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Identification of a novel nuclear localization signal of baculovirus late expression factor 11

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ABSTRACT

The baculovirus late expression factor 11 (LEF-11) has been reported to be involved in viral DNA replication and late/very late gene activation. In this study, serial N- and C-terminal truncations of *Bombyx mori* nucleopolyhedrovirus (BmNPV) LEF-11 protein were fused with DsRed to investigate the nuclear localization signal by which LEF-11 enters the nucleus. Results show that 72–101 residues at the C-terminus are essential for BmNPV LEF-11 nuclear localization. Sequence alignment of this NLS from multiple LEF-11 homologs revealed high conservation in general. Site-directed mutation analysis showed that five basic residue clusters, namely, K^{75}/R^{76}, H^{81}, K^{83}/R^{84}, R^{87} and K^{100}, were critical for the nuclear localization of BmNPV LEF-11. Co-IP analysis shows that LEF-11 binds directly to host importin α-3. Immunofluorescence analysis demonstrated that LEF-11 co-localizes with the immediate-early protein IE-1 at viral DNA replication sites in the nucleus. Further BiFC assays demonstrated the interaction of LEF-11 with LEF-3 and LEF-11 itself in the nucleus. Together, these results reveal a previous unknown mechanism for nuclear translocation of baculovirus LEF-11.

1. Introduction

The *Baculoviridae* family is a group of large, enveloped, double-stranded DNA-containing viruses that infect insects, particularly of the order Lepidoptera (Blissard and Rohrmann, 1990; Kool et al., 1995). During the infection cycle, baculoviruses produce two phenotypes: budded viruses (BVs) and occlusion-derived viruses (ODVs). The two virions contain identical genomic information and nucleocapsid structures but different viral envelopes, as they are produced at different stages of the virus life cycle. BVs are responsible for systemic infection throughout the host, while ODVs are embedded within polyhedral inclusion bodies and mediate vertical transmission between hosts (Rahman and Gopinathan, 2004). The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV) are two of the best characterized baculoviruses and are widely used as protein expression vectors (Maeda, 1989; Yao et al., 2012).

DNA replication, transcription and nucleocapsid assembly of baculoviruses occur within the host cell nuclei. Studies have demonstrated that baculovirus proteins are expressed in a temporally regulated cascade consisting of three sequential phases, namely, immediate-early, early and late. Baculovirus replication in host cells is controlled mainly at the transcriptional level and occurs in an ordered cascade of early and late phases, roughly divided by the initiation of viral DNA replication approximately 6–8 h post infection (h p.i.). The early phase occurs before the initiation of viral DNA replication, and viral protein products establish viral DNA replication and directly or indirectly regulate the expression of late/very late genes. These genes, which are involved in the transcription of the late-stage viral genes, are termed late expression factors (LEFs) (Hefferon, 2004; Passarelli and Miller, 1993). Studies have identified at least 11 proteins that are involved in baculovirus replication: LEF-1, LEF-2, LEF-3, LEF-7, LEF-10, LEF-11, IE-1, IE-2, P143, DNAPOL and P35 (Kool et al., 1994; Mitchell et al., 2013; Wang et al., 2006; Yu et al., 2013).

Abbreviations: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; BmNPV, *Bombyx mori* nucleopolyhedrovirus; BV, Budded viruses; CuniNPV, *Culex nigripalpus* nucleopolyhedrovirus; DAPI, 4′,6 diamino-2 phenylindole; BrdU, 5-bromo-2′-deoxyuridine; FBS, fetal bovine serum; LEF, late expression factor; MOI, multiplicity of infection; NLS, nuclear localization signal; NPV, nucleopolyhedrovirus; ODV, occlusion-derived virus; PBS, phosphate-buffered saline; VS, virogenic stroma.

