Poliovirus 2A\textsuperscript{pro} induces the nucleic translocation of poliovirus 3CD and 3C\textsuperscript{0} proteins

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Poliovirus genomic RNA replication, protein translation, and virion assembly are performed in the cytoplasm of host cells. However, this does not mean that there is no relationship between poliovirus infection and the cellular nucleus. In this study, recombinant fluorescence-tagged poliovirus 3CD and 3C\textsuperscript{0} proteins were shown to be expressed mainly in the cytoplasm of Vero cells in the absence of other viral proteins. However, upon poliovirus infection, many of these proteins redistributed to the nucleus, as well as to the cytoplasm. A series of transfection experiments revealed that the poliovirus 2A\textsuperscript{pro} was responsible for the same redistribution of 3CD and 3C\textsuperscript{0} proteins to the nucleus. Furthermore, a mutant 2A\textsuperscript{pro} protein lacking protease activity abrogated this effect. The poliovirus 2A\textsuperscript{pro} protein was also found to co-localize with the Nup153 protein, a component of the nuclear pore complexes on the nuclear envelope. These data provide further evidence that there are intrinsic interactions between poliovirus proteins and the cell nucleus, despite that many processes in the poliovirus replication cycle occur in the cytoplasm.

Keywords poliovirus; 2A\textsuperscript{pro}; 3CD; 3C\textsuperscript{0}; nuclear localization

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Introduction

Poliovirus is a member of the Picornaviridae family of viruses and contains a 7500-nucleotide single-stranded positive-sense RNA genome. After its entry into a host cell, a long polyprotein is produced and processed into functional viral proteins. The majority of viral reproduction events such as translation, RNA synthesis, and encapsidation take place in the host cell cytoplasm but this does not exclude viral interactions with the nucleus during its life cycle. Indeed, some poliovirus proteins localize in the nucleus and interact with nuclear proteins of the host cell [1–3].

Previous studies have indicated that the poliovirus 3CD protein localized in the cytoplasm when expressed alone, but localized to the cell nucleus during poliovirus infection [4]. The 3CD protein plays an important role in the poliovirus polyprotein processing by cleaving the P1 protein. The 3CD protein can stimulate the uridylylation of the VPg protein in the viral RNA replication complex [5]. The 3CD polyprotein is the precursor of both the 3C protease (3C\textsuperscript{pro}) and the 3D RNA polymerase. During poliovirus infection, active 3C\textsuperscript{pro} protease can act on some host nuclear factors, in particular host cell transcription factors such as TATA-binding protein (TBP) [6,7], cAMP response element-binding protein and octamer-binding transcription factor [8]. To function in the nucleus, 3C\textsuperscript{pro} is generated by auto-proteolysis of the 3CD precursor that was translocated into the cell nucleus. This process is important for the interaction between poliovirus and the host cell, but how 3CD is translocated into the cell nucleus is unknown. The 3C\textsuperscript{0} protein, being of unknown function, is a product of 3CD cleavage by 2A\textsuperscript{pro} [9] and contains the 3C\textsuperscript{pro} protein sequence. The 3C\textsuperscript{0} protein of rhinoviruses, analogous to poliovirus 3C\textsuperscript{0}, has been previously reported to localize to the nuclei of infected cells but the mechanism of this translocation is also unclear [10].

Poliovirus 2A\textsuperscript{pro} is an essential enzyme involving the proteolytic cleavage of viral and cellular proteins during virus infection [11,12]. It has been proven that poliovirus 2A\textsuperscript{pro} can cause host cell apoptosis [13] and 2A\textsuperscript{pro}-expressing cells show phenotypic characteristics of virus-infected cells [14]. Further, the 2A\textsuperscript{pro} protein can also induce some nuclear proteins to re-localize to the cytoplasm [15]. The present study investigated the localization and nuclear–cytoplasmic trafficking of poliovirus 3CD and 3C\textsuperscript{0} by fluorescence microscopy. When poliovirus 3CD and 3C\textsuperscript{0} proteins were expressed in Vero cells without poliovirus infection, the proteins localized in the cytoplasm. After poliovirus infection, many of the proteins re-localized to the nuclei. It was also found that the 2A\textsuperscript{pro} protein is responsible for the redistribution of 3CD and 3C\textsuperscript{0} proteins. Furthermore, a mutation that abolished the
protease activity of 2A\textsuperscript{pro} abolished the ability to cause the redistribution of 3CD and 3C\textsuperscript{'} proteins. The 2A\textsuperscript{pro} of poliovirus was found to co-localize with Nup153, which was an important component of the nuclear pore complexes (NPCs) on the nuclear envelope (NE). Therefore, these results highlight the important role of 2A\textsuperscript{pro} in poliovirus interactions with the host cell nucleus.

