Identification of the Nuclear Localization Signal Region of Duck Enteritis Virus UL14 and Its Interaction with VP16

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Keywords

Duck enteritis virus · pUL14 · VP16 · Interaction · Nonclassical nuclear localization signal

Abstract

Object: Duck enteritis virus (DEV) is a member of the Alpha-herpesvirinae viruses. VP16 and pUL14 are both predicted to be tegument proteins of DEV. **Methods:** An indirect immunofluorescence assay (IFA) was performed for preliminary analysis of the colocalization of pUL14 and VP16, which detected their subcellular localization in duck embryo fibroblasts (DEFs) during virus replication. The coexpression of pUL14 and VP16 was detected in transfected DEFs. A bimolecular fluorescence complementation (BiFC) assay was used to confirm a direct interaction between pUL14 and VP16. To better characterize the nuclear localization domain of pUL14, we designed a series of deletion mutants and transfected them with VP16. **Results:** Our IFA findings indicated that pUL14 binds to VP16 in the cytoplasm and that pUL14 leads VP16 import into the nucleus during DEV replication. The BiFC assay revealed the presence of pUL14 and VP16 complexes. Furthermore, 1–98 amino acid (aa) at the N-terminus of pUL14 played a role in the nuclear localization signal (NLS)

region and promoted translocation of VP16 into the nucleus to complete the virus life cycle. **Conclusions:** Our findings indicated that pUL14 could transport VP16 into the nucleus and that the N-terminal 1–98 aa may contain the NLS domain of pUL14.

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Introduction

Duck enteritis virus (DEV) can infect up to 100% of populations of birds, such as ducks, geese, and wild waterfowl, in the order Anseriformes and can cause an acute infectious disease [1, 2]. A high prevalence of DEV was found among free-ranging water birds in Poland [3]. The economic impact on waterfowl husbandry caused by DEV is considerable because the mortality, especially in young birds, can reach 100% [2, 3]. As a member of Alphaherpesvirinae, DEV contains 4 distinct structures: the core, capsid, tegument, and envelope [4, 5]. The tegument

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Table 1. Primers used for the *UL14* gene deletion mutants

Recombinant vectors	Primers	Fragment limits, aa
pEGFPN1-m1	F: <u>AAGCTT</u> AACATGCTATCGCGGCTTCAAGATC R: <u>GGATCC</u> GTGTCAACGAACACGGCACTATT	1–45
pEGFPN1-m2	F: <u>AAGCTT</u> ATGACTTTTACGTCAGCGAAAGC R: <u>GGATCC</u> GATAAAAAACCTTCTGTGCGCC	46–98
pEGFPN1-m3	F: <u>AAGCTT</u> AACATGCTATCGCGGCTTCAAGATC R: <u>GGATCC</u> GATAAAAAACCTTCTGTGCGCC	1–98
pEGFPN1-m4	F: <u>AAGCTT</u> ATGTCGCCAGGCCTAATTGAT R: <u>GGATCC</u> GCTAATAGGGCTTCGTCGTCTTC	99–152

The *Hind*III and *Bam*HI restriction sites are underlined.

is not highly structured and is distributed around the capsid [6]. It contributes to viral replication and immune evasion [6, 7]. Tegument assembly into a virion occurs via a series of processes that entails numerous interactions, including tegument-capsid, tegument-envelope, and tegument-tegument [8–13]. The tegument-tegument interactions constitute a dense network of associations among tegument proteins [8].

In DEV, the protein UL14 (pUL14) was predicted to be a minor component of the virion tegument protein, which is not essential for viral replication, and VP16 (UL48) was predicted to be a large tegument protein that is essential for viral assembly [14]. Although there is little information available about pUL14 and VP16 in DEV, they have been better characterized in herpes simplex virus type 1 (HSV-1) and HSV-2. The pUL14 has been shown to interact with tegument protein VP16 and capsid protein VP26 [15, 16]. Additionally, pUL14 may be required for efficient envelopment when particles fuse into cytoplasmic vesicles, promoting the spread of HSV-1 infection [17]. Alternatively, it has been reported that VP16 can interact with many viral tegument proteins and the cytoplasmic domains of glycoprotein, which indicates that VP16 has many potential interactions during virus infection [12, 13, 18–20]. By contrast, there is little information available about interactions between the 2 tegument proteins pUL14 and VP16 in DEV. The present study was designed to gain insight into the assembly of tegument protein pUL14 and VP16 during DEV infection.

