Identification and Characterization of a Baculovirus Occlusion Body Glycoprotein Which Resembles Spheroidin, an Entomopoxvirus Protein

Jorge E. Vialard, Leonard Yuen, and Christopher D. Richardson

Department of Microbiology and Immunology, McGill University, Montréal, Québec H3A 2B4, and Virology Group, Genetic Engineering Section, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Avenue, Montréal, Québec H4P 2R2, Canada

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A 37-kDa polypeptide specified by Autographa californica nuclear polyhedrosis virus was found to share significant homology with Choristoneura biennis entomopoxvirus spheroidin protein, which is the major component of entomopoxvirus occlusion bodies. Antibodies raised against spheroidin cross-reacted with the 37-kDa protein and confirmed its expression in the late phase of wild-type baculovirus infection. Immunoblot analysis and fluorescence microscopy demonstrated that the protein was associated with purified A. californica nuclear polyhedrosis virus occlusion bodies and was absent in purified virions. Immunofluorescence studies localized the protein to the periphery of occlusion bodies and the internal membranes of cells infected with wild-type baculovirus. The open reading frame encoding this spheroidin-like protein was inserted into a baculovirus expression vector, and recombinant protein was synthesized under control of the polyhedrin promoter. Studies of the recombinant protein demonstrated that it was heterogeneous in molecular mass as a result of N-linked glycosylation. Tunicamycin inhibited carbohydrate addition and yielded proteins of 34 and 33 kDa.

Autographa californica nuclear polyhedrosis virus (AcNPV) is a member of the family Baculoviridae, a group of double-stranded DNA viruses which infect insects from the Lepidoptera, Diptera, and Hymenoptera orders (reviewed in reference 5). A characteristic of this subgroup of baculoviruses is the occlusion of infectious virions in large proteinaceous structures termed polyhedra (17). The virions contained within these occlusion bodies (OBs) differ from nonoccluded virus (NOV), which bud from the plasma membrane, in both structure and mode of infection (27). NOVs are responsible for cell-to-cell infection, whereas the occluded virus (OV) is required for insect-to-insect transmission. OBs serve to protect the occluded virions from the outside environment for extended periods until they are ingested by insect larvae. The alkaline environment of the insect midgut dissolves the OBs, resulting in the release of virions and subsequent infection of the midgut epithelial cells.

To date, three proteins have been recognized to be associated with the structure of polyhedra. The most abundant component of polyhedra is a 29-kDa protein called polyhedrin, which constitutes the matrix of the OB (17) and accounts for up to 25% of the total protein in virus-infected cells (20). Another protein, p10, is expressed at high levels late in infection (9) and is also associated with polyhedra. This 10-kDa protein seems to play an important role in morphogenesis of polyhedra since mutants lacking the p10 gene produce fragile OBs (29). The third protein, pp34, is a phosphorylated polypeptide associated with the polyhedral envelope (PE), or calyx, a structure which surrounds the mature polyhedron (6, 7, 28). This envelope is composed primarily of carbohydrate (12). A 32-kDa protein, p32, produced by Orgyia pseudotsugata NPV (OpNPV) and pp34 have similar properties and exhibit 58% amino acid sequence homology (7, 13). Quant-Russell and Rohrmann (15) localized p32 to the PE of OpNPV polyhedra by immunogold staining.

In addition to NPVs, several other viruses produce OBs. These include granulosis viruses, cytoplasmic polyhedrosis viruses, and entomopoxviruses (EPVs) (17). Granulosis viruses form cytoplasmic OBs which contain granulin, a protein which is closely related to polyhedrin in both structure and function and bears 50% amino acid homology with polyhedrin. Cytoplasmic polyhedrosis viruses are double-stranded RNA viruses whose OB proteins bear no relatedness to those of baculovirus. Finally, the EPVs, which infect Lepidoptera and Diptera (1, 8), synthesize a major OB matrix protein called spheroidin (2). This protein is analogous to polyhedrin but does not possess significant amino acid homology with it. The gene encoding spheroidin in Choristoneura biennis EPV (CbEPV) has recently been identified and sequenced; it specifies a polypeptide of 38.5 kDa (31). The observed size of spheroidin is 50 kDa, and it was suggested that this size discrepancy was due to post-translational modifications such as glycosylation. Spheroidin was shown to be extremely rich in cysteine residues and formed high-molecular-weight complexes on sodium dodecyl sulfate (SDS)-polyacrylamide gels. These large species could be converted to a 50-kDa polypeptide in the presence of 0.5% B-mercaptoethanol and were shown to have identical patterns of proteolysis when compared with that of the monomer (31).

