ORIGINAL ARTICLE

Identification and characterization of *odv-e25* of *Spodoptera litura* multicapsid nucleopolyhedrovirus

Zhaofei Li · Lijin Pan · Hang Yu · Sainan Li · Guoping Zhang · Yi Pang

Abstract *Spodoptera litura* multicapsid nucleopolyhedrovirus (SpltMNPV) *odv-e25* is 684 bp long, potentially encoding 227 amino acids with a predicted molecular weight of 24.9 kDa. Homology analysis indicated that SpltMNPV ODV-E25 has 35–65% amino acid identity with that of other known baculoviruses. RT-PCR results revealed that the *odv-e25* is transcribed actively at the late stage of infection and the mRNA start site was mapped within a consensus baculovirus late promoter sequence (TTAAG). Western blot analysis of *odv-e25* expression with an antiserum made against 6 × His tagged ODV-E25 expressed in *Escherichia coli* indicated that it was present as a doublet of approximately 27 kDa from 24 h through 96 h in SpltMNPV-infected *Spli*-221 cells. Similar results were seen on Western blots of *Spodoptera exigua* (Se)MNPV-infected Se301 cells. Immunofluorescence analysis showed that ODV-E25 was predominantly present in the cytoplasm of SpltMNPV-infected cells and localized to the envelopes of occlusion-derived virus.

Keywords *Spodoptera litura* · Baculovirus · *Odv-e25*

Introduction

The *Baculoviridae*, a diverse family of more than 600 viruses, encompasses two genera, the *Nucleopolyhedroviruses* (NPVs) and the *Granuloviruses* (GVs) [1]. These viruses are very specific and mainly infect insects of the orders Lepidoptera, Hymenoptera and Diptera, but always within the phylum Arthropoda. The family is characterized by the occlusion bodies of virions into large proteinaceous capsules or occlusion bodies (OBs). In a single infection cycle, NPVs produce two functionally distinct virus progeny, the budded virus (BV) and the occlusion-derived virus (ODV). BVs are responsible for the spread of the virus from cell to cell in the larva host and ODVs initiate the infection in a susceptible host and are responsible for the horizontal spread of the virus in an insect population [1]. The structural components of BVs and ODVs, except for the circular double-stranded DNA, are different to accommodate their respective functions in the infection cycle [2].

Spodoptera litura multicapsid nucleopolyhedrovirus (SpltMNPV) is highly specific and infects only a single known host, the cotton leaf worm [3]. This insect is an economically important polyphagous pest in China, India, and Japan, causing considerable economic loss to many vegetable and field crops. SpltMNPV has been successfully applied in large scale as a commercial biological insecticide against the cotton leaf worm in China [4, 5]. A number of SpltMNPV genes have been characterized and the genome has been sequenced [6]. Sequence analysis of SpltMNPV genome revealed that a homologue of *Autographa californica* (Ac)MNPV ODV-E25 was present as 0RF85 [6]. Here, we described the transcriptional analysis of SpltMNPV *odv-e25*, prepared SpltMNPV ODV-E25 polyclonal antiserum and used it to characterize the expression pattern of ODV-E25 and its structural localization.

Materials and methods

Computer-assisted sequence analysis

SpltMNPV *odv-e25* [6] was analyzed using software of the EBI server (http://www.ebi.ac.uk) and the ExPASy server

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(http://www.expasy.ch) for the prediction of domains and motifs. Conceptual translation of *odv-e25* was compared with a variety of homologous proteins obtained from BLAST search of the updated GenBank/EMBL databases. Multiple sequences were aligned by using Clustal [7] and edited using Genedoc software.

Cells, insects, and virus

S. litura Spli-221 cells which was donated by Dr J. Mitsuhashi (Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan) were maintained in Grace's medium (Gibco BRL) supplemented with 10% fetal bovine serum. Larvae of *S. litura* were reared on an artificial diet at 28 °C. The SpltMNPV-G2 isolate was used. SpltMNPV polyhedra were proliferated by infecting third instar *S. litura* larvae and budded virus (BV) stocks were prepared by extracting hemolymph from infected insects 3 days post-infection (p.i.). The isolation and purification of occlusion-derived virus (ODV) and BV were carried out as described previously [9]. The US1 strain of *Spodoptera exigua* (Se)MNPV was used to infect *S. exigua* cells (Se301).