**Materials and Methods**

**Cells and virus**

Vero (African green monkey kidney) cells were purchased from China Center for Type Culture Collection (CCTCC), Wuhan University and grown in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Logan, USA) containing 10% fetal calf serum, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin and maintained in 5% \( \text{CO}_2 \) at 37\( ^\circ \)C. Poliovirus I Sabin I strain (a kind gift from Prof. Yunzhang Hu, Institute of Medical Biology, Chinese Academy of Medical Science, Kunming, China) was grown by infecting Vero cells at 37\( ^\circ \)C for 24 h. Cells were then washed with DMEM and subjected to freeze-thawing three times. The cell lysate was centrifuged and the supernatant was collected and stored at −80\( ^\circ \)C.

**Plasmids construction**

Viral mRNA was extracted from cell lysate supernatants using Trizol reagent (Invitrogen, Carlsbad, USA). Then the cDNA of viral mRNA from 3381nt to 7378nt was amplified by reverse transcription-polymerase chain reaction (PCR). The individual viral genes used in this study were amplified from the cDNA by PCR.

The plasmids, pEGFP-C1-3CD and pEGFP-C1-3C\textsuperscript{'}, were constructed by inserting the respective 3CD and 3C\textsuperscript{'}, amplified segments into the pEGFP-C1 plasmid (Clontech, Palo Alto, USA) at the 3\textquoteleft end of the EGFP gene using Xho\textsubscript{I} and EcoRI sites. The 3CD and 3C\textsuperscript{'} DNA was amplified by PCR using the same sense primer P3CD-S containing an Xho\textsubscript{I} site and a linker sequence encoding GGGGS and antisense primer PM2-A containing an EcoRI site. However, each gene was amplified using unique antisense primers. For 3C\textsuperscript{'} the antisense primer was P3C\textsuperscript{'}-A containing an EcoRI site and for 3CD the antisense primer was P3-A containing an EcoRI site.

The pEGFP-C1-2A\textsuperscript{pro} plasmid was constructed by inserting the 2A\textsuperscript{pro}-coding sequence into pEGFP-C1 at the HindIII and EcoRI sites. The 2A\textsuperscript{pro}-coding sequences were amplified by PCR using the sense primer P2A-S containing a HindIII site and a linker sequence encoding GGGGS and the antisense primer P2A-A containing an EcoRI site. The 2A\textsuperscript{pro}(H20A)-coding sequence was acquired by PCR using a long-sense primer PM2A-S, which contains a HindIII site and a H20A mutation and an antisense primer P2A-A, and then inserted into pEGFP-C1 to construct the plasmid pEGFP-C1-2A\textsuperscript{pro}(H20A).

For the construction of pmCherry-C1-2A\textsuperscript{pro} plasmid, the mCherry sequence was amplified from pRSET-B-mCherry (a kind gift from Prof. Roger Y. Tsien, Departments of Chemistry and Biochemistry, University of California at San Diego, USA) by PCR using sense primer PM1-S containing an Nhe\textsubscript{I} site, and antisense primer PM1-A containing a BgII site. The mCherry sequence was amplified and inserted into pEGFP-C1 to generate pmCherry-C1, which replaced the EGFP sequence with mCherry. The 2A\textsuperscript{pro} gene was amplified as described above and was inserted into pmCherry-C1, which was digested with HindIII and EcoRI. This vector allows the expression of the fusion protein mCherry-2A\textsuperscript{pro}.

To construct the mutant 2A\textsuperscript{pro}(H20A)-mCherry fusion protein expression plasmid pcDNA3-2A\textsuperscript{pro}(H20A)-mCherry, a sense primer of 2A\textsuperscript{pro}, P2AM-S containing a HindIII site, a Kozak sequence and the non-synonymous DNA mutations for H20A was used. The same P2A-A antisense primer was used for the construction of pEGFP-C1-2A\textsuperscript{pro}. The 2A\textsuperscript{pro}(H20A) DNA was inserted into pcDNA3.0 (Invitrogen) digested with HindIII and EcoRI, and the mCherry DNA (amplified using sense primer PM2-S containing an EcoRI site and a linker sequence encoding GGGGS and antisense primer PM2-A containing an Xho\textsubscript{I} site) was inserted with EcoRI and Xho\textsubscript{I}. The N-terminal linker sequence was necessary for the construction of 2A\textsuperscript{pro} expression vectors to prevent its own autocatalytic cleavage [11]. Sequences of the primers described above were all shown in Table 1. All the DNA sequences were verified by sequencing.

