trans*-rescue activity of 3CDpro.
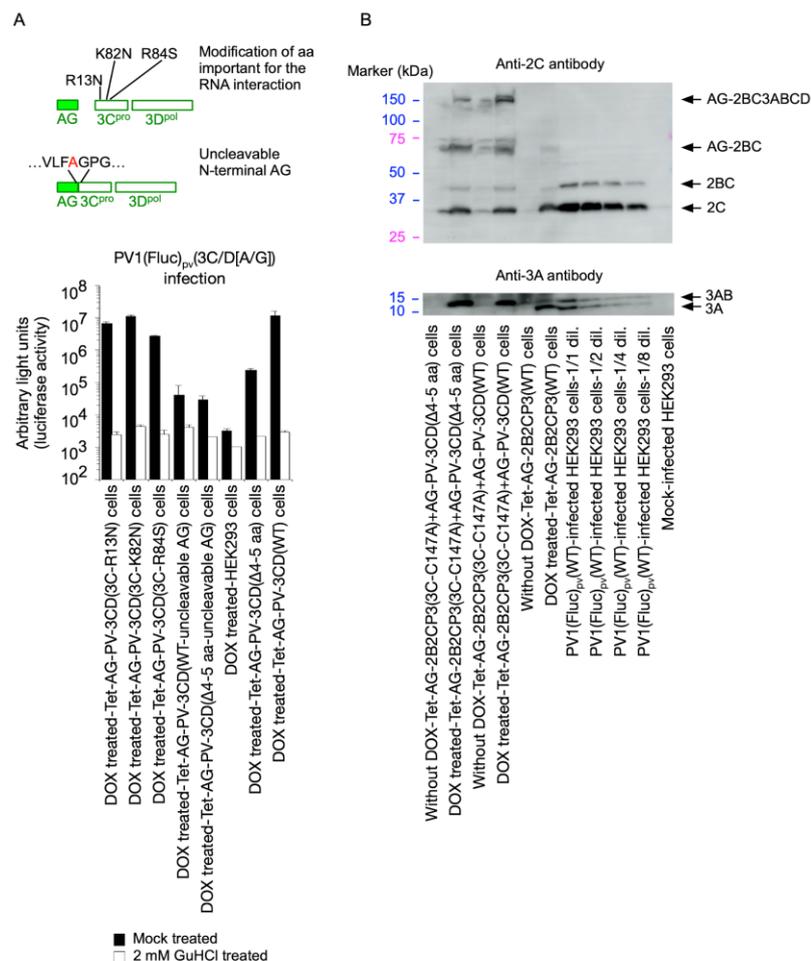

Figure 6. Effect of modifications of 3CDpro on *trans*-rescue activity. (A) Effects of amino acid substitutions that affect the interaction of 3CD with RNA or of N-terminal modification by addition of uncleavable AG on the *trans*-rescue activity. Cells expressing each AG-3CD variant were treated with DOX (1 mg/mL) at 37°C for 5 h, and then infected with PV1(Fluc)pv(3C/D[A/G]) at an MOI of 0.05, in the presence of absence of GuHCl. The luciferase signals measured at 17 h p.i. are shown. (B) *Trans*-cleavage activity of 3CD (WT and a Δ4-5 aa variant). Western blot analysis for the processing intermediates derived from a polyprotein are shown. Cells were treated with or without DOX for 17 h to co-express a polyprotein without the 3C protease activity conferred by a 3C-C147A substitution and AG-3CD (WT or a Δ4-5 aa variant). Processing intermediates derived from the polyprotein were detected by anti-2C or 3A antibodies. Lysates of Tet-AG-2B2CP3(WT) cells and serially diluted lysates

of PV1(Fluc)$_{pv}$ infected cells (dilution of 1/1 to 1/16) were taken as positive controls for the processing intermediates.

To analyze the effect of N-terminal modification of 3CD^{pro} on the *trans* activity, we generated HEK293 cell lines that could simultaneously express 3CD^{pro}(WT) or 3CD(Δ4-5 aa) variant with a PV polyprotein (2BC3ABCD), which lacks 3C^{pro} protease activity with a 3C-C147A aa substitution (59) (Tet-AG-PV-2B2CP3[3C-C147A]+AG-PC-3CD[WT] cells and Tet-AG-PV-2B2CP3[3C-C147A]+AG-PC-3CD[Δ4-5 aa] cells) (Figure 6B). Due to the lack of 3C^{pro} activity, the polyprotein remained intact without producing processing intermediates, and could not *trans*-rescue the replication of the 3C/D(A/G)) mutant (Figure S3). Simultaneous expression of this polyprotein with 3CD^{pro} (WT) or 3CD^{pro} (Δ4-5 aa) variant showed similar profiles of the precursor and processing intermediates AG-2BC, 2BC, 2C and 3AB; interestingly, no 3A but only 3AB was observed in these cells, suggesting that 3AB is the target of *cis* cleavage (Figure 6B). Both 3A and 3AB were observed in Tet-AG-PV-2B2CP3(WT) cells or in PV1$_{pv}$ infected cells. The 3AB protein was detected in the most diluted lysates of infected cells, at levels lower than those in the Tet-AG-PV-2B2CP3[3C-C147A]+AG-PC-3CD(WT) cells and Tet-AG-PV-2B2CP3[3C-C147A]+AG-PC-3CD(Δ4-5 aa) cells, confirming the absence of 3A in these cells. These results suggested that 3CD^{pro} (WT) and 3CD^{pro}(Δ4-5 aa) have similar *trans* cleavage activities and that processing of 3AB occurs exclusively in *cis* in the context of the polyprotein.

