Immunoelectron microscopic examination of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus-infected *Lymantria dispar* cells: time course and localization of major polyhedron-associated proteins

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Immunoelectron microscopy was employed to examine the temporal expression and localization of two proteins involved in baculovirus polyhedron assembly (polyhedrin and p10) of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) in infected *Lymantria dispar* cells. In addition, the association of p10 with the polyhedron envelope (PE) protein was studied. The major capsid protein (p39) was also examined to investigate the association of virion structural proteins with polyhedron formation. In infected cells, p39 did not show a concentrated association with any infected-cell structures other than nucleocapsids and appeared to be randomly distributed over the nucleocapsid surface. Likewise, polyhedrin showed no major concentrations outside of developing or mature polyhedra. The p10 antibody cross-reacted with a protein associated with condensed chromosomes in uninfected cells. In infected cells, p10 is a component of the body of fibrillar structures. The PE protein has been shown to accumulate around the periphery of fibrillar structures. Cells infected with a polyhedrin-minus virus expressing the β-galactosidase gene under the control of the polyhedrin promoter were examined to determine whether the lack of polyhedra would influence the localization of major polyhedron-associated viral proteins. High concentrations of PE protein accumulating on the periphery of fibrillar structures appeared to be the major difference from wild-type virus-infected cells. The β-galactosidase protein appeared to be distributed throughout the nucleus and cytoplasm, in contrast with the specific localization of the viral proteins.

Introduction

Baculovirus infection is spread between insects via virions which are occluded in polyhedron-shaped occlusion bodies, termed polyhedra. Polyhedra stabilize the virions, allowing them to remain viable for many years. After ingestion, the polyhedra are dissolved by the alkaline pH of the insect midgut and the released virions then infect the insect through the midgut epithelium. At the present time three proteins, polyhedrin, polyhedron envelope (PE) protein and p10, appear to be involved in the occlusion of baculovirus virions. Polyhedrin is the major component of polyhedra and forms the crystalline lattice which occludes virions. Surrounding a mature polyhedron is an envelope containing a 32K to 34K PE protein (Whitt & Manning, 1988; Gombart *et al.*, 1989; Zuidema *et al.*, 1990; Russell & Rohrmann, 1990; van Lent *et al.*, 1990). The PE protein has also been observed to associate with the periphery of prominent infected cell-specific fibril-like structures (Russell & Rohrmann, 1990). In addition, a hyperexpressed protein, p10, has been found associated with polyhedra (Quant-Russell *et al.*, 1987) and also with the fibrillar structures (Vlak *et al.*, 1988). P10 appears to play a role in the proper assembly of polyhedra, as it has been demonstrated that p10-minus virus recombinants produce polyhedra which are unstable and readily fracture (Williams *et al.*, 1989), with polyhedron envelopes that are incomplete or absent (Vlak *et al.*, 1988; Williams *et al.*, 1989). The p39 protein is a component of the virion nucleocapsid and is one of the major proteins occluded within polyhedra (Pearson *et al.*, 1988; Thiem & Miller, 1989). All four of these polyhedron-associated proteins are expressed as late or very late genes and all demonstrate nuclear localization except p10, which appears to be located in both the nucleus and cytoplasm. In *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV)-infected *Lymantria dispar* cells, p10 and p39 are detected by Western blot analysis at 24 to 28 h post-infection (p.i.), with p39 showing intense nuclear localization by 48 h p.i. using immunofluorescent staining (Quant-Russell *et al.*, 1987; Pearson *et al.*, 1988). Polyhedrin is not observed on Western blots until 48 h p.i. (Bradford *et al.*, 1990) and immunofluorescence examinations indicate that it is concentrated within the peripheral region of the nucleus.
(Quant-Russell et al., 1987). The PE protein is also a very late expressed protein and is not detected until 36 to 48 h p.i. (Gombart et al., 1989) on Western blots. It does not become associated with the periphery of polyhedra until after they are well developed (72 h p.i.) and appears as a part of the polyhedron envelope very late in infection (96 h p.i.) (Russell and Rohrmann, 1990).