**Plasmid transfection and virus infection**

Vero cells were cultured as described above. Cells were grown on glass coverslips in 35-mm tissue culture dishes and cultured to 70–80% confluence before transfection. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Before observation, the transfected cells were cultured at 37\( ^\circ \)C (5% \( \text{CO}_2 \)) for 20 h, unless indicated. Hoechst 33258 dye (Invitrogen) was used to stain cell nuclei.

For poliovirus infections, cells previously transfected with pEGFP-C1-3CD or pEGFP-C1-3C\textsuperscript{'} were cultured for 20 h and the medium was removed and replaced with serum-free DMEM. Cells were infected with poliovirus at approximately 30 plaque-forming units (PFU) per cell and cultured at 37\( ^\circ \)C (5% \( \text{CO}_2 \)) for 4 h before staining with Hoechst 33258 and visualization.

**Western blot**

Cells were harvested and lysed in the RIPA lysis buffer (Beyotime, Shanghai, China) according to the manufacturer’s
instructions. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes according to standard procedures. Blots were developed with rabbit anti-GFP polyclonal IgG (sc-8334; Santa Cruz Biotech, Santa Cruz, USA) and HRP-conjugated secondary antibodies. The blot was detected using Pierce ECL Western Blotting Substrate Kit (Pierce, Rockford, USA) and exposed with FUJI medical X-ray film (FUJI Photo Film Co., Shanghai, China).

Fluorescence microscopy
Cells were imaged with an epifluorescence microscopy system (Carl Zeiss, Oberkochen, Germany) using a cooled CCD camera (Photometrics, Tucson, USA). Images were acquired and manipulated using the MetaMorph software (version 6.0; Molecular Devices, Sunnyvale, USA).

Results

Intracellular localization of 3CD and 3C' with or without poliovirus infection
The recombinant fusion proteins, EGFP-3CD and EGFP-3C’, were first transiently expressed in Vero cells and the intracellular localization was imaged using fluorescence microscopy. As shown in Fig. 1, after the plasmid pEGFP-C1-3CD or pEGFP-C1-3C’ was transfected into Vero cells, the fluorescence of EGFP-3CD or EGFP-3C’ [Fig. 1(A)] was mostly distributed in the cell cytoplasm at 20 h post-transfection. And then these transfected Vero cells were infected with poliovirus and cultured for another 4 h. It was found that both EGFP-3CD and EGFP-3C’ proteins were distributed throughout the entire cell (cytoplasm and nucleus) after virus infection [Fig. 1(B)]. Western blot assay was carried out to verify whether the GFP fusion proteins were rightly expressed in live cells. Figure 1(C) showed that fusion protein EGFP-3CD expressed correctly in Vero cells and there was no degradation of the GFP fusion proteins to release GFP. It means that fluorescence imaging would display the true localization pattern of the viral protein. These results showed that upon poliovirus infection, the viral 3CD and 3C’ proteins re-localized to the nucleus in addition to remaining in the cytoplasm.

Poliovirus 2A pro causes distribution change of 3CD and 3C’
A previous report has shown that 2A pro can change the localization of some cellular proteins [15], which prompts the investigation of the localization of EGFP-3CD and EGFP-3C’ in the presence of 2A pro. Co-expression of mCherry-2Apro with either EGFP-3CD or EGFP-3C’ proteins caused the distribution of EGFP fluorescence throughout the whole cell (Fig. 2), similar to the localization pattern observed during poliovirus infection. Therefore, the poliovirus 2A pro protein can cause the re-localization of 3CD and 3C’ to the nucleus.

The protease activity of 2A pro is essential for inducing 3CD and 3C’ re-localization
A mutant 2A pro (H20A) that abolished the protease activity of 2A pro was constructed and fused to the fluorescent protein mCherry. When co-expressed with 2A pro(H20A)-mCherry, EGFP-3CD and EGFP-3C’ exclusively localized in the cytoplasm (Fig. 3), similar to the distribution when expressed in the absence of wild-type 2A pro. Therefore, the mutant 2A pro(H20A) did not change the localization of EGFP-3CD and EGFP-3C’, suggesting that the protease

