Analysis of p74, a PDV envelope protein of *Autographa californica* nucleopolyhedrovirus required for occlusion body infectivity in vivo

Peter Faulkner, John Kuzio,† Greg V. Williams‡ and Joyce A. Wilson§

Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada K7L 3N6

In nature, nuclear polyhedrosis viruses (NPV) are transmitted when susceptible insect larvae ingest viral occlusion bodies (OB). These dissociate in the alkaline environment of the midgut and release encapsulated virions (PDV) which bind to midgut epithelial cells and initiate an infection. A previous study showed that expression of the *Autographa californica* NPV (AcMNPV) p74 gene during replication is essential for the production of infectious OB. A set of p74 deletion and overexpression recombinants was used for the production and screening of monoclonal antibodies, and for an investigation of gross cytopathology and localization of p74. No differences in virus structure or morphogenesis were observed in infected cells when the p74 gene of AcMNPV was deleted, even though the infectivity of OB harvested from the cells was abolished when they were fed to *Trichoplusia ni* larvae. Mutant OB released virus particles and degraded insect peritrophic membrane as in infections with wild-type virus; in addition, virions purified from mutant OB were infectious when injected into the haemocoel of *T. ni* larvae. Western blot analysis confirmed that p74 was associated with the PDV and could not be detected in the budded form virion phenotype. The polypeptide was readily degraded by treatment of purified PDV with proteinase K, in the presence and absence of detergent, and could be extracted from PDV by a non-ionic detergent treatment. The data are consistent with p74 being a structural polypeptide of the PDV phenotype, most probably as a component associated with the outside surface of the virion envelope. Its presence is shown to be essential for primary infection of midgut cells of insect larvae.

Introduction

Replication of nuclear polyhedrosis viruses (NPV, family *Eubaculovirinae*; Francki *et al*., 1991) is biphasic in insects and involves two phenotypically distinct forms of virion. An occluded form referred to as polyhedra-derived virus (PDV) is utilized for primary infection in insect midgut cells (see Kozlov *et al*., 1986; Rohrmann, 1992) and is the entity required for spread of infection between insects. PDV are released from occlusion bodies (OB) in alkaline conditions in the midguts of many insect species, and attach to columnar epithelial cells (Granados & Lawler, 1981). Penetration of capsids through the epithelial cell membrane has been observed and eventually intranuclear replication of progeny nucleocapsids takes place (reviewed by Vaughn & Dougherty, 1985; Keddie *et al*., 1989). Progeny virions initially migrate to the baso-lateral plasma membrane, where they bud through and acquire a lipid envelope containing virus-encoded coat proteins. This second phenotypic form is called budded virus (BV) and is responsible for systemic infection in vivo, and for propagation in tissue culture.

Later in infection, maturation of the PDV phenotype occurs within the cell nucleus in a peristromal space (the ‘ring zone’), and mature particles are occluded within a paracrystalline matrix primarily composed of the viral protein polyhedrin (Summers & Smith, 1976). Both virion phenotypes share a common capsid structure 40–50 nm in diameter and 200–400 nm in length, comprising a nucleocapsid core (Tweeten *et al*., 1980; Wilson *et al*., 1987) encapsulated in a capsid shell (Rohrmann, 1992). Structural polypeptides of the nucleocapsid...
of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) include a 39 kDa major capsid species (Thiem & Miller, 1989; Russell et al., 1991) and a highly basic 6-9 kDa polypeptide (Tweeten et al., 1980; Wilson et al., 1987; Maeda et al., 1991) complexed to the viral genome. In addition, a 24 kDa polypeptide was found to be evenly distributed through the nucleocapsids (Wolgamot et al., 1993) and a 78 kDa phosphoprotein essential for virus propagation was localized to the end of the capsid (Vialard & Richardson, 1993). An 87 kDa capsid-associated polypeptide of unknown function was identified in *Orgyia pseudotsugata* NPV (Müller et al., 1990), and its 80 kDa homologue in AcMNPV has also been described (Lu & Carstens, 1992). Electron microscopy studies have shown that the rod-shaped nucleocapsids are enclosed in a lipid envelope acquired at the plasma membrane for BV (Fraser, 1986; Kawamoto et al., 1977) or within the nuclear ring zone in the case of the PDV (Stoltz et al., 1973; Mackinnon et al., 1974; Tanada & Hess, 1976).