Production of PV pseudovirus (PV$_{pv}$) with defective PV replicons. To substantiate the observed high replication level of the defective PV replicons, we attempted to produce PV$_{pv}$ with the defective PV replicons and PV capsid proteins (65) (Figure 7). Replication-competent PV WT replicon could produce PV$_{pv}$ to a titer (10^7 to 10^8 infectious units [IU] per mL) comparable to that of PV virus (65, 66).
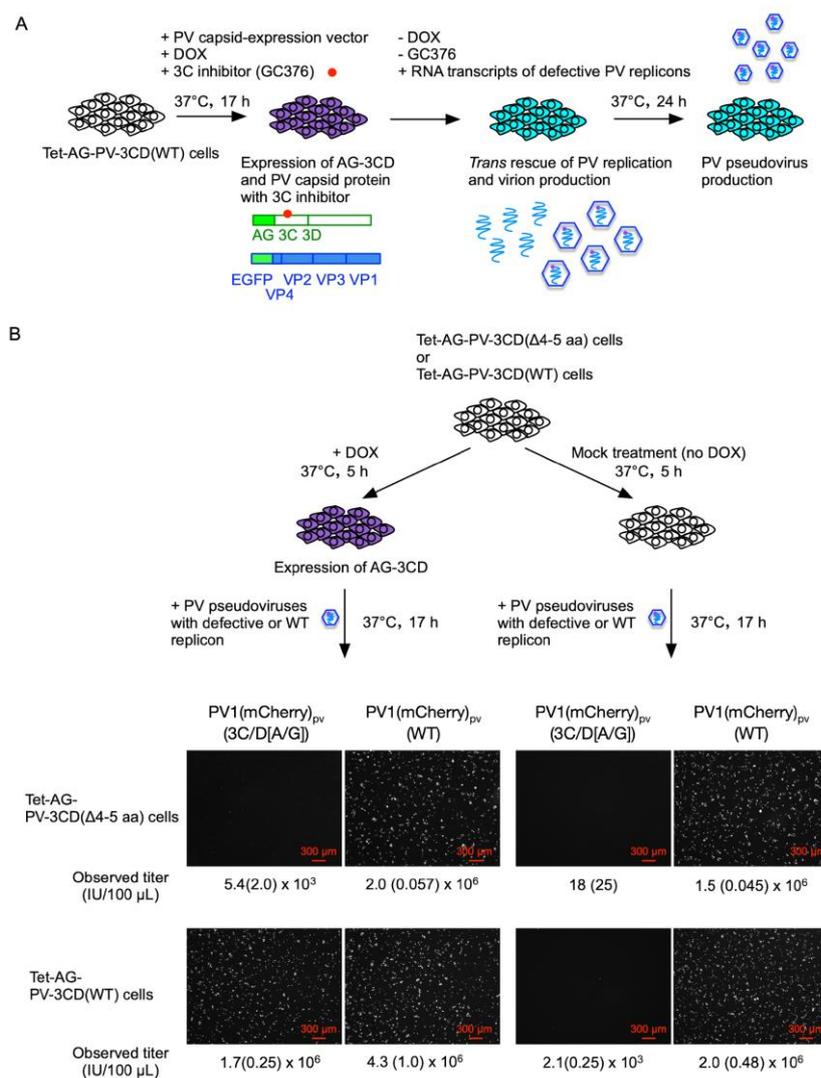
Figure 7. Production of defective PV_{pv}. (A) Experimental design for *trans* rescue of PV_{pv} production. Tet-AG-3CD(WT) cells were transfected with a PV capsid expression vector in the presence of DOX (1 mg/mL) and GC376 (100 μM) to express the 3CD protein at 37°C for 17 h. Then, RNA transcripts of each PV replicon (WT or 3C/D[A/G] mutant) were transfected to the cells in the absence of DOX and GC376. The cells were collected at around 24 h p.t. of the RNA transcripts. (B) Infectivity of PV_{pv} produced with a defective PV replicon with mCherry reporter. Tet-AG-3CD(WT) or Tet-AG-3CD(Δ4-5 aa) cells were treated with or without DOX (1 mg/mL) at 37°C for 5 h. The cells were infected with 10 μL of PV_{pv} solution and then incubated at 37°C for 17 h. Fluorescent microscope images of the cells for mCherry and the observed titers of PV_{pv} (infectious unit [IU]/100 μL) are shown. .

To produce PV_{pv}, a plasmid expression vector for type 1 PV capsid proteins was transfected into Tet-AG-PV-3CD(WT) cells in the presence of DOX and GC376 (Figure 7A). After expression of the PV capsid proteins and AG-PV-3CD(WT), RNA transcripts of the 3C/D(A/G) mutants, with mCherry or firefly luciferase reporters, were transfected into the cells in the absence of DOX and GC376 for the production of PV_{pv} (PV1[mCherry]$_{pv}$[3C/D{A/G}] or PV1[Fluc]$_{pv}$[3C/D{A/G}], respectively). As controls, PV_{pv} were produced with the WT replicons with mCherry or firefly luciferase reporters (PV1[mCherry]$_{pv}$[WT] or PV1[Fluc]$_{pv}$[WT], respectively). Typical cytopathic effects were observed in the cells on day 1 post-transfection of the RNA transcripts, then the cells were harvested to determine the titer of PV_{pv}. Collected PV_{pv} was inoculated into Tet-AG-PV-3CD(WT) cells or Tet-AG-PV-3CD(Δ4-5 aa) cells (a control) pre-treated with DOX (or no DOX treatment as a control) before the infection, and then the signals of reporters (fluorescence of mCherry or luciferase activity) in the infected cells were analyzed (Figure 7B). We observed fluorescence of mCherry in the cells infected