<table>
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<th>Primers</th>
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<tr>
<td>P3CD-S</td>
<td>5'-CAATCTCGAGGCGAGGCGCTCAGGGCGACAGGTTCGATTACGCAGTG-3'</td>
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<tr>
<td>P3C’-A</td>
<td>5'-GGAGGAATTCCTTAATATGGTGACAGTTTITTCGATT-3'</td>
</tr>
<tr>
<td>P3-A</td>
<td>5'-GACTGAATTCGTTGTTACTAAAAATGAGCTAAGCCACGCGGG-3'</td>
</tr>
<tr>
<td>P2A-S</td>
<td>5'-CAATAAGCTCGGAGGCGGCTCAGCCACATGGGATTTTGCA-3'</td>
</tr>
<tr>
<td>PM2A-S</td>
<td>5'-CAATAAGCTCGGAGGCGGCTCAGCCACATGGGATTTTGCA-3'</td>
</tr>
<tr>
<td>P2A-A</td>
<td>5'-TAACGAATTCGTTGTTACTAAAAATGAGCTAAGCCACGCGGG-3'</td>
</tr>
<tr>
<td>PM1-S</td>
<td>5'-AAAAGCTAGCCACATGGTGACAGTTTITTCGATT-3'</td>
</tr>
<tr>
<td>PM1-A</td>
<td>5'-AAAAAGATCTCAGGCTCGGCGACCTTTGTAACAGCTGTCATGCC-3'</td>
</tr>
<tr>
<td>P2AM-S</td>
<td>5'-CAATAAGCTCGGAGGCGGCTCAGCCACATGGGATTTTGCA-3'</td>
</tr>
<tr>
<td>PM2-S</td>
<td>5'-TAATGAATTCGCGGAGGCGGCTCAGTTGAGCAAGGGCGAGGAGGATA-3'</td>
</tr>
<tr>
<td>PM2-A</td>
<td>5'-TAATCTCGAGCTACTGTACAGCTGTCATGCAGGCGGGGT-3'</td>
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The enzyme cleavage sites are shown in bold.

Table 1 Sequences of the primers

Poliovirus 2A pro induces 3CD and 3C’ proteins re-localization

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activity of 2Apro might be essential for the ability of 3CD and 3C\textsuperscript{0} to enter the nucleus.

2Apro co-localized with nuclear pore complexes on the NE
To further explore the exact role of 2Apro in the translocation of EGFP-3CD and EGFP-3C\textsuperscript{0}, the intracellular localization of 2Apro was studied. It was noticed that when mCherry-2Apro was co-expressed with EGFP-3CD or EGFP-3C\textsuperscript{0}, the mCherry-2Apro mainly localized to the NE (Fig. 2). When mCherry-2Apro or EGFP-2Apro proteins alone were transiently expressed in Vero cells, they also localized primarily to the NE [Fig. 4(A)]. To further explore this localization, the Nup153 protein, a constituent marker of nuclear pore complexes, was fused to Cherry and co-expressed with EGFP-2Apro. As shown in Fig. 4(B), EGFP-2Apro could co-localize with mCherry-Nup153 at the NE in Vero cells.

2A\textsuperscript{pro}(H20A)-EGFP co-localized with Nup153-mCherry on NE and in annulate lamellae
Whether mutant 2A\textsuperscript{pro}(H20A) can co-localize with Nup153 on NE and in annulate lamellae (AL) was also tested. AL was thought to be the storage apartment of nucleoporins [16], and overexpression of Nup153 can induce the formation of the AL in some mammalian cells cytoplasm [17]. To test if AL can be induced in the Vero cells, mCherry-Nup153 was overexpressed and the result shows that the AL could be formed. When pEGFP-C1-2A\textsuperscript{pro}(H20A) was co-transfected with pcDNA3-2Apro(H20A)-mCherry in Vero cells, the 2A\textsuperscript{pro}(H20A)-EGFP could co-localize with mCherry-Nup153 on NE, while they could co-localize in the AL when overexpressed (Fig. 5).

Discussion
When expressed alone, the poliovirus 3CD and 3C\textsuperscript{0} proteins localized exclusively in the cellular cytoplasm but not in the nucleus. However, upon poliovirus infection these proteins can also be found in the nucleus. The viral 3CD precursor polyprotein can undergo auto-proteolysis to produce the 3C\textsuperscript{pro} protease, which is responsible for the inhibition of host cell transcription by digesting related cellular factors. It is believed that the 3C\textsuperscript{pro} protein might be delivered to the nucleus in the form of its precursor, which is cleaved and becomes active in the nucleus causing the cessation of host cell gene transcription and translation.