Plasmid construction and preparation of anti-ODV-E25 antiserum

The complete coding region of *odv-e25* was amplified by high fidelity polymerase chain reaction (PCR; Roche) from the genomic DNA of SpltMNPV. The following two primers, which have *Bam*HI or *Hin*dIII restriction enzyme site (underlined), were designed for PCR: P1, 5′-GGATCCATGATCGGAACGATCGTTTTCATATTG-3′, P2, 5′-AAGCTTTTATTGTAATCTAAATGATTTTT-3′. The obtained PCR product was cloned into pMD18-T (TaKaRa) to construct plasmid pMDODV25. The plasmid pQEODV25 was obtained by cloning the *Bam*HI/*Hin*dIII fragment from pMDODV25 into the *Bam*Hl and *Hin*dIII sites of the expression vector pQE30 (Qiagen) which contains the 6 × His tag at the N-terminus of the multiple cloning sites. The resulting plasmid pQEODV25 was checked by sequencing and transformed into *E. coli* M15.

The gene expression under control of Bacteriophage T5 promoter in *E. coli* M15 was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) as recommended by the manufacturer (Qiagen). Expressed 6 × His-ODV-E25 was purified from *E. coli* MI5 by Ni-NTA agarose under denaturing conditions as described in the handbook (QlAexpressionist™). Purified 6 × His–ODV-E25 protein was used to raise antiserum in rabbits according to the method of Sambrook et al. [8] using Freund's adjuvant (Gibco).

Total RNA isolation, reverse transcriptase (RT)-PCR and 5′ RACE analysis

Total RNA was isolated from 1.6×10^6 mock-infected and SpltMNPV-infected *Spli*-221 cells (multiplicity of infection (m.o.i.) was 5) at 0, 6, 12, 24, 48, 72 and 96 h p.i. Total RNA was also isolated from fat body tissue obtained after dissection of five mock-infected and five SpltMNPV-infected *S. litura* larvae at 0, 48 and 72 h p.i. RNA isolation was conducted using the RNA isolation kit (Omega).

RT-PCR was performed using the RNA PCR Kit (TaKaRa, Vet 3.0) employing 2 µg total RNA as the template per time point. First-strand DNA complementary to RNA (cDNA) synthesis was performed using *Avian Myeloblastosis* virus reverse transcriptase and the random 9 mers according to the manufacturer's instructions. The cDNA-mixtures were amplified by PCR using the gene-specific primers P1 and P2 (Fig. 2). The obtained PCR products were analyzed in 1% agarose gel.

The 5′end of *odv-e25* transcript was determined using the 5′ rapid amplification cDNA ends (RACE) kit (Roche) employing 2 µg 24 and 48 h p.i. total RNA as template. Briefly, first strand cDNA was synthesized with a gene-specific primer SPI: 5′-CATTGTACTGTCCCGCTTC-GTTCTG-3′. The tailed cDNAs were amplified by PCR using the oligo-dT anchor primer and the nested gene-specific primer SP2: 5′-GCTGAGCTGCCCCTCGCCAACGGTG-3′. All PCR products were gel purified and cloned into pMD18-T (TaKaRa), and sequenced with M13F(−47) or M13R(−48) primers. A total of 15 clones were sequenced.

Immunofluoresecence analysis

Spli-221 cells were seeded onto glass coverslips (2×10^4 cells per 15 mm dish) and incubated overnight at 27 °C. They were infected with SpltMNPV at an m.o.i. of approximately 5 and incubated at 27 °C for 2 days. Uninfected cells were analyzed in parallel as controls. The cells were fixed by immersion in 95% ethanol for 15 min at −20 °C and processed for immunofluorescence as described by O'Reilly et al. [9]. The primary antibody was either pre-immune serum or anti-ODV-E25 immune serum, both diluted 1:200. The secondary antibody was FITC-conjugated goat anti-rabbit IgG (Sigma), diluted 1:120. The samples were visualized by using a Leika Optiphot microscope with an MRC-600 laser scanning confocal imaging system (model Leika-TCS-SP2).