In this paper we report the discovery and characterization of a 37-kDa glycoprotein that is encoded by a baculovirus and appears to be associated with the PE. As this publication was in progress, another laboratory reported the DNA sequence of this 37-kDa polypeptide (30). We describe the localization, time of synthesis, and homology of this protein to spheroidin, the major OB component encoded by EPVs.
MATERIALS AND METHODS

Cells and virus. Spodoptera frugiperda (SF9) insect cells and AcNPV were obtained from Max Summers, Texas A&M University, College Station. Virus and cells were cultured in TNM-FH medium supplemented with 10% fetal bovine serum as previously described (22).

Construction and isolation of recombinant virus. A DNA fragment containing the spheroidinlike protein (SLP) open reading frame (ORF) was obtained from AcNPV DNA by using the polymerase chain reaction method (18). Two oligonucleotide primers were constructed which consisted of a pair of NdeI endonuclease recognition sites followed by several nucleotides corresponding to sequences near either the 5' or 3' end of the SLP ORF. The nucleotide sequences of the oligonucleotides were as follows: ATG oligo, 5'-GCTAGCGCTAGCAATATGATTGCATTATTA-3'; and transcription termination site oligo, 5'-GCTAGGCGCTA GCCTAAATATAATATGT-3'. The resulting polymerase chain reaction fragment was inserted into a baculovirus transfer vector under the control of the polyhedrin promoter. The DNA sequence of the polymerase chain reaction fragment was confirmed by the dideoxy-chain termination sequencing method (19).

This plasmid was transfected together with wild-type AcNPV DNA into SF9 cells by using the calcium phosphate precipitation technique (22). Recombinant virus was plaque purified by the rapid β-galactosidase screening assay as previously described (25).

Antiserum. Polyclonal antiserum from rabbits was prepared against proteins of CBEPV OBs by standard methods (16). The OBs were solubilized by alkali treatment and purified away from virions as previously described (2). The 50-kDa OB protein (spheroidin) was further purified by ion-exchange chromatography on QA Sepharose (Pharmacia, Uppsala, Sweden) and injected into rabbits with Freund incomplete adjuvant.

PAGE and immunoblots. Total cellular proteins and purified extracts were subjected to SDS-polyacrylamide electrophoresis (PAGE) by the method of Laemmli (10). Following electrophoresis, the gels were either stained with Coomassie blue or transferred to nitrocellulose membranes; proteins on the nitrocellulose were probed with antibody, and antigen-antibody complexes were detected with radiiodinated protein A (30 nCi/mg; Amersham Canada, Ltd., Oakville, Ontario, Canada) as previously described (3, 24).

Tunicamycin assay. SF9 cells were infected with the recombinant virus and subsequently grown in TNM-FH medium containing 10% fetal bovine serum and 10 μg of tunicamycin (Sigma, St. Louis, Mo.) per ml prepared from a 1-mg/ml stock in dimethyl sulfoxide. Proteins were collected 60 h postinfection in electrophoresis sample buffer and separated by SDS-PAGE.

Purification of OBs and virions. OBs were released from infected SF9 cells disrupted by treatment with 1% Triton X-100-0.01 M Tris hydrochloride (pH 7.0) and several strokes on a Dounce homogenizer. They were then purified on a discontinuous sucrose gradient as previously published (11), suspended in 0.1% (wt/vol) SDS, washed three times in distilled water, and pelleted each time by centrifugation at 3,000 rpm for 5 min. NOVs were purified by the method of Summers and Smith (22). OV's were purified away from OB proteins by alkali treatment followed by separation on a linear sucrose gradient as previously reported (2).