Immunohistochemistry-based techniques have been used to demonstrate that each form of virion has distinct protein species (Volkman, 1983) and marked differences in infectivity in vivo and in vitro (Keddie & Volkman, 1985; Volkman & Summers, 1977). BV gains entry to host cells by adsorptive endocytosis (Volkman & Goldsmith, 1985; Volkman et al., 1986). This form possesses an envelope fusion glycoprotein, gp64 (gp67) (Whitford et al., 1989; Monsma & Blissard, 1995; Blissard & Wenz, 1992), which is essential for infectivity (Monsma et al., 1996) and is the site of attachment of neutralizing monoclonal antibody AcV1 (Hohmann & Faulkner, 1983; Volkman et al., 1984; Keddie & Volkman, 1985). In contrast, PDV gains entry to insect epithelial cells by fusion at the cell surface (Granados & Lawler, 1981). Spikes that could participate in adsorption, fusion and penetration have not been identified on envelopes of PDV (see Rohrmann, 1992), but many polypeptides of unknown function [p25, PDV-E66, ODV-E56 (ODV-6E), ODV-E18 and ODV-E35] have been identified as constituents of the PDV envelope (Russell & Rohrmann, 1993; Braunagel & Summers, 1994; Hong et al., 1994; Braunagel et al., 1996a, b; Theilmann et al., 1996). A major glycoprotein of PDV (gp41) was found to localize to the tegument region of the virion (Whitford & Faulkner, 1992a, b), and although probably not involved in virus attachment or fusion, it may play a role in capsid entry or transport once fusion occurs.

In a previous study we reported that deletion of the C-terminus of the AcMNPV protein designated p74 abolished infectivity by *per os* inoculation of insects but did not interfere with virus replication in cell culture (Kuzio et al., 1989). We report here studies showing that p74 is a structural component of the PDV phenotype, associated with the outside of the virion envelope, and is not detected in the budded form virion. Histochemistry and mortality assays showed that while infectivity was abolished by *per os* inoculation of p74 OB, injection of p74− PDV into the haemocoel of *T. ni* larvae resulted in polyhedrosis with concomitant expression of marker gene (β-galactosidase). Using mutant viruses, the morphogenesis of PDV and polyhedra, as well as the release of mutant PDV from polyhedra, were indistinguishable from those seen with wild-type virus. Localization of p74 on the outside of the virion envelope suggests that p74 is important for virus entry into insect midgut cells. A potential role of the peptide as an intracellular effector or regulator of PDV or OB morphogenesis is not supported by this study.

### Methods

#### Cells and virus stocks.
*Spodoptera frugiperda* (BLP-SF21) cells (Vaughn et al., 1977) were maintained in BML-TC10 media (TC100; Gardiner & Stockdale, 1975) containing 10% FCS. The HR3 strain of AcMNPV (Brown et al., 1979), here designated wild-type, was the parental strain used for all recombinants produced in this study. Stocks were purified and titred by plaque assay (Brown & Faulkner, 1978) and maintained as culture supernatants. Phenotypic mixtures of mutant and wild-type virus were produced by co-infecting cells with an equal number of both mutant and wild-type virus with a total m.o.i. of 15 p.f.u. per cell.

#### Virus purification.
AcMNPV BV was clarified by centrifugation of cell culture supernatant (10 000 g, 10 min) then pelleted through a 20% (w/v) sucrose cushion at 100 000 g for 1 h (4 °C) and resuspended overnight in a small volume of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). PDV was released from OB (about 10⁶ OB per ml) by adding 0.08 vols of alkali buffer (1 M sodium carbonate, 0.4 M sodium thioglycolate; Arif & Brown, 1975). After 10 min at room temperature, 10 vols of distilled H₂O was added, and the solution incubated for 1 h at 4 °C. PDV was recovered by centrifugation as described above for BV.

#### Production of AcMNPV deletion/insertion and p74 overexpression mutants.
The recombinant viruses AcLP4 (a p74 deletion mutant) and AcNT25 (a virus which overexpresses the N-terminal third of a truncated p74 protein) are shown as a schematic in Fig. 1 A and were produced using standard methods (O'Reilly et al., 1992). Recombinant virus Ac744, a p74 overexpressor, has been described previously. AcNT25 was produced by altering the transfer plasmid containing AcMNPV *Hind*Ⅲ fragment in which the p74 gene had been disrupted by digesting with *Hind*Ⅲ, adding *Bam*HI linkers and then inserting the *β*-galactosidase ORF in frame with p74. Recombinant virus plaques were selected based on either polyhedrin-negative phenotype (Ac744 and AcNT25) or blue-white selection (AcLP4). These mutants, AcLP4 (Fig. 1 B), was constructed using a transfer plasmid containing AcMNPV *Hind*Ⅲ fragment in which the p74 gene had been disrupted by digesting with *Hind*Ⅲ, adding *Bam*HI linkers and then inserting the *β*-galactosidase ORF in frame with p74. Recombinant virus plaques were selected based on either polyhedrin-negative phenotype (Ac744 and AcNT25) or blue-white colour selection (AcLP4). These constructs were confirmed by sequence analysis. Ac744 and AcNT25 still carry their parental p74 gene under its own promoter. The AcMNPV deletion/insertion mutant Ac229z has been described previously (Williams et al., 1989); the deletion extends from the p74 region into the adjacent p10 gene sequence.