Separation of ODV into envelope and nucleocapsid fractions

The purified ODV was fractionated into envelope (E) and nucleocapsid (NC) using a modification of the protocol of

Braunagel and Summers [2]. In a 250 μl reaction system, 200 μg ODV was incubated in 1.0% Nonidet P40 (NP40), 10 mM Tris (pH 8.5), at room temperature for 30 min with gentle agitation. NC was sedimented by centrifugation at 150,000g for 60 min at 4 °C. The pellet was resuspended in 0.1 × TE. The E proteins in the supernatant were acetone-precipitated and concentrated by centrifugation (100,000g, 20 min), and the pellet was dissolved in 0.1×TE. ODV, envelope and nucleocapsid were sampled, and analyzed by Western blot.

Western blot analysis

Equal amounts (100 μg) of total cell pellets were separated in 15% SDS-polyacrylamide gels as described [8]. Following transfer to nitrocellulose membrane (Schleicher and Schuell), blots were blocked in 1% Blocking solution as described by the manufacturer (Roche Appl. Sc.). ODV-E25 antiserum and pre-serum were used at a dilution of 1:200. Immunoreactive proteins were visualized using goat anti-rabbit IgG and alkaline phosphatase conjugate (Roche Appl. Sc.).

Results and discussion

Sequence analysts of SpltMNPV odv-e25

The SpltMNPV odv-e25 is located between nt 80821 and 82021 of the genome [6], potentially encoding 227 amino acids with a predicted molecular weight of 24.9 kDa. The odv-e25 sequence displayed one baculovirus consensus late transcriptional initiation motif (TTAAG) [10] at nucleotide residues −61 to −65 and two host factor binding sites (GATA) [11] at nucleotide residues −5 to −8, and −68 to −71, with respect to the translational start codon, respectively. The canonical poly(A) signal (AATAAA) was found 58 nt downstream of the translational stop codon (TAA).

Blast search revealed that AcMNPV, *Bombys mori* (Bm)NPV, *Rachiplusia ou* (Ro)MNPV, *Orgyia pseudotsugata* (Op)MNPV, *Choristoneura fumiferana* (Cf)MNPV, *Epiphyas postvittana* (Eppo)MNPV, *Mamestra configurata* (Maco)MNPV, *Helicoverpa armigera* (Ha)SNPV, *H. zea* (Hz)SNPV, *Spodoptera exigua* (Se)MNPV, *Leucania separata* (Ls)NPV, *Adoxophyes honmai* (Ah)NPV, and LdMNPV contain homologues to SpltMNPV ODV-E25 (Fig. 1). However, it has not been found in *Culex nigripalpus* (Cuni)NPV and *Neodiprion sertifer* (Ns)NPV which were respectively isolated from Diptera and Hymenoptera [12, 13]. Analysis of the relationship of known ODV-E25 showed that these proteins contain a signal peptide spanning approximately 19–23 amino acids at N-terminus, and

within this region, there is a conserved 11 amino-acid hydrophobic domain (Fig. 1). It was proposed that the hydrophobic domain may interact with ODV membrane [14]. Homology analysis indicated that the aniino acid identity of SpltMNPV ODV-E25 with that of other known baculoviruses was 35–65%.

RT-PCR Analysis of odv-e25 Transcripts

Temporal regulation of the odv-e25 transcripts was examined by RT-PCR using total RNA isolated from SpltMNPV-infected *Spli*-221 cells as template. A band with an expected size of 0.68 kb was amplified at 24 h p.i., which was remained detectable up to 96 h p.i. (Fig. 2a). To investigate if odv-e25 was also transcribed during SpltMNPV infection in *S. litura* larvae (*in vivo* infection), RT-PCR was performed on RNA isolated from at body tissue. As shown in Fig. 2b, a band of about 0.68 kb was also amplified at 48 and 72 h p.i. The RT-PCR products obtained were cloned into pMDl8-T and sequenced. The obtained sequence matched, as expected, the odv-e25 sequence (data not shown). Thus, SpltMNPV odv-e25 was transcribed not only upon infection of cultured insect cells but also upon *in vivo* infection.