In this report, we describe electron microscopic investigations employing immunogold labelling and monospecific antibodies to polyhedrin, PE protein, p10 and p39. The time of appearance of these proteins, their location in infected cell nuclei or cytoplasm, and their association with virus-specific or cellular structures were investigated to elucidate their possible role in polyhedron formation. In addition, we have constructed a recombinant polyhedrin-minus OpMNPV in which the β-galactosidase gene is expressed under the control of the polyhedrin promoter. We have used this construct to determine the possible influence of polyhedron expression and polyhedra formation on the localization of major baculovirus proteins.

**Methods**

**Insect cell lines, antisera and virus.** The *L. dispar* (Ld) cell line and OpMNPV have been described previously (Bradford et al., 1990). The p10 monoclonal antibody (MAb; 210), p39 capsid MAb (236), polyhedrin MAb (61) and the PE polyclonal antibody have been described by Quant-Russell et al. (1987), Pearson et al. (1988), Quant et al. (1984) and Gombart et al. (1989), respectively. The polyclonal β-galactosidase antibody was purchased from Sigma.

Virus stocks were titrated by endpoint dilution assays as described by Summers & Smith (1987).

**Construction of OpLacZ recombinant and recombinant DNA techniques.** OpLacZ was constructed as shown in Fig. 1 and as follows. To obtain a *Bam*HI site downstream of the polyhedrin 3' flanking sequence, a 545 nucleotide (nt) *Hae*III fragment containing 524 nt of the 3' polyhedrin flanking sequence and the N-terminal seven codons of the polyhedrin gene was cloned into pUC18 digested with *Sma*I. The only clone isolated with the flanking sequence in the proper orientation contained a double insert of the *Hae*III fragment. The insert fragment abutting the *Bam*HI site was removed by *Bam*HI/*Hae*III digestion and cloned into a *Bam*HI/*Hind*III-cut plasmid (pGa11) which contains the β-galactosidase gene with a *Bam*HI site starting at the ninth codon (pGa11 was the generous gift of Dr Jerry Weir). The resulting clone was termed OpPHLacZ-S'. A deletion mutant of the OpMNPV polyhedrin gene containing an *Eco*RI site at nt 518 of the polyhedrin ORF was digested with *Nae*I which cut 213 nt downstream of the polyhedrin ORF and contains the polyhedrin mRNA 3' termination site. An *Eco*RI linker was added to the *Nae*I cut polyhedrin deletion mutant, then the linearized plasmid was digested with *Eco*RI producing a 508 nt fragment containing the 3'-terminal 277 nt of the polyhedrin ORF and 231 nt of the 3' polyhedrin flanking sequence. This was then cloned into the *Eco*RI site of OpPHLacZ-S' to produce OpLacZ. Upon sequencing the polyhedrin-β-galactosidase gene junction, it was found that a single nucleotide had been deleted during the original *Hae*III blunt-end ligation. To correct the reading frame, the construct was digested with *Bam*HI, filled in with the Klenow fragment and ligated. The final clone with the sequences of the junctions is shown in Fig. 1. All reactions utilized in the construction of this recombinant were done according to the methods of Maniatis et al. (1982). Enzymes were purchased from Bethesda Research Laboratories or United States Biochemical and used according to the manufacturers' instructions.

To produce the recombinant baculovirus, *L. dispar* cells were seeded at 1·3 × 10⁶ cells per well in six-well tissue culture plates. Transfections of these cells using 1 μg of OpMNPV DNA and 2 μg of the OpLacZ plasmid were performed as described by Summers & Smith (1987). After 5 days, cells and supernatants containing mixtures of wild-type and recombinant virus were collected. Recombinant virus was purified by a limiting dilution protocol (Fung et al., 1988) using a β-galactosidase assay for detection (see below) and an isolate was determined to be negative for polyhedrin production by Western blot analysis.

**Assays for β-galactosidase.** Cells infected with the recombinant virus containing the β-galactosidase gene were assayed for β-galactosidase activity by the method of Miller (1972) proportionately modified for small volumes. Infected cell cultures (200 μl) in 96-well microtitre plates were frozen and thawed to release intracellular enzyme. Eighty μl of sample was incubated with 40 μl of the chromogenic substrate o-nitrophenyl-β-D-galactoside (ONPG), in a total volume of 240 μl. After incubation for 1 h at 37 °C the yellow colour reaction was scored as plus or minus, or its absorbance at 420 nm was determined.

