Trypsin cleavage of the baculovirus occlusion-derived virus attachment protein P74 is prerequisite in per os infection

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Baculovirus occlusion-derived virions (ODVs) contain a number of infectivity factors essential for the initiation of infection in larval midgut cells. Deletion of any of these factors neutralizes infectivity by the per os route. We have observed that P74 of the group I alphabaculovirus Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is N-terminally cleaved when a soluble form of the protein was incubated with insect midgut tissues under alkaline conditions and that cleavage was prevented by soybean trypsin inhibitor (SBTI). Presently, biological assays were carried out that suggest SBTI inhibitors and trypsin enhances baculovirus per os infectivity. We developed a method to rescue per os infectivity of a P74 null virus involving co-transfection of viral DNA with a plasmid that transiently expresses p74. We used this plasmid rescue method to functionally characterize P74. A series of site-directed mutants were generated at the N terminus to evaluate if trypsin cleavage sites were necessary for function. Mutagenesis of R195, R196 and R199 compromised per os infectivity and rendered P74 resistant to midgut trypsin.

INTRODUCTION

Baculoviruses are a group of arthropod-specific viruses (Zanotto et al., 1993) that have been applied as insecticides and as vectors for the expression of exogenous genes. They have a biphasic replication strategy producing two distinct viral phenotypes: the budded virion (BV) and the occlusion-derived virion (ODV). Both virion phenotypes have bilayer lipid envelopes surrounding bacillus-shaped nucleocapsids and contain double-stranded, circular DNA genomes. However, the integral protein composition of their envelopes and their roles in infection are distinct.

BV spread viral infection throughout host tissues by attaching to and entering host cells via receptor-mediated endocytosis (Volkman & Goldsmith, 1985). Endosomal acidification triggers envelope fusion with the endosomal membrane to release the viral nucleocapsid into the host cell (Leikina et al., 1992). In the case of group I alphabaculoviruses (Jehle et al., 2006), budding, attachment and envelope fusion are mediated by the viral protein GP64 (Blissard & Wenz, 1992) and for other baculovirus types these processes are mediated by an F protein (Pearson et al., 2000; Westenberg et al., 2002).

ODVs are required in the horizontal transmission of baculoviruses between insect hosts (Kozlov et al., 1986). The ODVs of all baculoviruses are occluded into proteinaceous occlusion bodies (OBs) prior to release from the host (Rohrmann, 1986). The distinctive protein structure of OBs has a dual function. It serves to protect virions from deleterious environmental factors, and also acts as a delivery mechanism to transport the ODVs to the alkaline midgut where the cells are susceptible to infection.

ODV envelopes are derived from the inner nuclear membrane (Braunagel & Summers, 1994) and contain envelope proteins that can survive the protease-rich environment of the insect midgut. ODVs attach to midgut columnar epithelial cells and fuse their envelopes directly with the cell membrane (Kawanishi et al., 1972; Tanada et al., 1975; Granados, 1978; Horton & Burand, 1993). A number of ODV envelope proteins are essential per os infectivity factors (PIFs), including P74 (Kuzio et al., 1989), PIF1 (Kikhno et al., 2002), PIF2 (Pijlman et al., 2003) and PIF3 (Ohkawa et al., 2005). PIF1, PIF2 and P74 have been implicated to be involved in ODV attachment to midgut cells (Haas-Stapleton et al., 2004; Yao et al., 2004; Ohkawa et al., 2005).

P74, the first identified PIF (Kuzio et al., 1989), is N-terminally exposed on the ODV surface and C-terminally anchored in the ODV envelope (Faulkner et al., 1997; Rashidan et al., 2005). These investigations are the
continuation of previous ones that showed the N terminus of P74 to be specifically cleaved by insect midgut trypsins (Slack & Lawrence, 2005). In the present study we found that Trichoplusia ni larvae were more susceptible to per os infection by Autographa california multiple nucleopolyhedrovirus (AcMNPV) when trypsin was added to the diet and they were less susceptible in the presence of soybean trypsin inhibitor (SBTI).

We have developed a transient plasmid-based assay to evaluate the ability of P74 mutants to rescue per os infectivity of a P74 null virus. It has been established that a P74 fused with the green fluorescent protein can function in place of native P74 (Yao et al., 2004). In this assay, insect cells were co-transfected with P74 null virus DNA and a plasmid expressing the p74 gene fused in-frame with the enhanced green fluorescent protein (egfp) gene.