Fluorescence microscopy. SF9 insect cells were infected with wild-type AcNPV for 72 h in microchamber slides (Nunc, Naperville, Ill.). The cells were fixed and incubated for 2 h with a 1:100 dilution of rabbit antiserum directed against spheroidin as previously described (4). This antiserum was previously adsorbed against lysed SF9 insect cells to minimize background fluorescence. The infected cells, along with the bound primary antibody, were then incubated with a 1:100 dilution of biotinylated donkey anti-rabbit immunoglobulin G serum (Amersham Canada Ltd., Oakville, Ontario, Canada) for 1 h. The biotinylated secondary antibodies were detected by incubating the cells with a 1:100 dilution of fluorescein-conjugated streptavidin for 30 min.

OBs were heat-fixed to glass microscope slides. Some samples were solubilized in a solution of 50 mM sodium carbonate–50 mM sodium chloride for 2 min at room temperature and washed three times with phosphate-buffered saline. Untreated and alkali-treated OBs were subsequently fixed with formaldehyde and incubated with antibodies and fluorescent streptavidin as described above.

Spheroidin-antibody complexes were viewed by fluorescence microscopy on a Leitz microscope at ×400 to ×1,000 magnifications with a PL/APO 100 PHACO 3 oil immersion objective.

RESULTS

Homology between AcNPV SLP and CBEPV spheroidin. The CBEPV spheroidin gene was compared with GenBank sequence data, and homology was observed within an ORF found immediately adjacent to the AcNPV DNA polymerase gene (23). We sequenced this 909-nucleotide ORF and noted that a similar 927-nucleotide ORF had recently been reported by Wu and Miller (30). The translation products of AcNPV SLP and CBEPV spheroidin genes were aligned and compared by using the Microgenie Sequence Analysis Program (version 4.0). An overall homology of 39% at the protein level was observed. Although homology was observed throughout the two proteins, several regions of very high amino acid conservation were evident. Five conserved regions were identified and are illustrated in Fig. 1. All of these regions exhibited at least 75% homology between SLP and spheroidin, and where they differed, the general characteristics of the amino acids were retained. This was evident in region 4, which contained a string of 17 amino acids. Four nonidentical residues were apparent, but the general properties of these amino acids were conserved. The most striking example of amino acid sequence conservation was seen in region 5, which spanned 21 positions and contained only a single variation. In this case, the hydrophobic nature of the residue was maintained, i.e., a methionine in SLP was replaced by a leucine in spheroidin.

The distributions of proline and cysteine residues throughout the two proteins were highly conserved. SLP contained 18 prolines, and spheroidin contained 19; 11 of these residues were located at the same positions. More remarkably, all eight cysteines of SLP were paired to eight of the nine cysteines of spheroidin. Potential N-linked glycosylation sites also occurred at similar positions.

Finally, spheroidin was previously demonstrated to possess an N-terminal signal sequence of 20 amino acids. This hydrophobic sequence was posttranslationally cleaved during protein maturation (31). SLP contained a similar amino-terminal sequence which may also interact with membranes and be removed during posttranslational processing.

Expression and detection of SLP in insect cells infected with either wild-type or recombinant baculovirus. SF9 cells were infected with either wild-type AcNPV or recombinant virus

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which contained the SLP gene under control of the polyhedrin promoter. Northern (RNA) blot analysis of purified RNA with probes derived from the SLP gene indicated that mRNA was produced between 24 and 48 h postinfection. This observation was consistent for a gene expressed very late in infection (data not shown). Infected cells were also collected at various times postinfection, and proteins were separated by SDS-PAGE and were either stained with Coomassie blue or transferred to nitrocellulose membranes for immunodetection. The results of this time course study are presented in Fig. 2. In the wild-type AcNPV infection, SLP could not be visualized through Coomassie blue staining. However, a protein band migrating with a molecular mass of 37 kDa could be detected at 18 h postinfection with a polyclonal antiserum directed against spheroidin. This protein continued to be synthesized from 18 to 72 h postinfection and appeared to be produced in relatively small quantities compared with those in cells infected with the recombinant AcNPV. Cells infected with the SLP recombinant virus synthesized three major species which were evident by both staining with Coomassie blue and immunoblot detection. These protein bands migrated with molecular masses of 34, 35, and 37 kDa. We suspected that the smaller protein species might represent underglycosylated precur- sors of the 37-kDa protein. In addition to virus-derived proteins, the antibody directed against spheroidin reacted weakly with a 53-kDa protein which was present in uninfected cells. This represented a minor protein species which became less abundant late in wild-type baculovirus infection of SF9 cells.