Transcriptional mapping of the 5′ end of odv-e25 transcripts

The 5′ end of odv-e25 transcripts were determined by 5′ RACE analysis with total RNA isolated at 24 and 48 h p.i. from *Spli*-221 cells. A single cDNA was detected at all late times tested for odv-e25 transcripts. The start site of odv-e25 transcription maps 65 nt upstream of the ATG translation initiation codon at the first T in the sequence TTAAG. Thus, the odv-e25 transcript initiated within a baculovirus consensus late promoter motif. Taken together with the results of the RT-PCR analysis, these results led us to conclude that odv-e25 of SpltMNPV is transcribed actively in the late stage of infection as that of OpMNPV odv-e25 [14].

Immunodetection of ODV-E25 SpltMNPV- and SeMNPV-infected Cells

SpltMNPV ODV-E25 has predicted molecular weight of 24.9 kDa. Antibody was prepared by immunization of rabbits with purified ODV-E25 produced in *E. coli* Western blot analysis of extracts from SpltMNPV-infected *Spli*-221 cells revealed two specific polypeptides as a doublet with apparent sizes of 27 kDa and 28 kDa (Fig. 3a). The size of the immunoreacted proteins was larger than the predicted 24.9-kDa size of the putative odv-e25 translation product, suggesting that some posttranslational modifications occurred. This doublet was first detected at 24 p.i., stained