with PV1(mCherry)$_{pv}$(WT), irrespective of the DOX treatment and the cell types. In contrast, substantial replication of PV1(mCherry)$_{pv}$(3C/D[A/G]) was observed only in Tet-AG-PV-3CD(WT) cells after pre-treatment with DOX. The observed titer of PV1(mCherry)$_{pv}$(3C/D[A/G]) was about 10^7 IU per mL, thus similar to that of PV1(mCherry)$_{pv}$(WT). Replication in Tet-AG-PV-3CD(Δ4-5 aa) cells was significantly suppressed; about 1/100-fold or 1/100,000-fold lower than that of the WT replicon, in the presence or absence of DOX, respectively) (Figure 8A), supporting the weak *trans*-rescue activity of 3CD^{pro}(Δ4-5 aa). Infectivity of PV1(Fluc)$_{pv}$(3C/D[A/G]) showed similar cell-type specificity and dependency on DOX treatment than that of PV1(mCherry)$_{pv}$(3C/D[A/G]). To further confirm the presence of PV$_{pv}$ in the preparations, we performed neutralization tests for PV1(mCherry)$_{pv}$(3C/D[A/G]) with anti-PV antisera (Figure 8B). PV1(mCherry)$_{pv}$(3C/D[A/G]) was incubated with type-specific anti-PV antibodies (*i.e.*, anti-PV1, PV2, PV3 standard antisera), and then inoculated into Tet-AG-PV-3CD(WT) cells, pre-treated with DOX. Infection of PV1(mCherry)$_{pv}$(3C/D[A/G]) was inhibited by pre-incubation with anti-PV1 antiserum, but not with anti-PV2 or PV3 antisera, suggesting that the type 1 PV antigenicity is retained on PV1(mCherry)$_{pv}$(3C/D[A/G]) similar to PV$_{pv}$ produced with the WT replicon (66, 67). These results suggested that replication of defective PV replicons could be efficiently *trans* rescued and allow production of a high titer PV$_{pv}$.
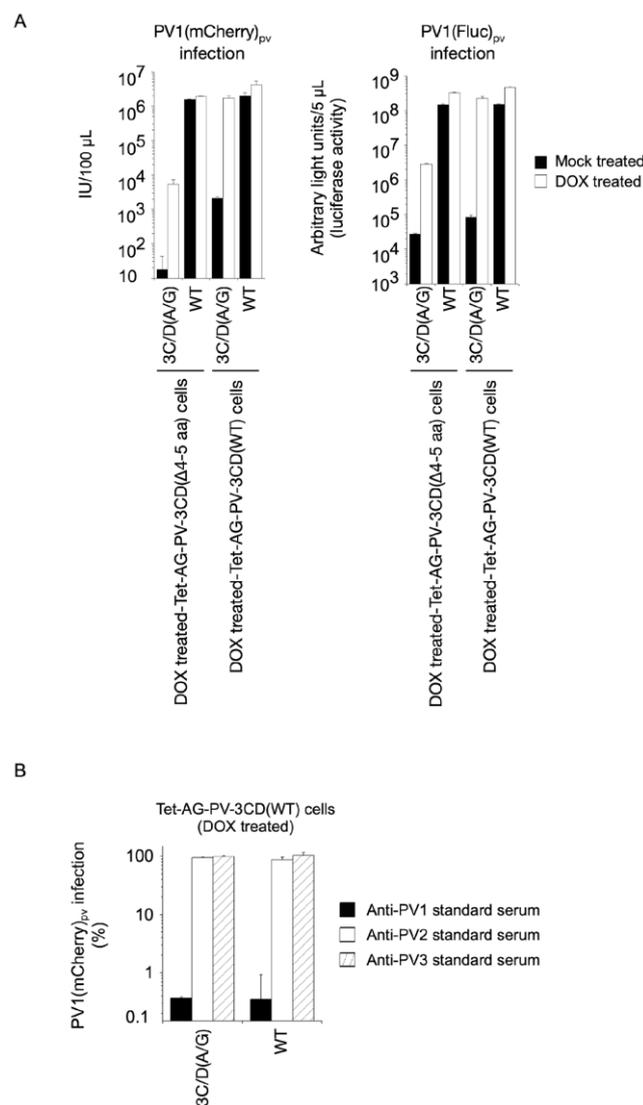

Figure 8. Titer and antigenicity of defective PV$_{pv}$. (A) Titer of defective PV$_{pv}$ (3C/D[A/G] mutant) with mCherry or firefly luciferase reporter. Observed titer of PV1(mCherry)$_{pv}$ (IU/100 μL) and luciferase

signals in the cells infected with 5 μL of PV1(Fluc)$_{pv}$ are shown. (B) Neutralization of PV1(mCherry)$_{pv}$. PV1(mCherry)$_{pv}$ (2.0 × 10^3 IU) were incubated with standard anti-PV1, PV2, or PV3 standard antisera (26 U for each type) at 4°C for 7 h, and then added to the DOX-treated Tet-AG-3CD(WT) cells. The numbers of the cells positive for mCherry fluorescence was counted at 17 h p.i. PV1(mCherry)$_{pv}$ infection in the absence of antisera is taken 100%.