A viral protein localized to the nucleus may contribute to host cell proliferation, development, apoptosis, and reactions [21, 22]. It has been determined that the proteins localized to the nucleus carry nuclear localization signals (NLS) [23]. There are 3 types of NLS, including classical NLS that contains a single peptide of basic amino acids,

bipartite NLS that consists of 2 stretches of basic amino acids that are separated by a linker region, and nonclassical NLS that has no obvious features of a basic amino acid signal sequence [24–29]. Although various NLS can differ in structure, they can perform the same function depending on protein recognition by soluble factors that mediate transport via the nuclear pore complexes [30]. To detect a presumptive NLS, a commonly used method is to assess the distribution of GFP [31]. There were no classical NLS in DEV pUL14, which was analyzed using the NLS prediction program of the PSORT II server. However, there were 4 α -helical segments in the 1–152 amino acid (aa) sequence of pUL14: 4–41, 55–103, 107–133, and 146–152 aa [32]. Subsequently, we studied the function of the secondary structure of DEV UL14 in nuclear import mediated by 1 of the 4 α -helical segments, which showed the same activity as nonclassical NLS. Immunofluorescence assay (IFA), coexpression, and bimolecular fluorescence complementation (BiFC) assessments were performed to gain insight into the assembly of tegument proteins pUL14 and VP16 during DEV infection. To determine the function of nonclassical pUL14 and its association with VP16, we constructed a series of UL14 mutants. This study will shed light on the molecular pathogenic mechanism of DEV.

Materials and Methods

Viruses and Cells

DEV was prepared at the Key Laboratory of Animal Disease and Human Health of Sichuan Province at Sichuan Agricultural University according to a method that has been previously reported. Duck embryo fibroblasts (DEFs) were cultured in Minimum Essential Medium (Gibco-BRL) with 10% fetal bovine serum (FBS). Then, cells were maintained in 2% FBS and incubated in 5% CO₂ and 37°C as previously reported for our laboratory [33].

Fig. 1. Intracellular localization of pUL14 and VP16 in DEV-infected cells. Cells were infected with DEV at a MOI of 2 PFU/cell and were fixed at 24, 36, 48, and 60 h.p.i. Cells were incubated with rabbit anti-VP16 primary antibody (PAb) and mouse anti-pUL14 PAb primary antibodies, and Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies. The subcellular localization of these proteins was assessed by visualizing the fluorescence. Scale bars, 10 μ m.

Plasmid Construction

To detect interactions between pUL14 and VP16, we constructed the plasmids pBiFCVC155-UL14 and pBiFCVN155-UL48. The primers for pBiFC-VC155-UL14 were F: GTCGACCATGCTATCGCGGCTTCAAGATC and R: GGTACCGCTAATAGGGCTTCGTCGTCTTC, with *SalI* and *KpnI* restriction sites, respectively. The primers for pBiFCVN155-UL48 were F: GTCGACTGTTCGTATTATTGGGTA and R: GGTACCTATGTAGGCTAATTATCTGGCGAG, with *SalI* and *KpnI* restriction sites, respectively.

To assess the colocalization of pUL14 and VP16, the eukaryotic expression plasmids pEGFP-UL14 and pDsRed-UL48 were constructed. The primers for pEGFP-UL14 were F: AAGCTTAA-CATGCTATCGCGGCTTCAAGATC and R: GGATCCGCTA-ATAGGGCTTCGTCGTCTTC, with *HindIII* and *BamHI* restriction sites, respectively. The primers for pDsRed-UL48 were F: GAGCTCATTATTGGGTAAATAGGTGTGGGTG and R: CCGCGGATTGGATCTCGTGATTAGTCACATG, with *SacI* and *SacII* restriction sites, respectively. Primers were designed according to sequences available in GenBank with accession No. JQ647509

Fig. 2. Subcellular localization of pUL14 and VP16. Cells were fixed at 48 h post-cotransfection. The pEGFP-UL14 alone (green fluorescence) and pDsRed-UL48 (red fluorescence) plasmids were used (colors refer to the online version only). Cells were transfected or cotransfected with pEGFP-UL14 and pDsRed-UL48. Scale bars, 10 μ m. YFP, yellow fluorescent protein.

[34]. The DEV genome was used as a PCR template. PCR products were sequenced (Invitrogen) and cloned into eukaryotic vectors according to the instructions for T4 DNA ligase (Takara).