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The nuclear import of macromolecules is a signal-mediated, energy-dependent and highly selective process (Gorlich and Mattaj, 1996). The regulated import of larger proteins (>40 kDa) usually involves the interaction of host shuttle proteins, including importin α, importin β and ran-GTPase, with a nuclear localization signal (NLS) embedded in the transported protein (Conti et al., 2006; Cook et al., 2007; Davis, 1992; Imamoto, 2000; Sorokin et al., 2007). Classic NLSs contain one or two clusters of basic residues. Monopartite NLSs consist of a simple sequence of 3–5 positively charged arginines or lysines (such as those found in large T antigens of simian virus 40), while bipartite NLSs such as nucleolin consist of a basic dipeptide upstream from a simple basic sequence (Efthymiadis et al., 1997; Rihs et al., 1991; Schwab and Dreyer, 1997). Studies have investigated the nuclear entry of several baculovirus proteins, and identified putative NLS elements involved in their transport (Au et al., 2009; Guo et al., 2010; Olson et al., 2002). However, the mechanism of baculovirus LEF-11 nuclear transport is not yet fully understood.

Here, we investigated the domains involved in the nuclear localization of BmNPV LEF-11. To this end, we constructed several deletion mutants and site-directed mutants expressing LEF-11 mutant fragments when transfected into silkworm cells. Immunostaining was used to determine the subcellular localization of fusion proteins. Co-IP assay was used to reveal that BmNPV LEF-11 whether interacts with the nuclear import receptor importin α-3. Further, we examined the nuclear localization of LEF-11 in relation to IE-1, a transcription factor localized at viral DNA replication sites in the nucleus. Finally, we investigated the interaction of LEF-11 with LEF-3 and LEF-11 itself in nucleus. Our data indicated that BmNPV LEF-11 is imported into nucleus *via* an importin α-dependent pathway, and its nuclear localization seemed essential in viral DNA replication.

2. Materials and methods

2.1. Cells and viruses

B. mori ovary-derived (BmN-SWU1) cells (Pan et al., 2010) were cultured at 27 °C in TC-100 medium (United States Biological) supplemented with 10% fetal bovine serum (FBS; Gibco), 200 U/ml penicillin and 200 U/ml streptomycin. The recombinant BmNPV $vBm^{lef11\text{-}REPMyc}$, which inserts one copy of a c-Myc-tagged *lef-11* gene with its native promoter into the *polh* locus, was constructed from a bMON7214bacmid containing the BmNPV genome (Zhang et al., 2014), according to the Bac-to-Bac manual (Invitrogen). The $vBm^{lef11\text{-}REPMyc}$ was plaque-purified and confirmed by analyses of PCR-amplified genomic segments. The viruses were then propagated in silkworm BmN-SWU1 cells. Viral titers were determined by end-point dilution assay (Reed and Muench, 1938).

2.2. Plasmids

The DsRed-encoding fragment without a termination cordon was inserted into the *Hin*dIII-*Kpn*I site of pIZ/V5-His to generate pIZ-DsRed. DsRed fusion protein expression was driven by an *Orgyia pseudotsugata* multi-capsid NPV OpIE2 promoter (Invitrogen). To generate the DsRed fusion plasmid pIZ-DsRed-LEF-11, the full-length BmNPV LEF-11 (residues 2–112), which was amplified from the BmNPV genome using the primer pair LEF-11/F–LEF-11/R, was digested with *Bam*HI and *Not*I and cloned into *Bam*HI-*Not*I–digested pIZ-DsRed. All the LEF-11 deletion plasmid constructs were derived from pIZ-DsRed. The gene coding the deletion fragment of LEF-11 was PCR-amplified from the pIZ-DsRed-LEF-11 plasmid, using the deletion mutation primers. The PCR products were digested with *Bam*HI and *Not*I, and the resulting fragments were ligated into *Bam*HI-*Not*I–digested pIZ-DsRed to generate various LEF-11 deletion constructs. Site-directed mutagenesis within LEF-11 was done using two complementary mutated nested primers as described previously (Au et al., 2009; Chen and Carstens, 2005). In the present study, we used $K^{75}L/R^{76}A$ for instance. Briefly, in the first round of PCRs, two separate PCRs were conducted using the primer pairs M-F and K75L/R76A-R, and K75L/R76A-F and M-F. The resultant PCR products were mixed, denatured and used as templates for the second round of PCR amplification, using the outside primers (M-F and M-R). The final PCR products were digested with *Bam*HI and *Not*I, and cloned into *Bam*HI-*Not*I–digested pIZ-DsRed, so that each coding sequence was fused in frame with DsRed at the N terminus. Finally, arginine (R) and histidine (H) were mutated to alanine (A), while lysine (K) was mutated to leucine (L).