#### Production of anti-p74 monoclonal antibodies.
SF21 cells were seeded in six-well plates at 1 x 10⁶ cells per well, infected with recombinant virus AcNT25 (m.o.i. of 10) and harvested at 4 days post-infection (p.i.). Cells were harvested and the proteins separated by SDS–PAGE (12% acrylamide). Protein bands were visualized by Coomassie brilliant blue staining, and the 25 kDa overexpression protein was recovered by electroelution from the cut band and used as immunogen. BALB/c mice were injected intraperitoneally with 5 μg protein in Freund’s complete adjuvant (primary immunization), or incomplete
Restriction enzyme sites are indicated with single or double letter codes. Those below are with respect to the polyhedrin gene start codon. Deletion/insertion mutant virus AcLP4 contains an insertion of the p74 peptide under control of the polyhedrin gene promoter. (B) The p74 polyhedron-negative and contains the N-terminal 194 amino acids of the promoter and the p74 ORF inserted at the position described by Kuzio et al. (1989). It contains the full-length polyhedron-BamHI, B. Restriction sites in bold type are those added by site-directed mutagenesis or through the addition of synthetic linkers. (C) Western blot analysis of p74 immunoreactive proteins in infected Sf21 total cell peptides (36 h p.i., m.o.i. of 10).

**Fig. 1.** (A) Schematic representation of AcMNPV p74 overexpression mutants Ac744 and AcNT25. Construction of the virus Ac744 has been described by Kuzio et al. (1989). It contains the full-length polyhedron promoter and the p74 ORF inserted at the position +1 relative to the polyhedrin start codon. The mutant virus AcNT25 is phenotypically polyhedron-negative and contains the N-terminal 194 amino acids of the p74 peptide under control of the polyhedrin gene promoter. (B) The p74 deletion/insertion mutant virus AclP4 contains an insertion of the β-galactosidase gene in frame with the p74 ORF. Nucleotide position numbers given above diagrams are with respect to the p74 start codon; those below are with respect to the polyhedrin gene start codon. Restriction enzyme sites are indicated with single or double letter codes. *Hin*II, Hc; *Hind*III, H; *Sal*I, S; *Bam*HI, B. Restriction sites in bold type are those added by site-directed mutagenesis or through the addition of synthetic linkers. (C) Western blot analysis of p74 immunoreactive proteins in infected Sf21 total cell peptides (36 h p.i., m.o.i. of 10). Immunodetection of overexpression peptides of 25 and 74 kDa adjuvant (subsequent immunizations). Mice were boosted at 2 weeks, and again at 4 weeks after primary immunization. After checking the antibody titre, spleen cells were fused with Sp2/0 myeloma cells 4 days after the second booster. Resulting hybrids were selected in hypoxanthine–aminopterin–thymidine (HAT) medium and cloned by limiting dilution using standard methods (Goding, 1987; Harlow & Lane, 1988). Antibody-producing clones were selected by ELISA using purified 25 kDa overexpression protein, and the specificity confirmed by Western blot, immunoprecipitation and immunofluorescence.

**Western blot analysis of recombinant virus-infected cell polypeptides.** Whole cell preparations, harvested at 48 h p.i., were heated for 5 min in a boiling water bath in electrophoresis sample buffer (ESB), then loaded on 10 or 12 % SDS–PAGE mini-gels and run at 60 V per gel for 3 h. Proteins were transferred onto nitrocellulose (Gelman Sciences) or Hybond C (Amersham) for 4 h at 150 mA, then membranes were washed in transfer buffer and pre-blocked with 1 % Tween 20 plus 2 % BSA overnight at 4 °C, rinsed for 1 h with Dulbecco’s PBS (DPBS; 1:0 g/l KH2PO4, 5:7 g/l Na2HPO4, 0:2 g/l KCl, 8 g/l NaCl, pH 7:2) and blocked for 1 h with 3 % normal goat serum (NGS) and 1 % BSA in DPBS. Hybridoma supernatants (1:20) were applied for 4 h at room temperature in the presence of 0:1 % NGS, then blots were washed (DPBS with 1 % NGS and 0:1 % Tween 20) and incubated for 2 h with goat anti-mouse IgG (Sigma), either conjugated to alkaline phosphatase (1:30000), horseradish peroxidase (1:5000) or biotin (5 µg/ml), then washed again. Streptavidin–horseradish peroxidase complex (SA–HRP; Amersham, 1:400) was applied to biotin-tagged blots for 25 min in washing buffer. All blots were washed several times following the last step and post-blocked for 15 min with DPBS containing 5 % NGS, 1 % BSA and 0:1 % Tween 20, then washed in distilled H2O and transferred to substrate buffer. For alkaline phosphatase, substrate comprised 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and nitro blue tetrazolium chloride (NBT) as described by the manufacturer (Bethesda Research Laboratories). Colour development for peroxidase-tagged blots was done using freshly prepared substrate [0:8 mM 3-amin0-9-ethyl-carbazole (AEC), 11 mM H3PO4, 29 mM sodium acetate, 4 mM saccharic acid, 0:2 mM thimerosal].