To identify the nonclassical NLS of pUL14, we designed deletion mutants of the UL14 gene. The secondary structure of pUL14 was analyzed using a protein prediction server (<https://www.predictprotein.org>) and 4 α -helices were predicted [32]. To analyze these helices in the nuclear targeting function, a series of deletion mutants were designed. Primers for the deletion mutant plasmids pEGFPN1-m1 (m1), pEGFPN1-m2 (m2), pEGFPN1-m3 (m3), and pEGFPN1-m4 (m4) were designed and contained *Hind*III and *Bam*HI restriction sites (Table 1). PCR products were ligated into pEGFP-N1, and all constructs were verified by sequencing (Invitrogen).

Indirect Immunofluorescence Assays

To examine the subcellular localization of pUL14 and VP16 during virus infection, an indirect IFA was used as previously reported [35]. First, DEV was used to infect DEFs that were grown on coverslips. Then, we harvested coverslips at 24, 36, 48, and 60 h post infection (h.p.i.) and fixed them with 4% formaldehyde for 30 min at 4°C. Cells were then permeabilized and blocked. The cells were incubated with mouse anti-UL14 IgG and rabbit anti-VP16 primary antibodies at 4°C. Then, we incubated them with Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (Bio-Rad). Cell nuclei were stained using DAPI (5 μ g/mL, Beyotime). Finally,

fluorescent images were viewed and recorded using a Nikon 80i fluorescence microscope (Nikon Inc., Kanagawa, Japan).

Transfection and Transient Expression

DEFs were grown on coverslips with 10% FBS (Gibco). After 24 h, DEFs were transfected with pBiFCVC155-UL14, pBiFCVN155-UL48, pEGFPN1-UL14, and pDsRed-UL48, according to the manufacturer's instructions for Lipofectamine 3000 (Invitrogen). After 48 h, cells were fixed, permeabilized, and stained with DAPI. Finally, fluorescence was visualized with a Nikon 80i fluorescence microscope [36].

To determine the nonclassical NLS of pUL14, deletion mutants (m1–m4) were transfected into DEFs. To analyze the transport function of the nonclassical NLS, deletion mutants of pUL14 and pDsRed-UL48 were cotransfected into DEFs. Cells on slides were treated as described above.

Results

Colocalization of pUL14 and VP16 by IFA

To detect the subcellular localization of pUL14 and VP16 during DEV replication in DEFs, IFA was performed. Cells were fixed at 24, 36, 48, and 60 h.p.i. Cells

Fig. 3. The BiFC assay was used to detect interactions between pUL14 and VP16. Cells were fixed and examined by fluorescence microscopy to confirm transfection at 48 h posttransfection. No fluorescence was detected in the pBiFCVN155-UL48/pBiFCVC155 and pBiFCVC155-UL14/pBiFCVN155 plasmid groups. By contrast, fluorescence was detected in the pBiFCVN155-UL48/pBiFCVC155-UL14 group. Bars, 10 μ m. YFP, yellow fluorescent protein.

were incubated with rabbit anti-VP16 and mouse anti-pUL14 primary antibody, followed by Alexa Fluor 594-conjugated goat anti-mouse and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies. Both red and green fluorescence could be detected in the cytoplasm at 24 h.p.i. Subsequently, fluorescence could be observed in the perinuclear region at 36 h.p.i. The fluorescence was both in the perinuclear region and nucleus at 48 h.p.i. Both red and green fluorescence could be observed in the nucleus at 60 h.p.i. (Fig. 1). Although the colocalization was not obvious from 24–36 h.p.i., it was more robust at 60 h.p.i.

pUL14 Is Involved in VP16 Nuclear Transport

As we observed colocalization of pUL14 and VP16 during infection, we decided to detect the subcellular localization of pUL14 and VP16 by overexpressing the 2 proteins. The DEFs were transfected with 3 groups of plasmids: pEGFP-UL14, pDsRed-UL48, and pEGFP-UL14/pDsRed-UL48. After 48 h, pUL14 was detected in the

nucleus by detecting green fluorescence, where pEGFP-UL14 was transcribed into DEFs alone. VP16 was detected in the cytoplasm by detecting red fluorescence, where pDsRed-UL48 was transcribed into DEF alone. In group 3, plasmids pEGFP-UL14 and pDsRed-UL48 were cotransfected, and the subcellular localization of pUL14 and VP16 were both nuclear (Fig. 2).