3. Discussions

The importance of *cis*-provided proteins for replication of PV was initially suggested from analysis of defective interfering (DI) particles (28), which have in frame deletions in the capsid-coding region (P1 region) of the genome and retain an intact non-structural protein coding region (P2P3 region) (29). These results suggested that the functions of viral proteins encoded in the P2P3 region could not be complemented by exogenous viral proteins, thus in *trans*. The *cis* and *trans* roles of the viral proteins in replication were intensively studied in the 1990s by *trans* complementation (or *trans* rescue) of replication of defective PV mutants (30-33, 37, 68-73). Main conclusions drawn from these studies includes 1) *trans* rescue of defective PV mutants is inefficient, and 2) a large intact precursor of the non-structural proteins is required for replication. Beside viral proteins, conserved RNA structures, encoded in the P2P3 region, were identified, including the CRE, RNase L ciRNA, α, and β (26, 74-78). The CRE is required for replication in *cis* as the template for uridylylation of 3B (26, 79), confirming the *cis* role of the P2P3 region. These properties/roles of non-structural proteins and RNA structures in viral replication are generally conserved in picornavirus (80-84).

To elucidate the role of processing in viral replication, we established cell lines that could conditionally express a large precursor of PV non-structural proteins (2BC3ABCD) and performed *trans* rescue of defective PV replicons in cells instead of using helper virus/replicon (Figure 1). One major advantage of using these cell lines is the high expression level of the precursor protein in the presence of inhibitors against viral proteins and controllable expression for a short period to avoid cytotoxicity, especially that caused by viral proteases 3C^{Pro}/3CD^{Pro} (85, 86). Processing of the 2BC3ABCD precursor gave both final and intermediate products, similar to those observed in PV-infected cells (27) (Figures 1 and 6). We found that 2BC3ABCD could produce 2BC, 2B, 2C$^{ATPas/hel}$ and 3AB (thus also 3CD^{Pro}) by *trans*-provided 3CD^{Pro}, but the processing of 3AB could occur only in *cis* (Figure 6), suggesting a possible role of the host PI4KB/OSBP family I pathway in the *cis* cleavage. In the *trans* rescue using the cell lines, replication was not detected for most of the mutants examined, except for a mutant with a disrupted cleavage site between 3C^{Pro} and 3D^{Pol} (3C/D[A/G] mutant), which showed comparable level of replication to that of the WT replicon (Figures 2 and 3). This allowed further analysis of the *cis* role of viral proteins in this mutant, and revealed that only the activity of 3D^{Pol}, but not those of 3B, 2C$^{ATPase/hel}$, or 3C^{Pro}, could be *trans* rescued (Figure 4). PV 3CD^{Pro} lacks RNA polymerase activity (87, 88), thus the 3C/D(A/G) mutant, which could express only 3CD^{Pro} but not 3C^{Pro} nor 3D^{Pol}, was predicted to be deficient in polymerase activity irrespective of the introduction of inactivating mutations for 3D^{Pol} activity (Figure 4). Previous reports suggested that 3D^{Pol} activity could be *trans* rescued but with low efficiencies, similar to the rescue of 2C$^{ATPase/hel}$ activity (30, 68, 69). The high expression level of the viral proteins in the cell lines might have improved the efficiency of *trans* rescue and provided all-or-none replication. *Trans* complementation of a PV mutant (EG mutant) with a partially defective cleavage site between 3B and 3C^{Pro} has been reported (89), but we could not detect significant replication or *trans*-rescued replication of the corresponding mutant (3B/C[A/G] mutant, Figures 2 and 3). This might suggest that partial or inefficient *cis* cleavage between 3B and 3C^{Pro} is sufficient for the *trans*-rescued replication. Studies on foot-and-mouth disease virus (FMDV), which belongs to the genus *Aphthovirus* in the family *Picornaviridae* (90, 91), suggested that the defects in 3B uridylylation and 3D^{Pol} activity could be *trans* rescued (34, 35). FMDV is unique in coding multiple copies of 3B (three tandem copies of 3B). In addition, the recently discovered mosavirus has two copies of 3B (92), which could not be stably maintained in the PV genome (93). FMDV does not depend on host PI4KB/OSBP family I pathway for replication (94) in contrast to EVs, thus the mechanism and/or the role of uridylylation of 3B might

be different from those in PV replication, in terms of *trans* rescue. Collectively, these results suggest a conserved *trans* role of 3D^{Pol} activity in picornavirus replication.