**Proteolysis of purified PDV.** Tissue culture-derived wild-type OB were purified on a 45–65% sucrose cushion at 50000 g for 1 h at 4 °C, then centrifuged at 50000 g for 1 h, 4 °C, and the pellets were resuspended in distilled H2O and transferred to substrate buffer. For alkaline phosphatase, substrate comprised 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and nitro blue tetrazolium chloride (NBT) as described by the manufacturer (Bethesda Research Laboratories). Colour development for peroxidase-tagged blots was done using freshly prepared substrate [0:8 mM 3-amin0-9-ethyl-carbazole (AEC), 11 mM H3PO4, 29 mM sodium acetate, 4 mM saccharic acid, 0:2 mM thimerosal].

Western blot analysis of recombinant virus-infected cell polypeptides. Whole cell preparations, harvested at 48 h p.i., were heated for 5 min in a boiling water bath in electrophoresis sample buffer (ESB), then loaded on 10 or 12 % SDS–PAGE mini-gels and run at 60 V per gel for 3 h. Proteins were transferred onto nitrocellulose (Gelman Sciences) or Hybond C (Amersham) for 4 h at 150 mA, then membranes were washed in transfer buffer and pre-blocked with 1 % Tween 20 plus 2 % BSA overnight at 4 °C, rinsed for 1 h with Dulbecco’s PBS (DPBS; 1:0 g/l KH2PO4, 5:7 g/l Na2HPO4, 0:2 g/l KCl, 8 g/l NaCl, pH 7:2) and blocked for 1 h with 3 % normal goat serum (NGS) and 1 % BSA in DPBS. Hybridoma supernatants (1:20) were applied for 4 h at room temperature in the presence of 0:1 % NGS, then blots were washed (DPBS with 1 % NGS and 0:1 % Tween 20) and incubated for 2 h with goat anti-mouse IgG (Sigma), either conjugated to alkaline phosphatase (1:30000), horseradish peroxidase (1:5000) or biotin (5 µg/ml), then washed again. Streptavidin–horseradish peroxidase complex (SA–HRP; Amersham, 1:400) was applied to biotin-tagged blots for 25 min in washing buffer. All blots were washed several times following the last step and post-blocked for 15 min with DPBS containing 5 % NGS, 1 % BSA and 0:1 % Tween 20, then washed in distilled H2O and transferred to substrate buffer. For alkaline phosphatase, substrate comprised 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and nitro blue tetrazolium chloride (NBT) as described by the manufacturer (Bethesda Research Laboratories). Colour development for peroxidase-tagged blots was done using freshly prepared substrate [0:8 mM 3-amin0-9-ethyl-carbazole (AEC), 11 mM H3PO4, 29 mM sodium acetate, 4 mM saccharic acid, 0:2 mM thimerosal].

**Proteolysis of purified PDV.** Tissue culture-derived wild-type OB were purified on a 45–65% sucrose gradient as described by O’Reilly et al. (1992), and PDV were liberated by incubating 5 × 106 OB per ml in 0:05 M sodium carbonate buffer for 20 min at 28 °C. Samples were diluted with 4 vols distilled H2O and incubated for 1 h at 4 °C, then centrifuged at 5000 g for 10 min. PDV was recovered from the supernatant by ultracentrifugation through a 20 % sucrose cushion at 70000 g (1 h, 4 °C), and the pellets were resuspended in distilled H2O at a high concentration (PDV from 105 OB per ml, described here as 105 OB eq) of PDV). Aliquots of PDV (20 µl) were probed by PK treatment using established methods (Pratt, 1986; Imanaka et al., 1987; Eskridge & Shields, 1986), and using a control duplicate series in the presence of 1 % NP40. Briefly, PK was added to a final concentration of 5 or 20 µg/ml, incubated at 4 °C for 5, 15 or 60 min, and digestions stopped simultaneously by addition of PMSF to 2 mM. Incubations were continued for 10 min on ice to ensure proteinase inhibition, then 0 µl of 4 × electrophoresis sample buffer (ESB) containing 4 mM PMSF was added and samples heated for 5 min in a boiling water bath. Additional
controls were done by immediately stopping the digest with addition of PMSF (to control), or by omission of PK, both in the presence and absence of NP40. Proteins were separated in 15% polyacrylamide gels (3.7 × 10^5 OB equiv of PDV per lane) and analysed by Western blot (described above) using MAAb hybridoma supernatants against p74 (clone N25-8C, diluted 1:5) and gp41 (clone 3.10, diluted 1:50; Whittford & Faulkner, 1992 a), anti-capsid ascites fluid (clone MAAb39, diluted 1:1000; Whitt & Manning, 1988), and polyclonal rabbit anti-p25 (diluted 1:4000; gift from M. J. Wilson) were used as additional controls. Blots were developed using the enhanced chemiluminescence system (Amersham) as described by the manufacturer.