Tunicamycin-inhibited glycosylation of SLP. To determine whether SLP was a glycoprotein, we performed experiments with tunicamycin (an inhibitor of N-linked glycosylation). SF9 cells were infected (in the presence and absence of tunicamycin) with recombinant AcNPV containing the SLP gene. Infected cells were harvested at 60 h postinfection, and cellular proteins were analyzed by SDS-PAGE. The results of this experiment are shown in Fig. 3. The addition of tunicamycin to cells infected with the SLP recombinant completely abolished the synthesis of both the 37- and 35-kDa polypeptides. The 34-kDa band remained apparent, and a 33-kDa protein band was also observed. These protein species may reflect other posttranslational modifications and could represent the nonglycosylated SLP polypeptide with and without its signal peptide.

Analysis of virus and OB proteins by using immunoblot.

FIG. 2. Immunoblot (A) and Coomassie blue-stained gel (B) of total proteins expressed in SF9 cells infected with either wild-type (WT) or SLP recombinant virus. Total proteins from 10⁶ cells were collected at 0, 12, 18, 24, 48, and 72 h postinfection and separated by SDS-PAGE. Panel A is an autoradiogram of an immunoblot prepared from a gel which was a duplicate of the Coomassie blue-stained gel shown in panel B. The immunoblot was probed with polyclonal antiserum directed against CbEPV spheroidin. Bound antibody was detected with protein A conjugated to 125I. The electrophoretic migrations of the various forms of SLP are indicated with arrows. PH refers to the electrophoretic migration of polyhedrin. Positions of protein mass standards (in kilodaltons) are indicated by numbers.
detected. Purified OBs, NOVs, and OVSVs were solubilized and subjected to SDS-PAGE. The resolved proteins were either stained with Coomassie blue (Fig. 4A) or transferred to nitrocellulose and probed with antibodies directed against the spheroidin protein (Fig. 4B). Although the OV preparation was not totally pure and contained some polyhedrin protein, it failed to react with the polyclonal antiserum used in these experiments. NOVs also failed to react with the spheroidin antibodies, whereas the OBs clearly contained the 37-kDa protein (SLP). The SLP was not a major component of OBs since it was not detected by Coomassie blue staining and was visible only through immunodetection techniques. A minor band migrating slightly faster than the 37-kDa protein was also detected and may represent a different level of N-glycosylation. Antiserum directed against spheroidin recognized two forms of protein in OBs derived from cells infected with CbEPV. Spheroidin protein has been shown to be a 50-kDa monomer which associates via disulfide bonds to form a 100-kDa dimer (31). We also constructed an AcNPV recombinant virus which contained the spheroidin gene of CbEPV. This recombinant virus produced a 50-kDa polypeptide as well as a 100-kDa form when the gene products were subjected to SDS-PAGE and analyzed by immunodetection techniques (data not shown). We conclude from these studies that SLP was associated with the OBs of wild-type AcNPV and had antigenic determinants in common with the spheroidin protein of EPVs.

Immunofluorescence studies of AcNPV-infected cells and OBs. Sf9 cells were infected with wild-type AcNPV and subsequently fixed and incubated with antispheroidin antibodies. Immune complexes were detected with biotinylated antibodies and fluorescent streptavidin. Uninfected Sf9 cells exhibited a small amount of background fluorescence (Fig. 5A), while cell membranes and OBs were clearly labeled at 72 h postinfection (Fig. 5C). Antibodies were localized to the cytoplasm early (12 to 24 h) in infection. However, fluorescence staining was minimal between 0 and 12 h infection and subsequently rose to maximum intensity between 60 and 72 h postinfection. Again, these results indicated that SLP was produced very late in infection. Sf9 cells infected with the SLP recombinant virus produced no OBs, and intense fluorescence was distributed throughout the cytoplasm, plasma membranes, and nuclear membranes (data not shown).