**Infection of cells, embedding, sectioning, immunogold labelling and electron microscopy.** Cells were infected with OpMNPV or OpLacZ at an m.o.i. of 100. Cells were fixed in 2·5% glutaraldehyde, dehydrated with ethanol, and embedded in LR White resin. Immunogold labelling was carried out as previously described (Russell & Rohrmann, 1990). Mouse ascites fluids containing MAbs against p39 (Pearson et al.,...
Fig. 2. Immunogold staining of the p39 protein in infected *L. dispar* cells: (a, b and c) 48 h p.i.; (d) cross-section of polyhedron from 84 h p.i. (e) Uninfected *L. dispar* cells. The p39 MAb was diluted 1:1000 and the second antibody was used at a dilution of 1:40 in (a) and (e), 1:30 in (b) and (c) and 1:50 in (d). The bars represent (a, b and e) 0.5 μm, (d) 0.25 μm and (c) 0.1 μm.
polyhedrin (Quant et al., 1984) and p10 (Quant-Russell et al., 1987) were used at dilutions of 1:1000, 1:32000 and 1:10000, respectively. Mouse polyclonal ascites fluid to β-galactosidase (Sigma) was used at a 1:10000 dilution. Rabbit polyclonal antiserum to the PE protein (Gombart et al., 1989) was used at a dilution of 1:2000. Goat anti-mouse IgG and goat anti-rabbit IgG conjugated to 10 nm gold particles were used at dilutions of 1:50 and 1:20, respectively, unless otherwise indicated. For the double immunogold staining, the p10 and PE antisera were mixed at the dilutions stated above for the first step. The second antibody staining step used goat anti-mouse IgG conjugated to 10 nm gold at a 1:50 dilution followed by goat anti-rabbit IgG conjugated to 20 nm gold at a 1:60 dilution.

Results and Discussion

P39: time course and localization in infected cells

The p39 MAb showed no significant cross-reaction with uninfected L. dispar cells (Fig. 2e). In infected cells, the p39 protein was observed by 24 h p.i. (data not shown) and was associated with the nucleocapsids of assembled virions (Fig 2a to d). The gold appeared to be randomly distributed over the surface of the nucleocapsid suggesting that p39 is a capsid protein rather than a component of a specialized structure (e.g. end structures). Although p39 is observed throughout the cytoplasm, virogenic stroma and nucleus, it is not found concentrated in specific areas or structures other than capsids. Within polyhedra, the protein appeared to be specifically localized to virions and did not stain other regions of polyhedra (Fig. 2d). In a previous study, Western blots detected p39 by 24 h p.i. and immunofluorescence examination showed a high degree of nuclear concentration of the protein after 24 h p.i. (Pearson et al., 1988). Our results agree with these data, suggesting that p39 is synthesized in the cytoplasm and is transported to the nucleus where it is assembled into virions without becoming concentrated in association with other prominent viral or host cell structures.

Polyhedrin: time course and localization in infected cells

The polyhedrin MAb did not cross-react with uninfected L. dispar cells (data not shown). Polyhedrin was not detected in sections of 24 h p.i. cells using the polyhedrin MAb at a 1:32000 dilution or in 36 h p.i. cells at a 1:32000 or 1:10000 dilution (data not shown). It was not until 48 h p.i. (Fig. 3a), when developing polyhedra were clearly present, that polyhedrin staining was observed. This staining was specifically localized to polyhedra. The areas surrounding developing polyhedra showed no concentrations of polyhedrin staining (Fig. 3a to c) even when the first antibody was used at a higher concentra-

![Fig. 3. Immunogold staining of polyhedrin in infected L. dispar cells: (a) 48 h p.i.; (b, c and d) 72 h p.i. The polyhedrin MAb was diluted 1:10000 in (a) and 1:32000 in (b, c and d). The second antibody was diluted 1:50. The bars represent 0.5 μm.](image)
Fig. 4. Immunogold staining of uninfected and infected *L. dispar* cells with the p10 MAb. (a) Cross-section of uninfected *L. dispar* cell in early anaphase; (b) immunogold staining of chromatin, same section as (a). Cells infected with OpLacZ virus (c) 48 h p.i., (d) 96 h p.i., (e) 48 h p.i., (f) 96 h p.i. The p10 MAb was diluted 1:10000 and the second antibody was used at 1:50. The bars represent (a) 5.0 μm, (b, c and d) 0.5 μm and (e and f) 0.25 μm.
virogenic stroma or fibrillar structures (Fig. 3c, d). These observations indicate that although polyhedrin is expressed at high levels, it is readily transported to the nucleus and assembled into polyhedra without accumulating elsewhere in the cell at levels detectable by our immunoelectron microscopy protocol. Concentrations of polyhedrin were not observed on the outer margins of enveloped virions in the process of being occluded (Fig. 3a, b). It is possible that the envelope may not have a specific role in polyhedrin nucleation or crystallization.