To provide *trans* 3D^{Pol} activity, expression of 3CD^{Pro}, but not of 3D^{Pol}, was essential and sufficient (Figures 5 and S1); a large precursor was not necessarily essential in *trans* rescue. Unexpectedly, substitution of aa residues of 3C^{Pro}, which are involved in binding to viral RNA elements or phospholipids (4, 24, 26, 62), did not abrogate the *trans* rescue activity. This is in contrast to requirements for 3CD^{Pro} *in an in vitro trans* complementation system; the interaction with RNA was essential for RNA synthesis and virus maturation (95, 96). This may suggest a critical difference between the pre-initiation complexes formed *in vitro* and *in vivo*, which could be *trans*-rescued by the 3CDpol activity, possibly via a different pathway. We found that the N-terminal region of 3C^{Pro} is essential for *in vivo trans* rescue (Figure 6). Primary structures of the N-terminal region of 3C^{Pro} are conserved within the viral species and protrude outside in the tertiary structure (Figure S4). PV non-structural proteins did not *trans* rescue a defective EV-A71 replicon, suggesting that the interaction is dependent on the viral species (Figure S2). We propose a model in which the *trans*-provided 3CD^{Pro} provides 3D^{Pol} activity to the viral pre-initiation complex, formed by the defective replicons, via virus species specific protein-protein interactions (80) (Figure 9).
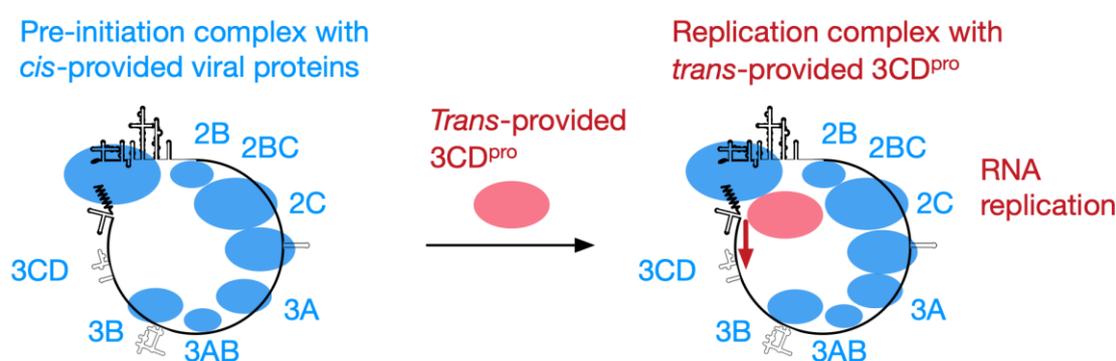

Figure 9. A model of PV replication by *trans*-provided 3CD^{Pro}. *Trans* provided 3CD^{Pro} provides 3D^{Pol} activity to the pre-initiation complex via virus species specific protein-protein interactions.

Based on the observations of DI particles, the capsid-coding region is dispensable for the replication of PV, PV replicons coding exogenous reporter genes in place of the capsid-coding region have been developed (24, 97). Subsequently, generation of PV pseudovirus (PV$_{pv}$) that encapsidates engineered replicon with *trans*-provided PV capsid proteins have been established by using helper PV (98, 99), recombinant vaccinia virus systems (100, 101), or virus-free capsid-protein expression plasmid vector (65). PV$_{pv}$ could serve as a safe alternative to live PV in biological tests, because no infectious virus is produced in the infected cells (65, 102). A limitation of these methods is that only replication-competent replicon RNA could be encapsidated (98, 103). In the present study, we partially solve this limitation to produce PV$_{pv}$ with a defective PV replicon after *trans*-rescued replication (Figures 7 and 8). This new generation of PV$_{pv}$ may be useful for biological tests with enhanced safety.

The limitations of this study include the detection limits of trans-rescued replication; defective replicons give signals of reporters (firefly luciferase or mCherry) derived from   initial protein synthesis before replication (about $1/10^2$ or $1/10^4$ of the signals at plateau, in the RNA-transfected cells or in PV$_{pv}$-infected cells, respectively). Therefore, inefficient initial replication resulting only in quasi infection (70) might be missed in this study. In processing of a polyprotein (2BC3ABCD), only the processing of 3AB could occur in *cis*. However, a requirement for 2A^{Pro}, which can be deleted from the genome but require a host factor SETD3 (104, 105), in *cis* processing remains to be elucidated.

Collectively, this work reveals potential roles of exogenous viral proteins in PV replication and offers insights into protein/protein interactions during picornavirus infection. This will aid elucidating the mechanism of multiple PV infection, including intra-species recombination that can

reduce the effectiveness of novel PV vaccines toward eradication (106, 107). Our findings might be useful for the development of effective antivirals targeting the polyprotein processing.

4. Materials and Methods

Cells. RD cells (human rhabdomyosarcoma cells) and HEK293 cells (human embryonic kidney cells) were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS). RD cells were used for virus titration. HEK293 cells were used for expression of PV non-structural proteins and for production of PV pseudovirus (PV_{pv}).

Viruses. PV_{pv} was produced with a firefly luciferase-coding or a mCherry-coding type 1 PV (PV1) Mahoney strain (GenBank: V01149) replicon and capsid proteins of PV1(Mahoney)(PV1[Fluc]$_{pv}$ or PV1[mCherry]$_{pv}$, respectively) (65).

Chemicals. Doxycycline (DOX) was purchased from FUJIFILM Wako Pure Chemical Corporation (049-31121). Guanidine hydrochloride (GuHCl, a 2C inhibitor) was purchased from SIGMA (G-9284). GC376 (a 3C inhibitor) was purchased from Selleck Chemicals (S0475). Rupintrivir (a 3C inhibitor) was purchased from SANTA CRUZ BIOTECHNOLOGY (sc-208317). Stock solutions of DOX (2 g/L) and GuHCl (2 M) were prepared in Milli-Q water. Stock solutions of GC376 (100 mM) and rupintrivir (10 mM) were prepared in dimethyl sulfoxide.