Confirmation of Interactions between pUL14 and VP16 by BiFC

To determine whether there was a direct interaction between pUL14 and VP16, we performed BiFC assays. DEFs grown on coverslips that were transfected with plasmids divided into 3 groups: pBiFCVN155-UL48/pBiFCVC155, pBiFCVC155-UL14/pBiFCVN155, and pBiFCVN155-UL48/pBiFCVC155-UL14. We found that there was no fluorescence in the pBiFCVN155-UL48/pBiFCVC155 group and pBiFCVC155-UL14/pBiFCVN155 group, whereas fluorescence was detected in the pBiFCVN155-UL48/pBiFCVC155-UL14 group

Fig. 4. The structures of the pUL14 deletion mutants. The 4 α -helices of pUL14 were predicted and are represented as blue helices. According to the helices, pUL14 mutants were designed. Blue strips (color in online version only) show the different regions of pUL14 and green strips show the GFP regions.

(Fig. 3). This finding suggested that green fluorescence was produced by an interaction between pUL14 and VP16 (Fig. 3).

Intracellular Localization of the UL14 Deletion Mutants

To identify the nuclear localization domain of pUL14, we designed a series of deletion mutants. The secondary structure of pUL14 was analyzed and revealed the presence of 4 α -helices (Fig. 4). DEFs were transfected with m1–m4 and pEGFPN1. We detected the localization of the pUL14 mutants via GFP fluorescence localization (Fig. 5). These findings indicated that the GFP fluorescence of UL14-m1 (1–45 aa), UL14-m2 (46–98 aa), and UL14-m4 (99–152 aa) were each diffuse, both in the nucleus and cytoplasm. UL14-m3 (1–98 aa) was distributed in the nucleus.

The Region 1–98 aa of pUL14 Has an NLS for VP16

As pUL14 could transport VP16 into the nucleus, we further confirmed that the NLS domain of pUL14 played a role in transporting VP16. UL14-m1, UL14-m2, UL14-m3, and UL14-m4 were coexpressed with VP16 in DEFs to determine the nuclear transport function of the nonclassical NLS in pUL14. Colocalization of m3 and VP16 was observed. No colocalization of the 2 proteins in the nucleus was detected in the other groups (Fig. 6). This finding confirmed that the 1–98 aa at the N-terminus of pUL14 plays a key role in the nuclear localization of VP16.

Discussion

The BiFC assay has been widely used to detect interactions between 2 proteins [37–39]. In this study, BiFC assays were used to determine whether there was an interaction between pUL14 and VP16. Expression plasmids that encoded pUL14 or VP16 fused to a C- or N-terminal fragment of yellow fluorescent protein were generated and transfected into DEFs. The 2 nonfluorescent fragments of yellow fluorescent protein could associate and form a fluorescent complex by the fusion of 2 proteins that interact with each other [40]. Our findings revealed that 2 fragments of yellow fluorescent protein interacted, as they produced fluorescence. In this study, the results of the BiFC assay suggested that interactions occur between pUL14 and VP16.

As DEV pUL14 localized to the nucleus, we sought to identify the nonclassical NLS of DEV pUL14. As it had 4 helices, we designed helix deletion mutants and then assessed the helix distribution in transfected DEFs. GFP distribution was used as a criterion to detect the presump-

Fig. 5. Schematic diagram of plasmids encoding mutants of DEV pUL14 fused with GFP. Subcellular localization of the DEV UL14 mutants in DEF cells transfected using Lipofectamine 3000, including pEGFP-UL14-m1 (m1), pEGFP-UL14-m2 (m2), pEGFP-UL14-m3 (m3), pEGFP-UL14-m4 (m4), and pEGFP-N1. GFP fluorescence of UL14-m1 (1–45 aa), UL14-m2 (46–98 aa), and UL14-m4 (99–152 aa) revealed diffuse staining in cells. UL14-m3 (1–98 aa) showed a nuclear distribution. Scale bars, 10 μ m.

(For figure see next page.)

Fig. 6. Subcellular localization of VP16 deletion mutants. Cells were fixed at 48 h post-cotransfection. Scale bars, 10 μ m.

tive nonclassical NLS of UL14. As previously reported, the molecular mass of GFP is 27 kDa and it can freely diffuse in the nucleus and cytoplasm [41, 42]. Our results indicated that the mutants, including UL14-m1 (1–45 aa), UL14-m2 (46–98 aa), and UL14-m4 (99–152 aa), diffused into the nucleus and cytoplasm because of their relatively small sizes (molecular mass ≤ 44.5 kDa), even if they did not contain a nonclassical NLS. By contrast, the mutant UL14-m3 (1–98 aa) showed exclusively nuclear staining, whereas UL14-m4 (99–152 aa) was diffuse both in the nucleus and cytoplasm. Therefore, we suggested

that the nonclassical NLS was contained within the m3 segment.