Bioassays. T. ni larvae (gift from R. P. Jacques, Agriculture Canada, Harrow, Ontario, Canada) were used in feeding assays (n = 50 or 75 insects per dose) based on procedures described by Jacques (1967) to determine infectivity of wild-type and recombinant virus OB. Insects were inoculated per os with OB coated on small blocks of diet. Once the dose was confirmed (> 2/3 of diet consumed), insects were transferred to fresh diet in individual dishes and monitored for polyhedrosis for 7–10 days p.i. Parallel groups were tested for expression of β-galactosidase due to infection with recombinant virus. At 4 days p.i., insects were injected with 0·3 μl DMSO containing X-gal (100 mg/ml). Insect larvae infected systemically with recombinant virus (lacZ') developed a blue colour within 2 h. Assays of BV or PDV infectivity when injected into the insect haemocoel were done by injecting third instar larvae (n = 20 or 30 insects per dose) with 0·3 μl medium containing approximately 5, 25 and 125 BV particles or 50, 500 and 5000 PDV particles per insect.

Computer analysis of polypeptide structure and promoter regions. Peptide secondary structure was predicted using the method of Garnier et al. (1978). Potential transmembrane regions (from hydrophathy profiles; Kyte & Doolittle, 1982) and amphipathic helices (Eisenberg et al., 1984) were identified using scanning windows of 19 and 11 amino acids, respectively. Profiles of late promoter regions were analysed using the programs of Gribskov et al. (1990) implemented into the UWGCG suite of sequence analysis software (Devereux et al., 1984).

Results

p74 mutants and hyperexpressed polypeptides

A set of recombinant viruses was constructed to study the loss of OB infectivity observed when insects were fed a p74 deletion mutant, Ac228z, having the phenotype p10− loss of OB infectivity observed when insects were fed a p74 deletion mutant, Ac228z, having the phenotype p10− (Williams et al., 1989; Kuzio et al., 1989). Mutant strains described here were engineered to overexpress polypeptides corresponding to the N-terminal one-third (AcNT25) or the complete p74 ORF (Ac744; Fig. 1 A). In addition a deletion/insertion mutant, AcLP4, was made in which the N-terminal 194 amino acids of the p74 ORF were fused in frame with β-galactosidase (Fig. 1 B). In AcLP4 the p10 gene remained intact and the deletion/insertion was confined to the p74 coding region. Predicted properties of the constructs were confirmed by sequence analysis and Southern blotting, and were phenotypically OB-negative (Ac744 and AcNT25) or formed blue plaques in the presence of X-gal (AcLP4). The 25 kDa N-terminal polypeptide was produced in greater quantity than the 74 kDa full-length protein and was easily identifiable on Coomassie blue-stained SDS–PAGE gels, and was selected for electroelution and used as the antigen for production of monoclonal antibodies.

Western blot analysis of intracellular specific proteins (ICSPs) using the monoclonal antibody clone designated N25-8C facilitated detection of all overexpression polypeptides, and specifically labelled a 74 kDa protein species in cells infected with wild-type virus, p74 overexpressing virus, or virus containing a partial deletion of the gene (Fig. 1 C; AcHR3, Ac744 and AcNT25, respectively), but not if the gene was deleted (Fig. 1 C; AcLP4). The data confirm that the native p74 gene was active and expressed the encoded polypeptide at or near its predicted molecular mass. These data also confirm that mRNA, previously identified as being transcribed from this region (Kuzio et al., 1989), is translated into a polypeptide. The specificity of N25-8C against p74 was shown by reaction with the hyperexpressed N-terminal polypeptide (Fig. 1 C), and by its ability to bind with the p74−β-galactosidase fusion protein produced in AcLP4-infected cells. This chimeric protein was difficult to detect, and required protein overloading to visualize the unique 130 kDa band (data not shown).