General methods for molecular cloning. *Escherichia coli* strain XL10gold (Stratagene) was used for the preparation of plasmids. Ligation of DNA fragments was performed using an In-Fusion HD Cloning Kit (Clontech). PCR was performed using KOD Plus DNA polymerase (Toyobo). DNA sequencing was performed using a BigDye Terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems) and then analyzed with a 3500xL genetic analyzer (Applied Biosystems).

5. Plasmids

5.1. Lentivirus Expression Vectors for PV Non-Structural Proteins

pTet-AG-PV-2B2CP3(WT):

A cDNA fragment of a polyprotein of PV non-structural proteins (2BC3ABCD) was obtained by PCR with pPV-Fluc mc (a plasmid encoding cDNA of a PV replicon) (108) as the template and following primer set 1. This DNA fragment was inserted into a DNA fragment of a lentivirus vector plasmid with a TRE3G promoter, which was obtained by PCR with pLJM1-TRE3G-His-AG-FLAG-PreScission-OSBP(406–807) (52) as the template and using primer set 2.

Primer set 1:

5' GAAGTTCTGTTCCAGGGCCTCACCAATTACATAGAGTCAC 3'

5' TCTGAGTCCGGATCAAAATGAGTCAAGCCAACGGCGGTAC 3'

Primer set 2:

5' CTGGAACAGAACTTCCAGCTTGTCGTCATC 3'

5' TGATCCGGACTCAGATCTCGAGCTCAAGC 3'

pTet-AG-PV-2B2CP3(3C-C147A):

Mutations for the 3C-C147A aa substitution (disruption of the catalytic triad of PV 3C at aa C147) (59) was introduced in pTet-AG-PV-2B2CP3(WT) by PCR with primer set 3.

Primer set 3:

5' ACCAGAGCAGGACAGGCUGGTGGAGTCATCACATGTACTG 3'

5' CTGTCCTGCTCTGGTTGGAAAGTTGTAC 3'

pTet-AG-PV-3CD(WT) and pTet-AG-PV-3CD(Δ4-5 aa):

A deletion of the 2BC3AB-coding region was introduced in pTet-AG-PV-2B2CP3(WT) by PCR with primer set 4. Unexpected deletion in the primer region, causing a deletion of aa 4 and 5 in 3C region, was found in a clone, which was designated as pTet-AG-PV-3CD(Δ4-5 aa).

Primer set 4:

5' GAAGTTCTGTTCCAGGGACCAGGGTTCGATTACGCAGTGG 3'

5' CTGGAACAGAACTTCCAGCTTGTCGTCATC 3'

pTet-AG-PV-3CD(3C-R13N, 3C-K82N, 3C-R84S):

Mutations for the aa substitutions that could affect interaction with negatively charged molecules (viral RNA and phospholipids) (3C-R13N, 3C-K82N, and 3C-R84S aa substitutions) (24, 62-64) were introduced in pTet-AG-PV-3CD(WT) by PCR with primer set 5 ,6, and 7, respectively.

Primer set 5:

5' AACATTGTTACAGCAACTACTAGCAAG 3'

5' TGCTGTAACAATGTTGTTTTTAGCCATAGCCACTGCGTAATCG 3'

Primer set 6:

5' TTCAGAGACATTAGACCACATATACC 3'

5' TCTAATGTCTCTGAAGTTTTCATTTCTCTTTAGAGTGATTATAG 3'

Primer set 7:

5' GACATTAGACCACATATACCTACTC 3'

5' ATGTGGTCTAATGTCGCTGAACTTTTCATTTCTCTTTAGAGTG 3'

pTet-AG-PV-3CD(WT-uncleavable AG, Δ4-5 aa-uncleavable AG):

A mutation that disrupts the cleavage site for the 3C protease between the AG and the 3CD was introduced in pTet-AG-PV-3CD(WT) or pTet-AG-PV-3CD(Δ4-5 aa) by PCR with primer set 8.

Primer set 8:

5' GGACCAGGGTACGCAGTGGCTATGGC 3'

5' TGCGTACCCTGGTCCGGCGAACAGAACTTCCAGCTTGTCGTC 3'

5.2. PV Replicon Mutants

pPV-Fluc mc (2A/B[A/G], 2B/C[A/G], 2C/3A[A/G], 3A/B[A/G], 3B/C[A/G], and 3C/D[A/G]) and pPV-mCherry mc (3C/D[A/G]):

Mutants with disrupted cleavage sites for the 3C protease: The mutations between each viral gene (2A/B[A/G], 2B/C[A/G], 2C/3A[A/G], 3A/B[A/G], 3B/C[A/G], and 3C/D[A/G] mutations) were introduced in pPV-Fluc mc with a hammerhead ribozyme at the 5'end of the replicon (109, 110) by PCR with primer sets 9, 10, 11, 12, 13, and 14, respectively. The 3C/D[A/G] mutation was also introduced in pPV-mCherry mc with a hammerhead ribozyme at the 5'end of the replicon (110) by PCR with primer set 14.