The Venus-based BiFC system, involving the fusion of one of two split yellow fluorescent protein termed N-terminus-Venus (nVenus, amino acids 1–173) and C-terminus-Venus (cVenus, amino acids 155–238), was used to investigate protein–protein interactions (Nyfeler et al., 2005). The nVenus and cVenus gene fragments were amplified from plasmids pBiFC-VC155 and pBiFC-VN-173 (Shyu et al., 2006) and were inserted into the *Hin*dIII-*Kpn*I site of pIZ/V5-His to generate pBiFC-nVenus/cVenus-vector, respectively. Finally, the LEF-11 and LEF-3 gene sequences were amplified from the BmNPV genome and then cloned into the Venus-based BiFC system to generate the final BiFC plasmids. To construct negative or positive control plasmid, the gene sequences of BmNPV LEF3 (aa 2–83) and LEF3 (aa 2–189) were also inserted into the Venus-based BiFC system, respectively.

All the plasmids were confirmed by restriction enzyme digestion and nucleotide sequence analyses. The primers used in this study are summarized in the Supplementary Table.

2.3. Transfection and confocal microscopy

BmN-SWU1 cells (10^6) were seeded onto coverslips (Fisher Scientific, Waltham, MA) in 24-well plates (Corning) and transfected with 1 μg of plasmid. At 36 h after transfection, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized in 0.1% Triton X-100 in PBS for 10 min and washed three times with PBS. The cells were then stained with 0.1 μg/ml 4′,6 diamino-2 phenylindole (DAPI; Sigma, St. Louis, MO) followed by washing six times in PBS. Auto-fluorescence signals were visualized using an FV1200 laser scanning confocal microscope (Olympus, Tokyo, Japan).

2.4. Co-immunoprecipitation (Co-IP) assay

Co-IP assays were performed as previously described (Lehiy et al., 2013; Wu et al., 2008). Briefly, BmN-SWU1 cells were inoculated with recombinant $vBm^{lef11\text{-}REPMyc}$ virus. The cells were incubated for 96 h at 27 °C and then washed twice with PBS. Cells were resuspended in 1 ml IP lysis buffer (25 mM Tris, 150 mM NaCl, [pH 7.2]) containing 10 μl phenylmethylsulfonyl fluoride (PMSF). The cell suspensions were lysed at 4 °C for 30 min. After centrifuging the lysates at 13,000 × *g* for 30 min at 4 °C, the supernatant was mixed with 2 μl of mouse α-c-myc antibody or mouse IgG and incubated at 4 °C overnight. Thereafter, 40 μl of protein A + G agarose beads (Beyotime) was added to the mixture and incubated at 4 °C for 4 h, followed by centrifugation at 3000 × *g* for 5 min at 4 °C. The beads were collected by centrifugation and washed six times with IP lysis buffer. After adding 10 μl 5× SDS loading buffer, the beads were boiled in water for 10 min and centrifuged at 13,000 × *g* for 5 min at room temperature. Precipitated proteins were subjected to Western blotting. The antibodies used were α-c-myc mouse monoclonal antibody (1:5000, Beyotime) and anti-Importinα-3 rabbit polyclonal antibody (1:5000, ABGENT).