The lack of Ac228z OB infectivity reported previously (Kuzio et al., 1989) is consistent with p74 being either an essential component of the virion or OB, or acting directly on such a component. We therefore examined the ultrastructure of Sf21 cells infected with p74 deletion and overexpression mutants, to look for gross disruption of PDV or OB morphogenesis. We did not observe any gross alteration in
Fig. 3. Western blot of PK-digested PDV with and without NP40 pre-treatment, as indicated. Lane markings are incubation times (min) relative to the control (t₀; see Methods). Polypeptides in lanes marked 60* were incubated with PK at high (20 µg/ml) concentration; all others were digested using 5 µg/ml. Probes used were (A) anti-p74; (B) anti-capsid; (C) anti-p25; (D) anti-gp41. Molecular markers are indicated on the left; positions of immunoreactive breakdown products are indicated by asterisks on the right of each panel.

cytopathology or virus morphogenesis in cells infected with AcLP4 (p74⁻), Ac744 (p74⁺) or AcNT25 by light and electron microscopic examination of tissue sections (data not shown). AcLP4 infection yielded mature OB containing enveloped virion bundles and mature calyx indistinguishable from wild-type OB. Ac744- and AcNT25-infected cells lacked OB and were indistinguishable from a polyhedrin deletion mutant control designated AcDEK1 (Kuzio et al., 1989). Structures associated with OB morphogenesis such as virions, de novo membrane patches, fibrillar bodies and bilamellar fibrous sheet appeared normal in all cases.

Localization of p74 on PDV membrane

Western blotting of purified PDV and BV proteins using the monoclonal N25-8C revealed the presence of p74 in PDV derived from wild-type OB (Fig. 2). The polypeptide was not detected in PDV from AcLP4 OB or in the BV phenotype from either strain of virus.

The localization of p74 within wild-type PDV was further examined by Western blot analysis of proteinase-treated virions (Fig. 3). The assay was titrated to demonstrate time-dependent changes, and PK concentrations of 5 and 20 µg/ml were found to give the best results for the polypeptides examined. Higher PK concentrations (40 and 100 µg/ml) yielded qualitatively similar results but digestions proceeded too quickly to observe intermediate polypeptide levels. Control (t₀) PK digests which were immediately stopped by addition of PMSF did not exhibit loss of signal or generation of breakdown products relative to controls lacking PK (not shown).

PK digestion of both intact and detergent-permeabilized PDV caused time-dependent loss of detectable quantities of p74 relative to the t₀ control (Fig. 3A). Immunoreactive breakdown intermediates were not observed at any enzyme concentration or digestion time; however, a small amount of p74 was detected after a 60 min digest at the high PK concentration. In the presence of NP40, degradation of p74 was more rapid and proceeded to completion. This may have been due to protein extraction by the detergent or to a change in system kinetics caused by the detergent. Under the same reaction conditions, the major capsid protein (p39) was not degraded by proteinase treatment of whole virions, but in the presence of NP40 the capsid protein was observed to shift from 40 to about 34 kDa within 5 min (Fig. 3B), and at 60 min yielded a minor breakdown product (about 15 kDa) seen only by overexposure of the blots. Furthermore, incubation for 60 min using a fourfold higher concentration of PK led to essentially complete degradation of capsid protein in the presence of NP40, but had no effect in the absence of detergent (lanes 60*, Fig. 3B). Thus sufficient enzyme was present to
Insects were injected with X-gal substrate at 4 days p.i. to demonstrate LD50. Insects were scored for mortality at 10 days p.i. and the number of dead insects over the number of insects infected is given for each dosage.

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Infectivity of mutant and wild-type viruses in vivo

Insects injected with either PDV or BV, or fed purified OB, were examined for polyhedrosis at 10 days p.i. or injected with X-gal at 4 days p.i. (Table 1). All viruses tested were infectious by injection (PDV and BV), and those with LacZ gene insertions were also positive for β-galactosidase activity. Insects expressing the enzyme turned blue when injected with X-gal. Mock-infected insects (injected with TC100 media or fed distilled H2O) survived to pupation, and displayed no detectable β-galactosidase activity. Wild-type virus caused a lethal infection when fed as OB or injected as PDV or BV, but no β-galactosidase activity was seen, thus baculovirus infection did not induce endogenous enzyme capable of X-gal hydrolysis. Insects fed OB harvested from cultures infected with AcLP4 (p74−β-galactosidase) failed to develop polyhedrosis and had no detectable β-galactosidase activity, although intraheamocoeloc injection of virions (PDV or BV) caused polyhedrosis which was β-galactosidase-positive. LD50 values for wild-type and AcLP4 PDV inoculated into T. ni larvae by injection were not statistically different, indicating that deletion of the p74 gene did not affect PDV infectivity by injection. Positive control infections were carried out using a deletion/insertion construct of the hyperexpressed very late p10 gene, designated Ac229z (p10+“p74”−β-gal)” (Williams et al., 1989). Substrate reactions in insects infected with this mutant were intense by 24 h p.i. Although some β-galactosidase activity was found to co-purify with OB preparations, it did not survive even brief exposure to midgut juices, and early time points (6 h p.i.) were negative for marker activity in Ac229z-infected larvae; thus we concluded that the marker activity observed was due to active replication of recombinant virus in insect tissues.