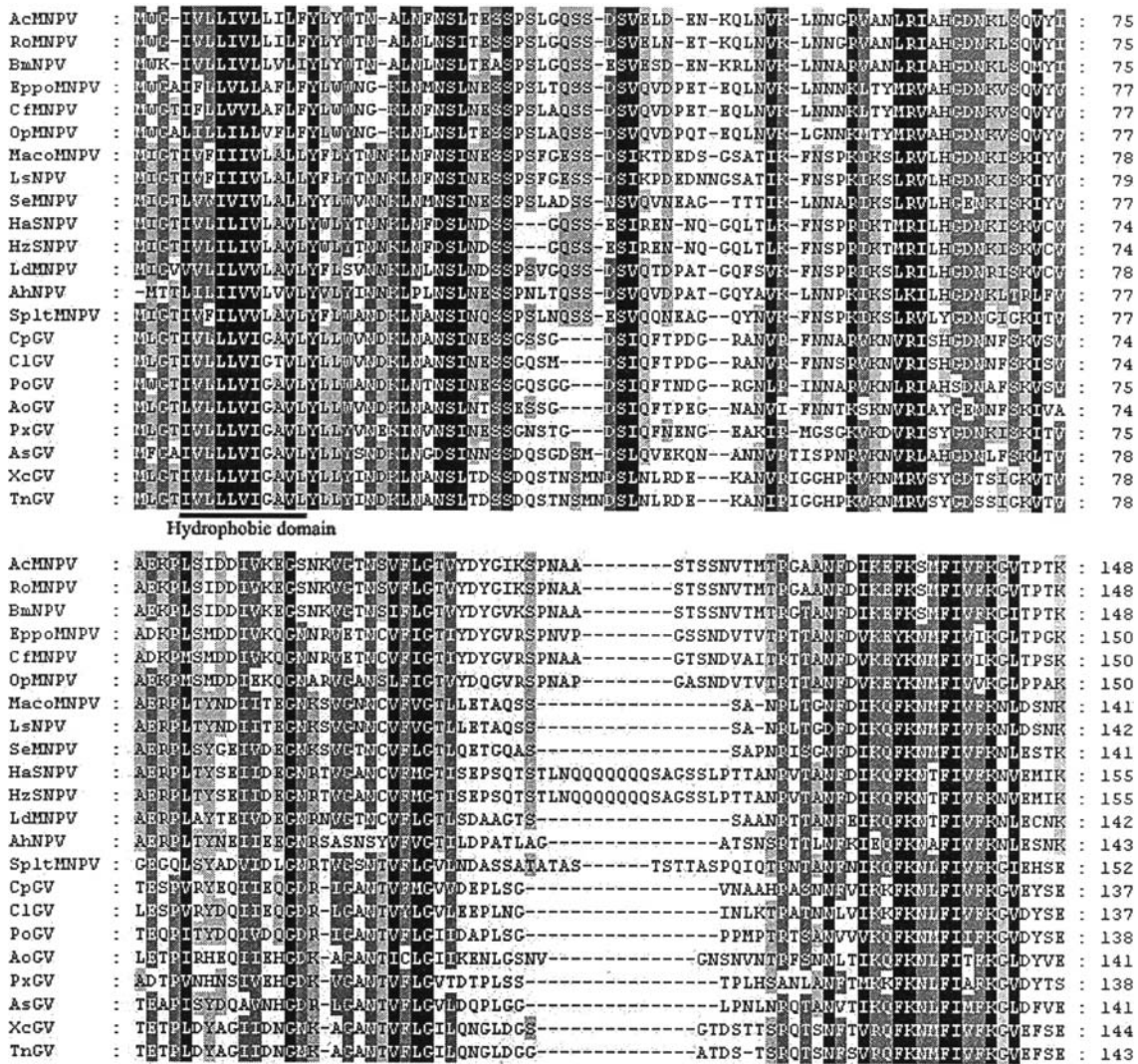
Fig. 1 Alignment of ODV-E25 proteins. The conserved hydrophobic domain is indicated by line below the alignment. Identical amino acids are indicated by shading and gaps that have been inserted to facilitate alignment are indicated by (-). The sequences used were: AcMNPV ODV-E25 (GenBank accession no. NC_001623), RoMNPV ODV-E25 (AY145471), BmNPV ODV-E25 (NC_001962), EppoMNPV ODV-E25 (NC_003083), CfMNPV ODV-E25 (NC_004778), OpMNPV ODV-E25 (NC_001875), MacoMNPV ODV-E25 (NC_003529), LsNPV ODV-E25 (BAA24236), SeMNPV ODV-E25 (NC_0021 69), HaSNPV ODV-E25 (NC_003094), HzSNPV ODV-E25 (NC_003349), LdMNPV ODV-E25 (AAC70282), AhNPV ODV-E25 (NP_818718), SpltMNPV ODV-E25 (AF325155), *Cydia pomonella* granulovirus (CpGV) ODV-E25 (NC_002816), *Crptophlebia leucotreta* (C1)GV ODV-E25 (NC_005068), *Phthorimaea operculella* (Po)GV ODV-E25 (AF499596), *Adoxophyes orana* (Ao)GV ODV-E25 (AF547984), *Plutella xylostella* (Px)GV ODV-E25 (NC_002593), *Agrotis segetum* (As)GV ODV-E25 (NC 005839), *Xestia c-nigrum* (Xc)GV ODV-E25 (NC_002331), *Trichoplusia ni* (Tn)GV ODV-E25 (AAC40850)