Heat shock proteins (HSPs) are the most abundant intracellular proteins. HSP70 is a highly conserved member of the HSP family that primarily acts as a molecular chaperone, mediating functions such as nuclear transport [43, 44]. In HSV-1, the 44–104 aa of pUL14 can interact with VP16, and it shows homology to HSP70 [15]. To assess whether DEV pUL14 had similarities with HSV-1 pUL14, we conducted a BLAST search using the NCBI server. We found that DEV pUL14 had 46% identity with HSV-1 and

HSV-2 [12]. Regarding the differences between these proteins, $_{60}\text{RLKSR}_{64}$ of HSV-1 pUL14 was similar to that of HSP70. An α -helix formed by this segment was integrated into the substrate-binding region [15]. In DEV, the sequence of pUL14 was $_{60}\text{QLRSN}_{64}$. The positively charged residues of R were replaced by the neutral residues Q and N at a critical position. This indicated that the characteristics of DEV pUL14 were different from those of HSV pUL14.

It has been investigated that NLS is a single peptide of basic amino acids [25–27]. Accordingly, only one cluster of basic residues $_{8}\text{RQRRLR}_{13}$ was found in pUL14. To assess whether similarity existed between DEV pUL14 and HSP70, we conducted a BLAST search of their sequences. We found that the segment of $_{8}\text{RQRRLR}_{13}$ was similar to that of the $_{259}\text{AVRRLR}_{264}$ of HSP70. Moreover, this fragment, $_{259}\text{AVRRLR}_{264}$, is contained in the bipartite NLS of HSP70 [44–46]. Thus, we proposed that the cluster of basic residues, $_{8}\text{RQRRLR}_{13}$, played a key role like the NLS. The consensus sequence has been reported [47]. Furthermore, we found that the mutants could not localize to the nucleus unless mutants UL14-m1 (1–45 aa) and UL14-m2 (46–98 aa) recombined with each other. This finding indicated that the combined actions of the 2 α -helix fragments might account for the nuclear localization pattern. Additionally, there were 2 α -helices in 1–98 aa (4–45 aa and 46–98 aa) that contained 34 and 26% basic amino acids, respectively. In 1–98 aa of pUL14, 9 protein-binding sites were identified. Thus, we suggested that the 2 α -helices acted as a non-classical NLS by forming a unique spatial conformation. However, it cannot be excluded that the deletion mutants disrupt the overall structure generated by the 2 proteins. It may also be likely that both proteins required a third unidentified partner to undergo relocation.

The IFA and cotransfection assay results demonstrated that pUL14 interacted with VP16 in perinuclear regions, then both proteins entered into the nucleus during DEV assembly. It has been reported that tegument pro-

tein, which is distributed in the nucleus, can be incorporated into the capsid during viral assembly [9]. In HSV-2, pUL14 has been established to interact with the capsid proteins pUL35 and pUL33, while VP16 contributed to capsid assembly [15]. In the present study, pUL14 and VP16 were both localized to the nucleus, suggesting that the 2 proteins might interact with capsid protein. Moreover, pUL14 and VP16 have been reported to be associated with glycoprotein, which indicates that a similar function might present in DEV during virion assembly. The time required for the relocation of pUL14 and VP16 into the nucleus was 48–60 h, indicating that relocation occurred at a late stage. The first and secondary envelopment events occurred during this stage. The first envelopment occurred near the nuclear membrane. It has been previously reported that unenveloped capsids accumulated in the cytoplasm of UL14D-infected cells [17]. Moreover, the same authors suggested that pUL14 facilitated the addition of tegument and envelope for virion assembly. The findings of Yamauchi et al. [16] also indicated that pUL14 was required for the events that followed capsid formation. Those 2 DEV proteins might associate with the mutation of tegument and envelope proteins, and this possibility merits further investigation.

In summary, the NLS was initially identified by constructing pUL14 truncation mutants. Moreover, pUL14 may be involved in the replication of viral DNA and assembly of both tegument and envelope.

Acknowledgements

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