Purified PDV or BV of AcLP4 (p10+“p74”−LacZ”) were infectious when injected into the haemocele of T. ni larvae (Table 1), and β-galactosidase activity could be demonstrated in insects at all time-points from 14 to > 72 hours

* 20 or 30 fourth-instar T. ni larvae per dose were injected with the indicated doses of BV p.f.u. per insect. Mock injections consisted of TC100 media only.
† 30 fourth-instar T. ni larvae per dose were injected with the indicated OB equivalents of alkali-liberated PDV.
‡ 50–75 second-instar T. ni larvae per dose were fed the indicated numbers of OB on a small diet sample.
§ Insects were scored for mortality at 10 days p.i. and the number of dead insects over the number of insects infected is given for each dosage.
LD50 values and 95% confidence intervals (c.i.) were determined using probit analysis in the SPSS statistical software package.
∥ Insects were injected with X-gal substrate at 4 days p.i. to demonstrate β-galactosidase activity.
p.i., hence the PDV form of these viruses was competent at least within some cell types in the insect host. These data, together with the localization of p74 to the outside of the PDV particle, suggest a role in the early events of midgut infection, most probably in virus attachment or penetration. To exclude the possibility that lack of infectivity of AcLP4 OB might be due to aberrant release of PDV, we examined alkali-treated wild-type and mutant OB by negative staining and TEM, but saw no difference in release of virus bundles (data not shown). Examination at high magnification failed to reveal any anomalies compared with wild-type virus, such as unusual polyhedrin residuals or signs of virion disruption. In addition, peritrophic membranes of AcLP4 OB-fed insects were dissected from larvae 2 h after feeding and exhibited the same degradation as those of wild-type OB-fed insects when examined microscopically (data not shown), thereby confirming dissolution of OB and access to the target midgut cells.

Discussion

Analysis of AcMNPV-infected Sf cells using the monoclonal antibody N25-8C confirmed that the p74 coding region was expressed during virus replication and encoded a peptide very similar to the size indicated from its ORF (Fig. 1C). Recombinant viruses AcNT25 and Ac744 (Fig. 1A, B) hyperexpressed polypeptides which were derived from the p74 coding region, and these existed in infected cells at or near 25 and 74 kDa, respectively (Fig. 1C).

Western blotting of purified virions showed that p74 was a structural polypeptide in wild-type PDV but not BV (Fig. 2), and by proteinase assays was found to be associated with the PDV envelope rather than the nucleoprotein core (Fig. 3). p74 also contains a potential membrane-anchoring region (Fig. 4), suggesting that it is probably an integral membrane protein of the PDV membrane. p74 is synthesized as a very late protein (Kuzio et al., 1989), probably timed to the morphogenesis of the occluded form of virion. Progeny capsids develop within the virogenic stroma and migrate to the intranuclear ring zone, where they either egress toward the cell surface (BV) or acquire an envelope and become occluded within the ring zone (PDV). Since the production and infectivity of BV was unimpaired by p74 deletion or overexpression, a direct role in the BV morphogenetic process is unlikely. In addition, each phenotype of virion derives its envelope from a different source within infected cells, and carries distinct polypeptide profiles (Volkman, 1983; Braunagel & Summers, 1994) which optimize the virions for infectivity in their native site of attachment and entry (Keddie & Volkman, 1985; Volkman & Summers, 1977). PDV polypeptide profiles on SDS–PAGE gels appear the same regardless of whether p74 is present or absent (Fig. 1C), and OB of AcLP4 could not be distinguished from wild-type based on TEM examination of tissue sections or by negative stain preparations of alkali-released virions. Although this does not

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**Fig. 4.** Predicted secondary structure (Garnier et al., 1978) and hydropathy plot (19-residue window; Kyte & Doolittle, 1982) of AcMNPV p74, oriented with the N terminus to the left. Regions of amphipathic $\alpha$-helix were determined using the method of Eisenberg et al. (1984). Four helical domains predicted to be weakly amphipatic are indicated (H1, H2, H3, H4). NT25 peptide is shown for reference.
exclude the possibility that p74 affects subtle tertiary or quaternary envelope-related structures during virion maturation, we could not detect changes to virion morphogenesis or gross cytopathology in deletion or overexpression mutant infections as compared to wild-type or polyhedrin-negative virus-infected cells.