Primer set 9:

5' GGCCTCACCAATTACATAGAGTCACTTGGG 3'

5' GTAATTGGTGAGGCCGGCTTCCATGGCTTC 3'

Primer set 10:

5' TATGTCATCAAGGCCGGTGACAGTTGGTTGAAGAAGTTTACTG 3'

5' GGCCTTGATGACATAAGGTATCTCCAGAACATCGC 3'

Primer set 11:

5' GGACCACTCCAGTATAAAGACTTGAAAATTG 3'

5' ATACTGGAGTGGTCCGGCAAACAAAGCCTCC 3'

Primer set 12:

5' CTGTTTGCTGGACACGCCGGAGCATACACTGGTTTACCAAAC 3'

5' GGCGTGTCCAGCAAACAGTTTATACATGAC 3'

Primer set 13:

5' GGACCAGGGTTCGATTACGCAGTGGCTATG 3'

5' ATCGAACCCTGGTCCGGCTACCTTTGCTGTC 3'

Primer set 14:

5' TTCACTCAGAGTGCCGGTGAAATCCAGTGGATGAGACCTTCG 3'

5' GGCACTCTGAGTGAAGTATGATCGCTTCAGGG 3'

pPV-Fluc mc (Δ2A, Δ2B, Δ2C, Δ3A, Δ3B, Δ3C, and Δ3D):

Mutants with deletion of each viral gene: Deletion of each viral gene (Δ2A, Δ2B, Δ2C, Δ3A, Δ3B, Δ3C, and Δ3D) were introduced in pPV-Fluc mc by PCR with following primer set 15, 16, 17, 18, 19, 20, and 21, respectively.

Primer set 15:

5' GAAGCCATGGAACAAGGCCTCACCAATTAC 3'

5' TTGTTCCATGGCTTCTGTGGTGAGCTCCAATTTG 3'

Primer set 16:

5' GGTGACAGTTGGTTGAAGAAGTTTACTG 3'

5' CAACCAACTGTCACCTTGTTCCATGGCTTCTTCTTCGTAGGCATAC 3'

Primer set 17:

5' GGACCACTCCAGTATAAAGACTTGAAAATTG 3'

5' ATACTGGAGTGGTCCTTGCTTGATGACATAAGGTATCTCCAGAAC 3'

Primer set 18:

5' GGAGCATACACTGGTTTACCAAACAAAAAACC 3'

5' ACCAGTGTATGCTCCTTGAAACAAAGCCTCCATACAATTGCCAATG 3'

Primer set 19:

5' GGACCAGGGTTCGATTACGCAGTGGCTATG 3'

5' ATCGAACCCTGGTCCCTGGTGTCCAGCAAACAGTTTATACATGAC 3'

Primer set 20:

5' GGTGAAATCCAGTGGATGAGACCTTCGAAG 3'

5' CCACTGGATTTCACCTTGTACCTTTGCTGTCCGAATGGTGGGCAC 3'

Primer set 21:

5' TAGTAACCCTACCTCAGTCGAATTGGATTG 3'

5' GAGGTAGGGTTACTATTGACTCTGAGTGAAGTATGATCGCTTCAG 3'

pPV-Fluc mc (3C/D[A/G-2C-K153A], 3C/D[A/G-3B-Y3F], and 3C/D[A/G-3D-D328N/D329N]):

Mutants encoding functionally inactive viral proteins (2C, 3B, and 3D) with a disrupted cleavage site for 3C protease between 3C and 3D: functionally inactivating mutations in PV 2C (a 2C-K153A aa substitution) (6), PV 3B (a 3B-Y3F aa substitution) (58), PV 3D (3D-D328N/D329N aa substitution) (60) were introduced in pPV-Fluc mc (3C/D[A/G]) by PCR with primer sets 22, 23, and 24, respectively.

Primer set 22:

5' CCCGGAACAGGTGCCTCTGTAGCAACCAACCTGATTGCTAG 3'

5' GGCACCTGTTCCGGGGCTGCCATGTACTAGC 3'

Primer set 23:

5' ACACCAGGGAGCATTCACTGGTTTACCAAACAAAAAACCCAACG 3'

5' ATGCTCCCTGGTGTCCAGCAAACAGTTTATAC 3'

Primer set 24:

5' AATAATGTAATTGCTTCCTACCCCCATGAAG 3'

5' AGCAATTACATTATTACCATAGGCAATCATTTTTAGG 3'

5.3. EV-A71 Replicon Mutants

pEV-A71-Fluc mc (WT and 3C/D[A/G] mutant):

A hammerhead ribozyme (109) was introduced in pEV71(Fluc-mc) (111) at the 5'end of an EV-A71 BrCr-TR strain (GenBank: AB204852) replicon coding firefly luciferase reporter by PCR with primer set 25.

Primer set 25:

5' CGGTATCCCGGGTTCTTAAAACAGCCTGTGGGTTGCACCC 3'

5' GAACCCGGGATACCGGGTTTTCGGCCTTTCGGCCTCATCAGTTAAAACACCCTATAGTGAGTCGTATTAATACGTAATTTCG 3'

The mutation between 3C and 3D (3C/D[A/G] mutations) were introduced in the above plasmid by PCR with primer set 26.

Primer set 26:

5' GGCCTCGCTGGCAAAATAACTCCTCTTTAG 3'

5' TTTGCCAGCGAGGCCGGAGAGATCCAGTGGATGAAGCCTAACAG 3'

RNA transfection. RNA transcripts of PV replicons were obtained using a RiboMAX Express Large Scale RNA Production System (Promega, P1320) with *Dra*I-linearized plasmids of PV replicons.

RNA transcripts (0.025 μL) were transfected into the cells (4 ·10^4 cells per well in 100 μL medium) in a 96-well plate (Corning Incorporated, 3595) using *Trans*IT-mRNA Transfection Kit (Mirus, MIR 2250).

