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The nuclear localization signal of the NS1 protein is essential for *Periplaneta fuliginosa* densovirus infection

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ABSTRACT

The regulatory protein NS1 is a key molecule in life cycle of *Periplaneta fuliginosa* densovirus (*Pf*DNV). When we ectopically expressed the *Pf*DNV NS1 protein in non-*P. fuliginosa* insect cells, the NS1 protein could not enter the nucleus and remained in the cytosol. However, the NS1 was localized to both the cytosol and nucleus of cockroach hemocyte cells. So we investigated the abilities of the potential nuclear localization signal (NLS) of *P. fuliginosa* Densovirus non-structural protein 1 (NS1) to translocate NS1 and a carrier protein to the nucleus following transfection into insect cells. Possible nuclear localization sequences were chosen from the NS1 on the basis of the presence of basic residues, which is a common theme in most of the previously identified targeting peptides. Nuclear localization activity was found within the residues 252–257 (RRRRRR) of the NS1, while replacement of a single arginine in this region with glycine abolished it. The targeting activity was enhanced with the arginine residues added.

1. Introduction

Densoviruses (DNV) are autonomously replicating parvoviruses that are highly pathogenic for invertebrates and are frequently isolated from arthropod hosts (Bergoin and Tijssen, 1998; Fédière, 2000). The DNV genome consists of a single-stranded linear DNA that is 4–6 kb in length (Tijssen and Bergoin, 1995; Bergoin and Tijssen, 2000). Based on the size, organization of coding sequences, and structure of the ends of their genome, DNVs can be classified into four genera within the subfamily *Densovirinae* (Tattersall et al., 2005). *Periplaneta fuliginosa* densovirus (*Pf*DNV) has a 5.5-kb genome, inverted terminal repeats (ITRs), and an ambisense organization. ITRs are on both ends of the genome. ORF1, ORF2, and ORF3, which encode the three structural proteins VP1, VP2, and VP3, respectively, are located in the 5′ half of one strand. ORF4, ORF5, and ORF6, which encode the three non-structural (NS) proteins NS1, NS2, and NS3, respectively, are located in the 5′ half of the complementary strand (Dumas et al., 1992; Tijssen and Bergoin, 1995; Berns et al., 2000; Guo et al., 2000). The *ns* and *vp* genes are transcribed from the P3 and P97 promoters, respectively (Yang et al., 2006).

The regulatory protein NS1 is a key molecule in viral replication. It is a multi-functional protein that has several catalytic activities, including ATP binding and hydrolysis (Walker et al., 1982; Jindal et al., 1994), oligomerization (Pujol et al., 1997), site-specific DNA binding to a cognate recognition motif (Owens et al., 1993), site- and strand-specific nicking (Walker et al., 1997), helicase activity (Wilson et al., 1991), and promoter trans regulation (Yang et al., 2006; You et al., 2006). These activities allow this pleiotropic protein to execute the different functions necessary for progeny virion production, including regulation of viral DNA amplification and gene expression (Nüesch, 2005). Additionally, some of the latter functions may also act upon the host cell genome and may contribute to the cytotoxic activities of NS1 (Liu et al., 2005). Moreover, the ability of NS1 physically interacts with a variety of host cell proteins, including components of the DNA replication machinery (Christensen and Tattersall, 2002) and transcriptional regulators (Krady and Ward, 1995; Lorson et al., 1998) may represent another "scavenging" mechanism by which NS1 negatively interferes with essential cellular processes (Corbau et al., 1999).

This study demonstrates that the NS1 protein contains a region that determines its ability to translocate into the nucleus of permissive cells in order to regulate the propagation of *Pf*DNV. As the nuclear localization is necessary for the function of NS1, this finding also helps to explain the strict host range of *Pf*DNV. We focused on the nuclear localization activity and nuclear localization region of the *Pf*DNV NS1 protein. Immunofluorescence and Western blot analyses were used in this work to study the nuclear localization activity. The NLS showed high conservation in mutagenesis assay. Furthermore, cell-type specificity of the nuclear localization activity was also determined in this study.

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2. Materials and methods

2.1. Plasmids

The *NS*1 coding sequence (nt 1122–2738 of the *Pf*DNV genome, AF192260) was amplified by PCR extension from the infectious plasmid pUCA. The primer pair was designed by the program, primer 5.0. The segment *ns*1 was amplified with forward primer of NS1F: 5′-TATTTGGAATTCACTATGCAAGATGGAAAGCAGCCAGACA-3′; containing EcoRI site (underlined) and reversed primer of NS1R: 5′-GAATGACTCGAGATTTTATTGTTCAATTATCCTTGG-3′; containing XhoI site (underlined), and cloned into the same sites of insect expression vector pAc5.1/V5-His (Invitrogen) to generate pAcNS1. The positively charged regions from 151 to 163 (KAK region) and 251 to 257 (6R region) of NS1 were deleted by PCR-based mutagenesis from the infectious plasmid (pUCA) and cloned into the pAc5.1/V5-His vector, respectively (pAcΔKAK, pAcΔ6R). The 6R region was mutated to nine Arg residues by PCR-based mutagenesis, with pAcNS1 as the template, and cloned into the pAc5.1/V5-His vector (pAcNS1R9). The 6R region was ligated to hemagglutinin (HA) using long primer PCR extension. The segment *ha gene* was amplified with forward primer of HAF: 5′-GAATTCATGGCAGGGAATGGTAGATGG-3′; containing EcoRI site (underlined) and reversed primer of HAR: 5′-GAATTCACGTCGTCTTCTCCTTCTGCCCAAATTCTGCATTGTAACGATC-CATTGGAGC-3′; containing EcoRI site (underlined), and cloned into the same sites of insect expression vector the pAc5.1/V5-His (Invitrogen) to generate pAcHA6R. Additionally, the eGFP gene was cloned into pAc5.1-His (pAcGFP). The arginine → glycine substitutions in the 6R region were created by PCR-based mutagenesis and cloned into the pAcGFP vector (pAcNS1a-eGFP) (Table 1).