BV acquire their envelope at the plasma membrane while budding from the cell, at modified patches rich in a virus-encoded glycoprotein gp67 (Volkman et al., 1984; Whitford et al., 1989; Blissard & Rohrmann, 1989), and enter target cells primarily by adsorptive endocytosis (Volkman & Goldsmith, 1985; Volkman et al., 1984). In contrast, PDV acquires a distinct envelope within the nucleus (Braunagel & Summers, 1994) and attaches to and fuses with microvilli of insect midgut cells (Gr מערכת et al., 1981), simultaneously infecting columnar epithelial and regenerative cells (Keddie et al., 1989). When insects are fed a high dose of OB, the nucleocapsids that enter midgut cells may migrate to the nucleus and uncoat, or rapidly (0.5 h p.i.) bud through the basolateral surface and initiate systemic spread of infection (Gr découvert et al., 1981). Infection may then spread quite rapidly throughout the insect by utilizing the tracheolar system of the insect (Engelhardt et al., 1994). AcLP4 BV and PDV were able to cause lethal polyhedrosis when injected into T. ni larvae, but failed to cause infection when inoculation was by an oral route even with very high doses; hence both midgut and systemic infection (by pass-through of nucleocapsids) were blocked. In addition, using β-gal histochemistry there was no apparent infection of the insect gut with AcLP4 virus. The most likely explanation is that mutant virus did not enter midgut cells, and since p74 localized to the outside of the PDV envelope, the protein has a role in PDV attachment or fusion. Three putative domains in p74 are predicted to be helical and weakly amphipathic (Fig. 4): H1 (residues 191–206), H2 (residues 460–478) and H3 (residues 491–502). Such regions have been implicated as initiators of membrane fusion (Eisenberg & Wesson, 1990; Fuji et al., 1992) and could be involved in a mechanism of p74-mediated fusion of PDV membranes with those of gut epithelial cells. The failure of even very high doses of AcLP4 OB to initiate infection in all instars tested supports the contention that primary infection relies on a single mechanism of entry, and that p74 is essential to that process.

Alternative functions of a PDV membrane polypeptide in primary infection might include actions prior to attachment to target cells, such as assistance in OB dissolution (PDV release) or alteration of peritrophic membrane (PM). Our data show that p74 is not required for either of these processes, since virus release from OB and dissolution of PM were both unaffected by the absence of p74. A viral factor active against the PM is associated with a soluble polyhedrin fraction of dissolved OB (Derksen & Granados, 1988); by contrast p74 co-localized exclusively with the virion fraction. The very small quantity of p74 present in virion envelopes has rendered this polypeptide undetectable by standard methods, and raises the issue of how many proteins are actually associated with the envelope. Major species recently identified with the PDV envelope include a 25 kDa species (Russell & Rohrmann, 1993), a 66 kDa and a 43 kDa species (Braunagel & Summers, 1994). However, p74 is the first PDV envelope protein demonstrated to have a functional significance in primary infection.

The process of virus attachment and subsequent adsorption is not yet resolved with baculovirus PDV. Our physical data and computer analysis indicate that the polypeptide p74 has the properties characteristic of a virion attachment protein (see Crowell & Lonberg-Holm, 1986) and does not have physical domains, localization patterns or temporal expression patterns, which would suggest an alternative function such as transcriptional or translational regulation. These observations are further strengthened by the uninhibited replication of p74 mutants in cell culture, and unaltered LD50 values of p74 mutant BV and PDV when injected into target insects. Recently, the p74 gene was identified in Choristoneura fumiferana multicapsid NPV, and comparison of p74 from AcMNPV with this virus indicates that this protein is highly conserved: 77% amino acid identity overall (Hill et al., 1993), with most mismatches occurring at the C terminus, and 88% identity in the N-terminal 240 amino acids (Hill et al., 1993). By comparison, there is about 80% amino acid sequence identity between polyhedrins of baculoviruses which infect lepidopterans, and 42% or less between p10 polypeptides (Rohrmann, 1992). The high conservation of p74 between these two species of NPV and its absolute requirement for infectivity of AcMNPV PDV indicate that p74 is a molecule of fundamental importance in primary infection, and may indicate a conserved target of entry into midgut cells common to many NPVs.

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