![Figure 3](http://jvi.asm.org/)

**FIG. 3.** Analysis of N-linked glycosylation of SLP in Sf9 cells infected with recombinant virus. The figure shows an autoradiogram of an immunoblot (A) and a Coomassie blue-stained gel (B) of total proteins produced by recombinant virus infection in the absence (−) and presence (+) of tunicamycin. Proteins were collected at 60 h postinfection. The immunoblot was probed with antisera directed against CbEPV spheroidin and detected with $^{125}$I-protein A. Numbers indicate migration of protein mass standards in kilodaltons.

![Figure 4](http://jvi.asm.org/)

**FIG. 4.** Immunodetection of SLP associated with AcNPV OBs. Virus and OB proteins were separated by SDS-PAGE and detected by Coomassie blue stain (A) or immune autoradiography (B). Lanes: 1, purified CbEPV OB proteins; 2, AcNPV OV proteins; 3, AcNPV NOV proteins; 4, purified AcNPV OB proteins. The immunoblot was probed with antisera directed against spheroidin, and immune complexes were detected with $^{125}$I-protein A. SLP, SPH monomer and dimer, and polyhedrin (PH) proteins are indicated by arrows. Numbers indicate size (in kilodaltons) of protein mass standards (lane STD).

![Figure 5](http://jvi.asm.org/)

**FIG. 5.** Immunofluorescence localization of SLP in Sf9 cells infected with wild-type AcNPV and immunofluorescence localization of SLP in purified AcNPV OBs. The figure shows immunofluorescence (A) and phase-contrast microscopy (B) of uninfected cells and immunofluorescence (C) and phase-contrast microscopy (D) of infected cells at 72 h postinfection. Insect cells were fixed and incubated with antibodies directed against spheroidin as described in Materials and Methods. Magnification, ∼380 (panels A to D). Also shown are immunofluorescence detection (E) and phase-contrast microscopy (F) of purified OBs and immunofluorescence analysis (G) and phase-contrast microscopy (H) of purified OBs treated with sodium carbonate. OBs were heat-fixed and stained as described in Materials and Methods. Magnification, ∼950 (panels E to H).
Purified OBs were fixed to glass slides and incubated with antibodies directed against spheroidin. Exterior regions of the OBs were fluorescently labeled in Fig. 5E. Other OBs were subsequently dissolved and permeabilized with sodium carbonate buffer. These solubilized OBs were washed in phosphate-buffered saline and stained with spheroidin antibodies. Alkaline treatment converted the normally opaque OBs to transparent sacs which probably represented the PE. These sacs were intensely labeled by the spheroidin antibodies.

The fluorescent-antibody studies appear to indicate that SLP is synthesized in the cytoplasm and may be transported to the nuclear membrane to become associated with the OBs in the nucleus of infected cells. More specifically, SLP is probably associated with the PE. Further electron microscopy and immunogold-labeling studies may substantiate this observation.

**DISCUSSION**

In this communication, we report the identification and characterization of a 37-kDa glycoprotein (SLP) which appears to be associated with the AcNPV OB proteins. We sequenced an ORF immediately adjacent to the AcNPV DNA polymerase gene which encodes a polypeptide that exhibits 39% amino acid homology to CbEPV spheroidin, the major EPV OB protein. Polyclonal antiserum produced against purified spheroidin reacted with a 37-kDa protein encoded by this ORF, and we demonstrated that this protein was expressed late in infection and localized to the envelope surrounding the OB matrix.

OBs are structures produced by several groups of viruses, including the NPVs, granulosis viruses, cytoplasmic polyhedrosis viruses, and EPVs. Their function is to protect virions embedded within them from the outside environment. Each baculovirus OB is composed of a proteinaceous matrix (17) surrounded by an envelope or calyx (6, 7, 28) which is composed primarily of carbohydrate (12). The major component of AcNPV OBs is polyhedrin (p29), a matrix protein which is hyperexpressed late in the course of infection, as reviewed by Rohrmann (17).