maximally by 48 h p.i. and then remained constant. Western blot analysis of SeMNPV-infected *S. exigua* cells showed similar results (Fig. 3b) to SpltMNPV. The SeMNPV ODV-E25 was not detected until 24 h p.i. and was present as a doublet of approximately 25 kDa at all subsequent time points.

Subcellular and Structural Localization of ODV-E25

The subcellular localization of SpltMNPV ODV-E25 was examined by indirect immunofluorescence analysis. As shown in Fig. 4b, uninfected *Spli*-221 cells exhibited no detectable fluorescence. Use of the pre-immune serum as primary antibody also resulted in no detectable fluorescence in either infected or uninfected cells (Fig. 4c, d). In contrast, after staining of infected *Spli*-221 cells with the anti-ODV-E25 antiserum, ODV-E25 was clearly visible in the cytoplasm of infected cells (Fig. 4a). Thus, ODV-E25 differs from OpMNPV ODV-E25, which is present in the infected cells' nucleus [14].

To determine if SpltMNPV ODV-E25 is associated with a structural component of virions, preparations of ODV and

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AcMNPV     : TVEDNGLPFEVDNHIVCLIDPNTAPLSEREWPELRKSNCTWYTRWAAAQVLLENNFTVIWAEQTAYLKWYRSYREMN : 228
RoMNPV     : TVEDNGLPFEVDSHIVCLIDPNTAPLSEREWPELRKSNCTWYTRWEAAQVLLENNFTAILADQTAYLKWYKSYREMN : 228
BmNPV      : TVEDNGLPFEVDSHIVCLIDPNTGPLSEREWPELRKSNCTLWYTKWEAAKQVLLENNPTAILADQTAYLKWYKSYREMN : 228
EppoMNPV   : WTKEDNLLCFTVDSLHVCLIDANAAPLSERELPELRSSCLWYTRWSAAWQILIENGFTVWNTEHTAYLKWQKSYREL- : 229
CfMNPV     : WTKEDSWLYFTVDCLHVCLIDANAAPLSERELRELRRSACTLWYTRWSAAWQILLENGFTVWWAENTAYLKWQKSYREL- : 229
OpMNPV     : WTKEDNWLCFTVDCLHVCLVDANAAPLSERVFARLPPSACTLWYTRWSAAWQLLLENGFTVWWAEHTAFLKWHRSYREL- : 229
MacoMNPV   : IKESVNWTFEADCHWYCLIDASTTSWPD--LRDTS--YPITWYTWANVQLKWREWDWTQIWDSGTLFIKWEKSFRLQ- : 216
LsNPV      : IKESVNWTRYEADCHWYCLIDASTTSWPD--LRDLR--TPS-------------------LCTLQTL---------- : 186
SeMNPV     : IKESVNWCFEADCHWYCLIDSTTTSWPD--LRDAS--YPITWYTWANVQLKWREWDWTQIWDAGTLFIKWEKSFRLQ- : 216
HaSNPV     : IKESANWWRYESDCHWYCLIDSQSTTWPD--LRFWS--YPIVWYTWANVQLKWREWWSAQIWDAGTMFVWMETSFRIQ- : 230
HzSNPV     : IKESANWWRYESDCHWYCLIDSQSTTWPD--LRFWS--YPIVWYTWANVQLKWREWWSAQIWDAGTMFVWMETSFRIQ- : 230
LdMNPV     : IKENNNWWPYESEGHWYCLIDASASPWPD--LRFWS--YPIVWYTAGPNVQLKWREWDWVQVWDAGTLFVWMEKSFRLQ- : 217