**P10: cross-reaction of the p10 MAb with a cellular protein, time course and localization in infected cells; association with PE protein**

In a previous report, it was demonstrated that the p10 MAb 210 cross-reacted with cytoskeletal structures of uninfected *L. dispar* cells (Quant-Russell et al., 1987). In addition, the cross-reacting structure appeared to be highly conserved as MAb 210 also reacted with similar structures in rat fibroblasts. Specific cross-reaction of uninfected cells by the p10 MAb was also observed by immunoelectron microscopy (Fig 4a, b). When dividing cells were examined (Fig. 4a) the p10 MAb appeared to bind to mitotic figures (Fig. 4b). The OpMNPV p10 epitope with which MAb 210 combines has been determined and is located within eight amino acids near the carboxy terminus of the protein (Quant-Russell et al., 1987). Screening of the protein database with the sequence of this epitope failed to show homology with any known chromatin-associated or cytoskeletal protein. In a previous report (Quant-Russell et al., 1987), we suggested that because of the cross-reaction of the p10 MAb with the cytoskeleton protein, p10 may share homology with a host microtubule-associated protein (MAP) and that during infection p10 may displace the MAP, and profoundly affect the organization of the cytoskeleton. It has recently been shown that if *Spodoptera frugiperda* cells are infected with an *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) p10 deletion mutant, no major differences in c.p.e. between the p10 mutant and the wild-type (wt) virus are observed (Volkman & Zaal, 1990). However, this mutant encodes the amino-terminal 29 amino acids of the p10 gene. The only highly conserved region of p10 is located at amino acids 1 to 14 (Leisy et al., 1986). If this highly conserved N-terminal region is not involved in cytoskeletal interactions, then the results of Volkman &
Zaal (1990) suggest that p10 may not influence the cytoskeletal reorganization observed in infected cells.

Although the p10 MAb cross-reacted with uninfected cells, its reaction with infected cells showed localization to viral-specific structures. The p10 MAb demonstrated specific binding to fibrillar structures, which appear late in OpMNPV infection (48 h p.i. and thereafter) (Fig. 4c). The association of p10 with fibrillar structures has also been reported for the AcMNPV by van der Wilk et al. (1987). The p10 MAb also was associated with virions and polyhedra (Fig. 4e). An association had been suspected because a high proportion of MAbs produced against gradient-purified polyhedra-derived virus (PDV) were specific for p10 (Quant-Russell et al., 1987). The p10 MAb also stained granular chromatin-like material present in close association with the nuclear membrane (Fig. 4d) and p10 appeared to be scattered throughout infected cells.

To investigate the influence the lack of polyhedra might have on the localization and accumulation of major polyhedron-associated structures, cells were examined after infection with a polyhedrin-minus virus (OpLacZ) expressing the β-galactosidase gene under the polyhedrin promoter (Fig. 1). Late in infection, p10-staining fibrillar structures were observed. By 96 h p.i., these fibrils were associated with condensed polyhedron envelope-like material (Fig. 4f). The periphery of the fibrillar structures stained intensely with the PE antibody (Fig. 5a, b), indicating a close association between the p10 and PE proteins. Foci of the PE protein associated with fibrillar structures were also observed (Fig. 5c). To confirm this association, double immunogold staining of the fibrillar structures with PE and p10 antisera was performed using second antibodies conjugated to 20 nm and 10 nm gold, respectively. This experiment confirmed the association of p10 with the body and the PE protein with the periphery of fibrillar structures (Fig. 5d). Such an intense concentration of the PE protein on the periphery of p10 fibrillar structures was not observed in wt virus-infected cells (Russell & Rohrmann, 1990), although there was no obvious difference in the appearance of the fibrillar structures themselves in OpLacZ-infected cells. This extensive concentration of the PE protein at the periphery of p10 structures may be due to the lack of polyhedra which would normally provide a substrate for PE protein deposition in wt OpMNPV-infected cells. In combination with the observation that p10-minus recombinant AcMNPV lacks or forms an incomplete polyhedron envelope (Vlak et al., 1988; Williams et al., 1989), these immunoelectron microscopic data indicate the presence of a functional relationship between p10 and the PE protein. It is likely that p10 plays a role in the assembly and proper association of the PE protein with polyhedra.