2.2. Cells

Schneider line 2 (S2) cells were cultured in Shneider's Drosophila medium containing 10% FBS at 27 °C. Aedes albopictus C6/36 (C6/36) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 27 °C. *P. fuliginosa* nymph hemocyte cells were isolated from third-instar *P. fuliginosa* nymphs and maintained in Grace's medium supplemented with 15% FBS, penicillin (200 IU/ml), streptomycin (200 μg/ml), and amphotericin (50 IU/ml) and incubated at 27 °C in 24-well plates.

2.3. Virus propagation and purification

Viruses were prepared as previously described (Junping et al., 2005; Jiang et al., 2007).

2.4. Infection and transfection

Insect cells were *plated* in 24-well plates (approximately 10^5 cells per well) and, after four hours, the cells were inoculated with purified virus stocks (10^{10} virions/ml). Cells were incubated with 15 μl of the viral inoculum, which was diluted into 200 μl of serum-containing culture-medium, for five hours at 27 °C, after which the media was replaced with complete medium.

Table 1
Location of the arginine → glycine mutation in the 6R region.

Plasmid	Mutating region	Localization
pAcNS1GFP	-RRRRRR-	Nucleus
pAcNS1aGFP	-RGRRRR-	Cytoplasm
pAcNS1bGFP	-RRGRRR-	Cytoplasm
pAcNS1cGFP	-RRRGRR-	Cytoplasm
pAcNS1dGFP	-RRRRGR-	Cytoplasm
pAcNS1eGFP	-RRRRRG-	Cytoplasm

Transfection of insect cells was carried out using CELLFECTIN Reagent (Invitrogen). A solution containing 1 μg of plasmids (pUCA and pAcNS1) and 3 μl of CELLFECTIN reagent diluted in 200 μl of serum-free Grace's medium (concentration of cellfectin: 15 μl/ml) was added to the S2, and C6/36 cell lines and the hemocytes for five hours at 27 °C, after which the media was replaced complete medium. Following transfection, the cells were cultured at 27 °C for 48 h. S2 cells were transfected with plasmid pAcNS1R9.

2.5. Immunofluorescence analysis

The transfected cells were fixed with PBS/2% paraformaldehyde 48 h post-transfection. Following fixation, the cells were permeabilized with PBS/0.2% Triton X-100 and blocked with PBS containing 3% bovine serum albumin (BSA) overnight at 4 °C. The cells were then incubated with rabbit anti-NS1 serum (diluted 1/500) at 4 °C for two hours, followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (diluted 1/200) in the dark. Nuclei were counterstained with PBS containing 10 μg/ml of propidium iodide, and the cells were washed thoroughly. Fluorescence was detected using a laser scanning confocal microscope.

2.6. Nuclear extracts and Western blot analysis

Insect cells were washed three times in cold Dulbecco's phosphate-buffered saline and placed in lysis buffer (0.25 mol/l sucrose, 10 mmol/l Tris–HCl pH 8.0, 3 mmol/l $MgCl_2$, 0.1 mmol/l phenylmethyl sulfonylfluoride (PMSF) and 0.1% (v/v) Triton-X100) for 10 min on ice. The cells were pelleted by centrifugation at 2000 × *g* for ten minutes and the pellets were resuspended in separating buffer (2.2 mol/l sucrose, 10 mmol/l Tris–HCl pH8.0, 3 mmol/l $MgCl_2$, 0.1 mmol/l PMSF) and centrifuged at 46,000 × *g* for 60 min. Nuclei and undiluted supernatants were analyzed by Western blot using NS1 antisera, as previously described (Chen et al., 2007).

3. Results

3.1. P. fuliginosa nymph hemocyte cells can be infected with PfDNV

*Pf*DNV was isolated from sick nymphs. Transmission electron microscopic analysis demonstrated that a large number of virions could be detected in the hindgut of the infected sick nymphs. The rod-shaped virions are tobacco mosaic virions, which are used as molecular rulers (15 nm in width). The purified *Pf*DNV virions were icosahedrons, approximately 20 nm in diameter (Fig. 1.I). The viral genome extracted from sick nymphs was approximately 5.5 kb in length (Fig. 1.II).

To understand clearly the sensitivity of insect cells, we infected the insect cells of hemocyte, S2 and C6/36 by *Pf*DNV strains. The characteristic cytopathic effect typical for *Pf*DNV, in the form of clumping and ballooning of cells and syncytia formation, was observed in hemocyte cells at around 48 h post-infection. Hemocyte showed an extensive CPE (60–70%) at 72 h post-infection. And the cells were split seriously after 72 h post-infection. Characteristic cytopathic effect was shown in (Fig. 1.III), while no difference was found between the mock-cells and the insect cell lines, S2 and C6/36 (data not shown).

3.2. PfDNV NS1 protein is expressed and localizes to the nucleus of P. fuliginosa nymph hemocyte cells

To understand clearly the localization of NS1, we constructed a plasmid, pAcNS1, by PCR the ns1 sequence from pUC-*Pf*DNV.