A second protein (p10) synthesized very late in infection and at high levels seems to be involved in the morphogenesis of the PE (29). Fibrillar structures containing p10 associate with electron-dense spacers to form what is thought to be nascent PE (26).

In addition to p10, a phosphoprotein (pp34) is required for the formation of PEs (32). Like polyhedrin and p10, this protein is also a late gene product. It seems to be associated with the PE via thiol linkages (28). Immunogold detection techniques were used to localize p32, an OpNPV protein homologous to pp34 from AcNPV (6, 7), to the envelope surrounding OpNPV polyhedra (15). The integrity of the PE and the presence of p32 were maintained even after dissolution of the polyhedra in dilute alkali. As with pp34, dissociation of p32 from the PE required the presence of a reducing agent (7).

Although SLP and pp34 share several common characteristics, such as molecular mass, timing of expression, and association with the PE, they do not appear to be the same protein. They exhibit different immunoreactive properties and possess different amino acid sequences encoded by separate regions of the baculovirus genome. The predicted amino acid sequence of p32 shared 58% homology with a translation product (pp34) encoded by an analogous region of the AcNPV genome (7). These two proteins (pp34 and p32) displayed immune cross-reactivity. In contrast, the amino acid sequence of spheroidin bears no significant homology to that of pp34 or p32. A radioactive probe constructed from the EPV spheroidin or SLP gene hybridized to a distinct region of the AcNPV genome. In addition, antibodies directed against spheroidin reacted with the gene product specified by SLP recombinant baculovirus as well as wild-type AcNPV. Therefore, the nucleotide and protein sequences of pp34 and p32 appear to be quite different when compared with SLP (6, 7, 13, 30).

The spheroidin antibodies also cross-reacted weakly with a cellular protein. This reactivity disappeared later in infection owing to shut-off of host protein synthesis. Reaction of virus-directed antibodies with cellular proteins is not uncommon (21), and molecular mimicry has previously been observed with a p10-directed monoclonal antibody which cross-reacts with cytoskeletal elements (14). For this reason, antiserum directed against spheroidin was preadsorbed to lysed insect cells to minimize background fluorescence during microscopy studies.

The structure of EPV OBs differs significantly from that of NPV OBs with respect to the major protein component. This is not unexpected since these viruses are not closely related; NPVs replicate in the nuclei of infected cells and produce nuclear OBs, whereas EPVs replicate in the cytoplasm and yield cytoplasmic OBs. The major protein of CbEPV OBs is spheroidin. It is a 50-kDa polypeptide that is rich in cysteines and capable of forming dimers (31). Although the amino acid sequence of spheroidin does not exhibit significant homology to polyhedrin, it is quite similar to the less abundant SLP found in baculovirus OBs. This amino acid conservation is especially evident in five distinct regions of the two proteins. These may represent functionally or structurally relevant motifs. Conservation of prolines, cysteines, and N-linked glycosylation sites indicates structural homology between the two proteins.

The similarities between the amino acid sequences of SLP and spheroidin suggest that the two proteins were derived from a common ancestor. This hypothesis may be supported by the identification of similar proteins in other OVs in the future. Two distinct possibilities come to mind. The genes encoding these proteins may have been acquired from the host cell genome or they may be induced by transposition or recombination from other viral genomes. It would be interesting to identify the gene encoding the cellular polypeptide which cross-reacted with the antibodies directed against spheroidin and to determine whether it bears significant homology to SLP or spheroidin. On the other hand, the SLP gene could be obtained from other viruses. Acquisition of the SLP gene by AcNPV from a donor EPV could be supported by the codon usage bias observed in the translated product. Codon usage in SLP favors A and T residues at the wobble position of most amino acids (30). This resembles codon bias in poxviruses whose genomes are A+T rich as opposed to the G+C-rich genes of NPVs.

In future studies, our observation that SLP is a protein associated with the PE will be corroborated by immunogold electron microscopy and biochemical separation of the PE from the matrix of the OBs. Finally, the significance of SLP in OB formation may be determined by the deletion of the gene encoding SLP and analysis of this mutated phenotype during baculovirus replication.

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LITERATURE CITED