Earlier, we proposed that P74 is cleaved at arginine residue R156 (Slack & Lawrence, 2005) and this study examines the functional effects on P74 of mutating R156 and nearby R156 (Slack & Lawrence, 2005) and this study examines the

METHODS

Cell lines and viruses. Spodoptera frugiperda Sf9 cell lines were propagated in 10 % v/v fetal bovine serum, 2.5 % w/v trypose broth-supplemented Grace’s (BFS/Grace’s) media (Sigma-Aldrich). The parental AcMNPV virus used in this study was isolate HR3 (Brown et al., 1979). The P74 null (P74A) AcMNPV virus used in this study was AcLP4 (Faulkner et al., 1997). This AcLP4 virus has the β-galactosidase reporter gene inserted into the middle of the native p74 ORF of AcMNPV and it produces the N-terminal 194 aa of P74 fused in-frame with β-galactosidase.

Plasmid constructs. The plasmids, pBAC-5-EGFP and pBAC-5-p74-EGFP, were previously described as pBAC-5-GFP and pBAC-5-p74-GFP (Slack et al., 2001). pBAC-5-p74-EGFP contains the AcMNPV p74 ORF fused at the 3’ end in-frame with the jellyfish EGF ORF (Zhang et al., 1996). pBAC-5-p74-EGFP is based on the pBAC-5 baculovirus transfer plasmid (Novagen).

Site-directed mutagenesis. All mutants were made using mutagenic PCR primers and the Deep Vent polymerase (New England Biolabs). PCR amplifications were done with 35 cycles of 95 °C 1 min, 45 °C 1 min 30 s and 72 °C 1 min 45 s.

The seven P74 mutants, R114Q, R119Q, R132Q, K138Q, R156Q, RK184/186QQ and RRR195/196/199QQ, were generated by amplifying two PCR products that, when ligated together, yield an 823 bp fragment of the 5’ end of the p74 ORF. This 823 bp fragment contained Ncol and BstXI sites that permitted cloning in place of the corresponding p74 ORF region in pBAC-5-p74-EGFP. The first or ‘left’ PCR products were made with a common Ncol site (underlined)-containing primer, p74M1Ncol-5’ (5’-AAGCACCA-TGCGGTTTAAAAACCCGT-3’) and phosphorylated mutagenic primers. The second or ‘right’ PCR products were made with a common BstXI site (underlined)-containing primer, p74D274BstXI-3’ (5’-AAGCACCATGCGGTTTAAACAGCGT-3’) and primers that corresponded to the positions immediately on the 5’ end of the mutant primers (see Supplementary Table S1, available with the online version of this paper).

Left and right PCR products were fractionated by agarose gel electrophoresis and purified using Qiagen glass milk (Qiagen). The corresponding left and right PCR products were ligated using T4 DNA ligase and the ligation was amplified by PCR using the primers p74M1Ncol-5 and p74D274BstXI. The pBAC-5-p74-EGFP plasmid contained two BstXI sites and was partially digested with BstXI prior to Ncol digestion. The resulting vector minus the original p74 Ncol/BstXI fragment was fractionated by agarose gel electrophoresis and purified by glass milk. The PCR product was cut with Ncol and BstXI and ligated into the corresponding sites in the p74 ORF.

The P74 mutants R195Q, R196Q, R199Q, R195Q/R196Q, R199Q/R199Q and R195Q/R199Q were made within a 395 bp EcoRI/SacII fragment of the p74 ORF. All of these mutagenic PCR products were amplified with the SacII-containing primer p74R319SacII-3’ (5’-CGTTACCGCGTAAATTTGATCGATC-3’) and an EcoRI site containing mutagenic primer (see Supplementary Table S1).

PCR products were fractionated by agarose gel electrophoresis and purified using glass milk. The pBAC-5-p74-EGFP plasmid and PCR products were cut with EcoRI and SacII, fractionated by agarose gel electrophoresis, glass milk-purified and ligated together.

All p74 mutants were sequenced (University of Guelph Molecular Supercentre) in both directions using primers p74Sequencing1LP36 (5’-AAACCCCAAGTTTCAGTCTGC-3’) and p74Sequencing2RP37 (5’-AAGTGACAAAGATGCTGTC-3’).

OB preparation. OBs were obtained from infected Sf9 insect cells. Infected cells were suspended in media and then centrifuged for 5 min at 1000 g. Pellets were suspended in PBS (120 mM NaCl, 10 mM Na2HPO4, 2.5 mM KCl, pH 6.2) and the OBs were released with SDS (0.3 % w/v) and centrifuged for 10 min at 2000 g. OB pellets were washed several times in PBS (pH 6.2), counted with a haemocytometer and diluted to desired concentrations.

Insect bioassays. Bioassays were all done in 128-well assay trays (Costar International). Artificial insect diet and T. ni larvae were obtained from the Great Lakes Forestry Centre, Insect Production Service, Ontario, Canada. Assay tray wells contained 1 ml volumes of diet. OBs were suspended in PBS (pH 6.2) and 20 µl were applied to the diet surface (2.3 cm2) and allowed to dry onto the diet surface for 1 h under shaded light. Larvae were placed onto diet, incubated at 28 °C and mortality data were collected at 7 days.