AhNPV      : IKENLYWSRFESDCHWFCLIDSNTTTWPD--LRDWS--YPIAWYWTTWPAVWLKWREWDWTEIWDSGSLYIKWEKSFRIQ- : 218
SpltMNPV   : IKVTSNWWARYEAECWWYCLIDSSSSSAPD--LRDWS--YPITWGLWWANVWTQIWDSGTVFIKWEKSFRLQ- : 227
CpGV       : IDSNNLWWRYESWRWWYALIDASNSTLPEL-LRDWS--YPICWLIWWSSAQLVWREWGWTTQIWDSATLFVWMEKSFRLQ- : 213
ClGV       : IDSNNLWWRYESWRWWYALIDASNSTLPEL-LRDWS--YPICWLIWWSSAQLVWREWGWTTQIWDNATLFVWMEKSFRLQ- : 213
PoGV       : IDNNTLWWPYESWRMWYALVDASNSTLPEL-LRDWS--YPICWLIWWSSAQLVWREWGWTQVWDASTLFVWMEKSFRLQ- : 214
AoGV       : IDSNMYWWRYEWWRWWYALLDASNSTIPDL-LRDWS--YPICWLVNWSSAQLVWREWGWTTQIWDSGTLYVWMEKSFRLG- : 217
PxGV       : IDSNNIWWRYEAWDWWYALWTNSTIPEL-LRFWN--YPIVWLWWSSAQLVWREWGWTTQIWDSATLFVWMEKSFRPFY- : 214
AsGV       : IDNNNLWWPYEVGSLWYALIDASNATLPDL-LRDWN--YPIVWLWWSSAQLVWREWGWTTQIWNSGTLFVWMEKSFRLQ- : 217
XcGV       : IDSSNYWLRFESWRWWYALVDASNSTLPEL-LRDWS--YPIVWLINWSSAQLVWREWGWTQVWDSATLFVWMEKSFRLN- : 220
TnGV       : IDSSNYWLRFESWRWWYALVDASNSTLPEL-LRDWS--YPIVWLINWSSAQLVWREWGWTQVWDSATLFVWMBRSFRLN- : 219
```
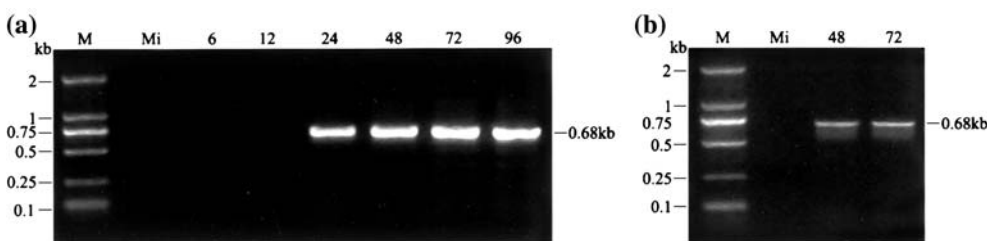
Fig. 1 Continued

Fig. 2 Temporal expression of *odv-e25* transcripts. (**a**) RT-PCR analysis of *odv-e25* performed on total RNA extracted from SpltMNPV-infected *Spli*-221 cells. (**b**) RT-PCR analysis performed on total RNA extracted from SpltMNPV-infected *S. litura* fat body tissue. Times p.i. are indicated above the lanes (Mi, mock-infected)
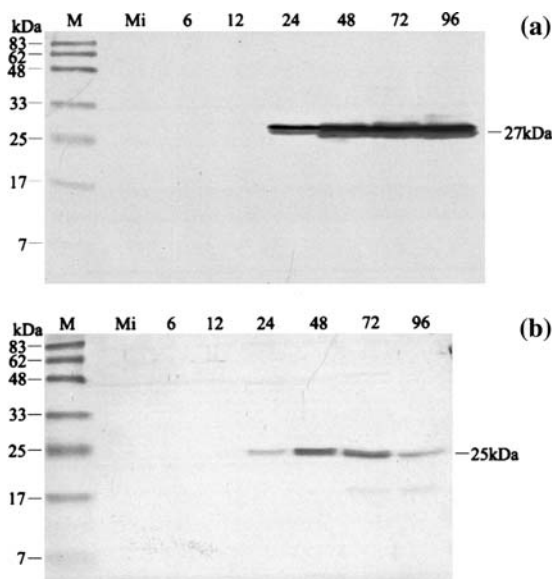

Fig. 3 Western blot analysis of *odv-e25* in infected cells. (**a**) Time course of *odv-e25* expression in SpltMNPV-infected *Spli-221* cells. (**b**) Time course of *odv-e25* indicated expression in SeMNPV-infected Se301 cells. The corresponding times p.i. are indicated above the lanes (Mi, mock-infected.). The sizes (in kilodaltons) of the prestained protein standards are indicated to the left and the positions of protein are indicated to the right of the Western blots

BV from SpltMNPV were subjected to Western blot analysis. The result indicated that ODV-B25 is present as a doublet in ODV but not in BV (Fig. 5a), while the pre-immune serum did not show any positive reaction (data not shown). The detected molecular weight of SpltMNPV ODV-E25 was 27 kDa and 28 kDa, which was as that detected in infected cells. The precise location of ODV-E25 in