Besides cleaving cellular transcription factors, it was found that 3C\textsuperscript{pro} protein can also cleave poly(A)-binding proteins (PABPs) [18,19]. There are two classes of PABPs...
in mammalian cells, PABPC1 (PABP1) and PABPN1 (PABP2) [20,21]. PABPN1 primarily localizes in the nucleus and plays a role in the synthesis of poly(A) tails [22,23]. PABPC1 primarily localizes in the cytoplasm and influences translation and mRNA decay [24]. Recent studies, however, have indicated that PABPC1 can also localize in the nucleus and bind nuclear pre-mRNA poly(A) tails, thereby affecting pre-mRNA processing, stability, and mRNA trafficking [25]. The 3Cpro protein is, therefore, an important regulator of host gene expression acting primarily in the nucleus [4]. Hence, the nuclear localization of its precursors, 3CD and 3C′, is likely to be

Figure 4 Images of 2Apro Localization in Vero cells (A) Vero cells were transfected with pmCherry-C1-2Apro or pEGFP-C1-2Apro for 20 h. MCherry fused Nup153 was expressed in Vero cells, which were transfected with pmCherry-Nup153 for 8 h. The fusion protein mCherry-2Apro and EGFP-2Apro localized to the NE in Vero cells. DIC, differential interference contrast. Scale bar = 20 μm. (B) The cells were co-transfected with plasmids pEGFP-C1-2Apro and pmCherry-Nup153 for 20 h. Fusion proteins EGFP-2Apro and mCherry-Nup153 co-localized to the NE in Vero cells. Hoechst pointed the localization of cell nuclei. Scale bar = 10 μm.

Figure 5 Co-localization of 2Apro(H20A) and Nup153 in Vero cells The plasmids pEGFP-C1-2Apro(H20A) and pmCherry-Nup153 were co-transfected for 8 and 32 h, respectively. AL was induced by overexpression of mCherry-Nup153 and co-localized with EGFP-2Apro(H20A). Hoechst pointed the localization of cell nuclei. DIC, differential interference contrast. Scale bar = 20 μm.
an important step for 3Cpro to induce host cell transcription shut-off.

The present study showed that the nuclear re-localization of 3CD and 3C' could be induced by 2Apro alone. It is reported that 3CD and 3C' of poliovirus have nuclear localization signal, which only functions under poliovirus infection [4]. Previously studies have indicated that NPCcs were altered during poliovirus infection [15,26] and this could not be simply explained as virus-induced leakiness of the NE because some functions of cellular NPCcs remained [26]. The degradation of Nup98, Nup153, and p62 by 2Apro has also been reported as important for poliovirus infection [26,27]. It is likely that NPCcs are altered by 2Apro and then induce the re-localization of 3CD and 3C' to the cell nucleus. A variety of nucleo-cytoplasmic trafficking pathways are inhibited in poliovirus-infected cells such as importin α/β and transportin, which are the targets of the SV40 large T antigen and heterogeneous nuclear ribonucleoprotein, respectively. However, the transportation of some other proteins that use the transportin-serine/arginine-rich (SR) pathway, such as the SR protein SC35, is not affected by poliovirus infection [26]. The re-localization of 3CD and 3C', therefore, may be involved in a special cytoplasmic–nucleic pathway that is induced during poliovirus infection or 2Apro expression. Further studies should be dedicated to determine the pathway used by poliovirus 3CD and 3C' to enter the nucleus.

Poliovirus 2Apro can cleave the eIF4G directly or indirectly and suppress the cap-dependent translation [28,29]. To overexpress Nup153-mCherry during investigating, the co-localization of 2Apro and Nup153 in the AL, the mutant 2Apro (H20A), was used. His20 of 2Apro is one of the important residue in this protease putative catalytic triad and His117 is part of the putative substrate-binding pocket [30], and it is reported that the mutant 2Apro(H20A) cannot cleave eIF4G, which plays an important role in the cap-dependent translation [31]. Thus, this mutant 2Apro will not suppress the expression of Nup153-mCherry.

NPCcs can actively conduct macromolecular translocations across the NE. The 2Apro protein of poliovirus co-localizes with NPCcs and induces protein re-localization, which suggests that NPCcs are altered by 2Apro during poliovirus infection, similar to a recent report that the matrix protein of vesicular stomatitis virus co-localizes with host cell NPCcs and alters protein nucleo-cytoplasmic trafficking [32]. These results suggest that some RNA viruses can alter the nucleo-cytoplasmic trafficking of viral or cellular proteins to benefit their reproduction.

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