Fig. 6. Characterization of L. dispar cells infected with the OpLacZ virus. (a) Uninfected cells; (b and c) 72 h p.i. The β-galactosidase antibody was diluted 1:10000 and the second antibody was used at 1:50. The bars represent 0.5 μm.
dispar reactions were carried out as described in Methods.

Fig. 7. Time course of β-galactosidase activity in OpLacZ-infected L. dispar cells at m.o.i. 5 (lower trace), 10 (middle) and 100 (upper). The reactions were carried out as described in Methods.

OpLacZ: characterization of β-galactosidase expression

In the recombinant virus OpLacZ, the β-galactosidase protein is expressed as a fusion with the N-terminal seven amino acids encoded by the OpMNPV polyhedrin gene (Fig. 1). Both light and electron microscopic examination of infected L. dispar cells failed to detect polyhedra. In addition, restriction enzyme analysis of the recombinant OpLacZ viral DNA indicated that the polyhedrin gene was missing (data not shown). By immunoelectron microscopy, the β-galactosidase antibody showed no significant cross-reaction with uninfected cells (Fig. 6a) or cells infected with the wt virus. In cells infected with OpLacZ, β-galactosidase staining was observed by 36 h p.i. (data not shown) as would be expected for a gene under the control of the polyhedrin promoter. In contrast to the viral polyhedron-associated proteins, it showed no specific localization and appeared to be present in both the cytoplasm and nucleus (Fig. 6b, c). In the cytoplasm (Fig. 6b), it stained some granular material but was not observed associated with mitochondria or any other structures. In the nucleus (Fig. 6c), it appeared to be randomly distributed.

To investigate further the expression of β-galactosidase, its relative concentration in cellular extracts at different times p.i. was determined by an ONPG reaction (Fig. 7). These investigations indicated that at high m.o.i. (10 and 100) β-galactosidase activity was detectable as early as 24 h p.i. (Fig. 7). At all m.o.i. (5, 10 and 100) a major increase in β-galactosidase concentra-

tion was seen by 36 h p.i. These results indicate that β-galactosidase activity is detected earlier than the presence of polyhedrin, which is not observed until 48 h p.i. by immunoelectron microscopy or by Western blot analysis (Bradford et al., 1990). This is probably due to the fact that the β-galactosidase enzyme assay is based on the ONPG reaction which may be more sensitive than Western blot analysis.

Summary: time course of OpMNPV infection

These investigations on the temporal expression of several major OpMNPV proteins involved in polyhedron formation confirm parallel studies characterizing major events in the OpMNPV life cycle. It has been demonstrated by Bradford et al. (1990) that in OpMNPV-infected L. dispar cells, a high level of budded virus is produced between 12 and 36 h p.i. In a previous study (Pearson et al., 1988), the p39 capsid protein was detected at 24 h p.i. which corresponds to the time of its appearance as observed by immunoelectron microscopy. The results of these three studies agree as to the initial presence of p39 and its relationship to virion production. At 48 h p.i., polyhedrin protein and polyhedra are detected (Bradford et al., 1990; Russell & Rohrmann, 1990; and this study). Our results suggest that polyhedrin does not accumulate in the cell but is transported to the nucleus and forms polyhedra immediately. The polyhedron envelope protein is observed at low concentration by 36 h p.i. (Gombart et al., 1989) but is not associated with polyhedra until 72 h p.i. (Russell & Rohrmann, 1990). The polyhedron envelope does not develop until very late in infection (e.g. 96 h p.i.) (Russell & Rohrmann, 1990) and appears to have a close association with the fibrillar structures which are composed of p10.

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