SpltMNPV ODV was determined by Western blot analysis of NC and E fractions (see Materials and methods). Two bands of 27 kDa and 28 kDa were also clearly detected in E fraction. No detectable bands were present in NC fraction (Fig. 5b). These results suggested that SpltMNPV ODV-E25 was an ODV-specific envelope protein. To determine if SpltMNPV ODV-E25 was N-glycosylated, an ODV preparation was digested with N-glycosidase F (NEB) according to the manufacturer's instructions. After digestion, treated and untreated ODV preparations were analyzed by Western blot using the ODV-E25 antiserum. These preparations were also reacted with antiserum to an N-glycosylated protein (GP37) that is present in ODV preparations [15] to confirm that the N-glycanase was active. Although the GP37 protein was reduced in size from 34 kDa to about 29 kDa by digestion with N-glycanase, ODV-E25 was
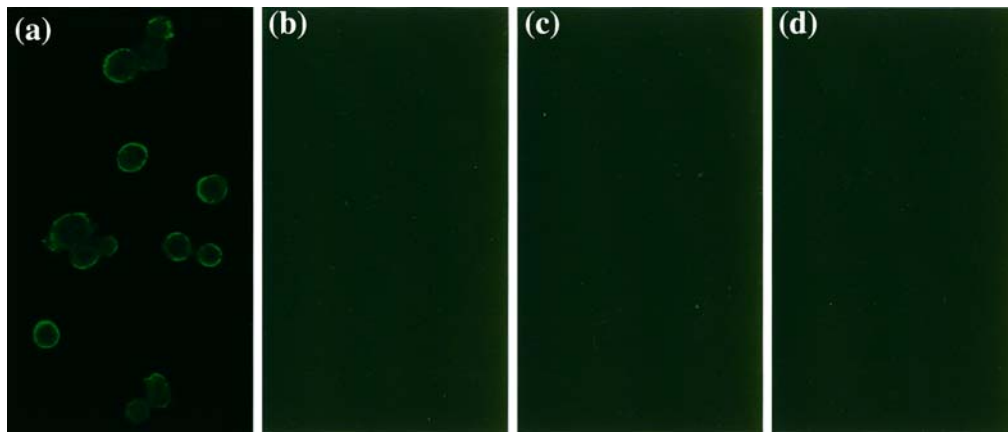
Fig. 4 Immunofluorescence analysis of subcellular localization of Sp1t NPV ODV-E25, SpltMNPV-infected (48 h p.i.) (**a** and **c**) or uninfected (**b** and **d**) *Spli*-221cells were fixed on glass coverslips and incubated with anti-ODV-E25 antiserum (**a** and **b**) or pre-immune serum (**c** and **d**). Bound antibody was detected by staining with FITC-conjugated goat anti-rabbit antibodies and visualized by confocal microscope
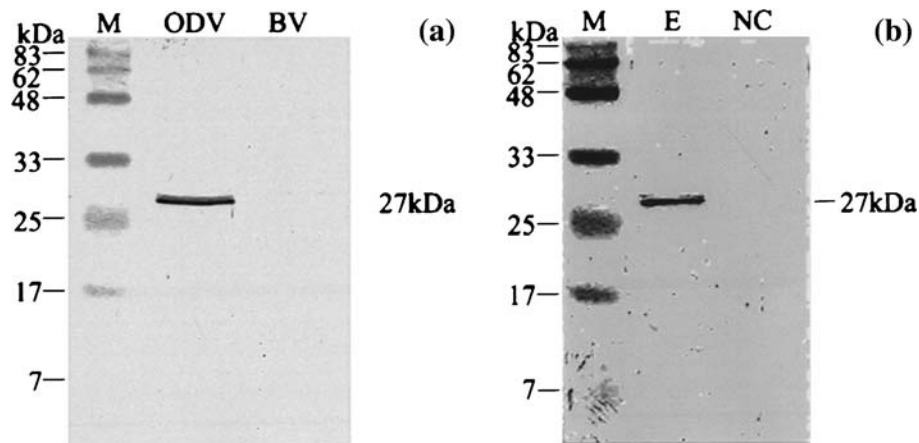
Fig. 5 Immunobloting analysis of structural localization of ODV-E25 in BV and ODV. ODV, BV, and B and NC structural components of ODV are indicated above the lanes (20 μg total proteins/lane). The sizes (in kilodaltons) of the prestained protein standards are indicated to the left and the positions of protein are indicated to the right of the Western blots

unaffected, indicating that it is not N-glycosylated (data not shown). Similarly, no evidence of N-glycosylation was found when virus was grown in the presence of tunicamycin (data not shown). In AcMNPV- and OpMNPV-infected cells, the ODV-E25 protein also appeared as a doublet with about 1 kDa separating the two bands. No glycosylation modification was found in these ODV-E25 proteins [14]. This suggested that some other forms of processing occurred in baculoviruses ODV-E25.

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