Preparation of PV pseudovirus (PV$_{pv}$) with a defective PV replicon. Tet-AG-PV-3CD(WT) cells (1.6 ·10^6 cells per well in 4 mL medium) in a six-well plate (Corning Incorporated, 3516) were transfected with 4 μg of a PV1(Mahoney) capsid-expression vector (pKS435-EGFP-PV1(Mahoney) (108) per well using *Trans*IT-PRO transfection kit (Mirus, MIR 5700) in the presence of DOX (1 mg/L) and GC376 (100 μM). The cells were incubated at 37 °C for 24 h, and then washed with the medium without DOX and GC376. RNA transcripts (1 μL) of defective PV replicons (3C/D[A/G]) with mCherry or firefly luciferase reporters were transfected into monolayers of Tet-AG-PV-3CD(WT) cells transiently expressing the capsid proteins and the 3CD protein in the absence of DOX and GC376. The cells were harvested at 24 h post-transfection of the RNA transcripts, and then stored at -20 °C.

Titration of defective PV$_{pv}$. For the titration of PV1(mCherry)$_{pv}$ (3C/D[A/G]), Tet-AG-PV-3CD(WT) cells (4 ·10^4 cells per well in 100 μL medium) or Tet-AG-PV-3CD(Δ4-5 aa) cells (a negative control of the infection) in a 96-well plate (Corning Incorporated, 3595) were incubated at 37 °C for 5 h in the presence of DOX (1 mg/L). The cells were inoculated with 10 μL of serially diluted PV1(mCherry)$_{pv}$ (3C/D[A/G]) solution (dilution of 1/1 to 1/10^5), and then incubated at 37 °C for 17 h. Infectious units (IU) of PV1(mCherry)$_{pv}$ (3C/D[A/G]) were determined by counting the number of the mCherry-fluoresence positive cells (44). Images were collected at 4× magnification using a BZ-9000 fluorescence microscopy (Keyence), and then analyzed by using CellProfiler software (112). For the titration of PV1(Fluc)$_{pv}$ (3C/D[A/G]), Tet-AG-PV-3CD(WT) cells (8 ·10^3 cells per well in 20 μL medium) or Tet-AG-PV-3CD(Δ4-5 aa) cells (a negative control of the infection) in a 384-well plate (Greiner Bio-One, 781080) were incubated at 37 °C for 5 h in the presence of DOX (1 mg/L). The cells were inoculated with 5 μL of serially diluted PV1(Fluc)$_{pv}$ (3C/D[A/G]) solution (dilution of 1/1 to 1/10^5), and then incubated at 37 °C for 17 h. A 15 μL of the supernatant was removed from each well, and then 10 μL of Steady-Glo Reagent (Promega, E2520) was added to each well. Luciferase signals were measured using a 2030 ARVO X luminometer (Perkin-Elmer).

Neutralization of defective PV$_{pv}$. PV1(mCherry)$_{pv}$ (WT or 3C/D[A/G] mutant) (2.0 × 10^3 IU in 10 μL medium) were mixed with 10 μL of standard anti-PV1, PV2, or PV3 antisera (26 U for each type of PV) at 4°C for 7 h. Tet-AG-PV-3CD(WT) cells (4 ·10^4 cells per well in 100 μL medium) in a 96-well plate (Corning Incorporated, 3595) was incubated at 37 °C for 5 h in the presence of DOX (1 mg/L). PV1(mCherry)$_{pv}$ (WT or 3C/D[A/G] mutant) (2.0 × 10^3 IU in 10 μL medium) was mixed with 10 μL of standard anti-PV1, PV2, or PV3 antisera (26 U for each type) at 4°C for 7 h, and then added to the DOX-treated Tet-AG-PV-3CD(WT) cells. The cells were incubated at 37 °C for 17 h, and then the number of the mCherry-fluoresence positive cells was counted as described the titration of PV1(mCherry)$_{pv}$.

Western blot. The cells (8 · 10^5 cells) were collected in 20 μL of cell lysis buffer (21 mM HEPES buffer [pH 7.4], 0.7 mM disodium hydrogenphosphate, 137 mM NaCl, 4.8 mM KCl, 0.5% Nonidet P-40 and 5 mM EDTA, supplemented with complete-mini protease inhibitor cocktail tablet [Roche, 04 693 159 001]), and then were subjected to e-PAGEL 5-20% gradient polyacrylamide gel electrophoresis (Atto Corporation) in a Laemmli buffer system. Proteins in the gel were transferred to a polyvinylidene difluoride filter (Millipore, Immobilon) and blocked in iBind™ solution (Thermo Fischer Scientific). Filters were incubated with anti-PV 2C, 3A, and 3D antibodies (37) (rabbit antisera, 1:500, 1:200, and 1:250 dilution, respectively), then with secondary antibodies (Thermo Scientific, 32460, goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase, 1:200 dilution) in iBind™ Western System (Thermo Fischer Scientific). Signals were detected with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, 34095), then analyzed with ImageQuant 800 (cytiva).

Statistical analysis. Results of experiments are shown as means with standard deviations. Presented data are representative of at least two independent experiments with two or three

biological replicates. Values of $P<0.05$ by one-tailed t test were considered to indicate a significant difference, and were indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Disclosure: The author declares no conflict of interest.

List of Abbreviations

aa: amino acid
EV: enterovirus
PV: poliovirus
PV1$_{pv}$: type 1 PV pseudovirus

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