DNA purification and transfections. Transfections were done with P74A virus DNA and plasmid DNA. Plasmid DNA was purified by CsCl density-gradient centrifugation (Slack & Lawrence, 2002). P74A virus DNA was purified from ODVs by methods adapted from O’Reilly et al. (1992). Six 150 cm2 T-flasks, each containing 2×107 Sf9 cells, were infected with AcLP4 at a m.o.i. of 1 p.f.u. per cell and OBs were purified at 5 days post-infection, cells were harvested and OBs were purified. Suspensions of OBs were pelleted for 45 min at 1200 g and suspended in 20 ml H2O and were released by the addition of 0.5 ml of 500 mM EDTA and 2.5 ml freshly made 2 M Na2CO3. After 10 min, 2 ml Tris-OH (1 M, pH 6.8) was added and the insoluble debris was removed at 1200 g centrifugation for 5 min. The supernatant was centrifuged at 112500 g for 1 h at 15 °C. The ODV...
pellet was suspended in 400 μl TE buffer (1 mM EDTA, 10 mM Tris-OH pH 8.0) and disrupted by addition of 20 μl EDTA (500 mM), 10 μl SDS (10% w/v) and 12.5 μl proteinase K (10 mg ml⁻¹). After 2 h at 37 °C, viral DNA was extracted by phenol and chloroform/isoamyl alcohol (24:1). The DNA was dialysed against TE at 4 °C in 10 K molecular weight cut-off Slide-A-Lyzer cassettes (Pierce).

Transfections were done with the transfection reagent, ExGen 500 (Fermentas) using methods adapted from Oegy et al. (2006). S9 cells were seeded onto 6-well plates at a density of 1.25 × 10⁶ cells per well in 2 ml FBS/Grace’s media. Medium was supplemented with antibiotics/antimycotics (100 U penicillin G ml⁻¹, 100 μg streptomycin sulfate ml⁻¹ and 250 ng amphotericin B ml⁻¹) (Sigma-Aldrich). The transfection mixtures included 1600 ng plasmid DNA, 600 ng viral DNA, 8 μl NaCl (1M), 1077 μl NaCl (150 mM) and 55 μl ExGen500 transfection reagent. A transfection mixture volume of 200 μl was added to each well and then plates were centrifuged for 5 min at 500 g. At 5 days post-transfection, cells and media were harvested and processed for either OB purification or SDS-PAGE.

Brush border membrane vesicles preparation. BBMV were prepared from 80 fourth instar T. ni larvae using methods described previously (Slack & Lawrence, 2005). Final BBMV preparations were suspended in buffer B (8.5 mM MgCl₂, 150 mM d-sorbitol, 5 mM EGTA, 17 mM Tris-OH, pH 7.4), diluted to 1 mg ml⁻¹ total protein and stored at −80 °C. BBMV total protein was determined with a Bradford reagent assay (Bio-Rad Laboratories).

ODV purification and interactions with BBMV. ODVs were produced in S9 cells that had been co-transfected with P74Δ virus DNA and P74–EGFP plasmid DNA. Five 150 cm² T-flasks (1.3 × 10⁸ S9 cells) were transfected with 160 μg plasmid DNA and 3.6 μg P74Δ virus DNA. DNA was suspended in 11 ml NaCl (150 mM) and 550 μl ExGen500 was added and incubated for 15 min. The solution was added to 110 ml FBS/Grace’s media-suspended S9 cells (1.2 × 10⁶ cells ml⁻¹). Cells were then distributed into five 150 cm² T-flasks (22 ml per flask). After 5 days, OBs were purified as described, but with three additional PBS washes to ensure complete removal of SDS. The final yield was 5 × 10¹¹ OBs per transfection group.

ODVs were released from OBs by alkali treatment prior to experiments and were prepared on a small scale in 2 ml polypropylene tubes. To a total of 1.3 × 10¹⁰ OBs in 165 μl PBS (pH 6.2), 300 μl freshly prepared carbonate-buffered potassium was added (CBK) (500 mM KCl, 100 mM Na₂CO₃, pH 10.5). The final solution was incubated at room temperature for 5 min. Tubes were placed on ice and then centrifuged in an Eppendorf 5417R microcentrifuge for 45 min, 21 000 g, at 4 °C. The supernatant was aspirated off and ODV pellets were suspended by brief vortexing and stored at −80 °C. ODV pellets remained on ice no more than 30 min until their suspension in BBMV solutions.

Frozen BBMV were thawed on ice and then diluted 50% in CBK buffer. The resulting BBMV incubation (BI) buffer contained 250 mM KCl, 50 mM Na₂CO₃, 75 mM d-sorbitol, 1.25 mM EGTA, 4 mM Tris-OH and 4 mM MgCl₂. The pH of BI buffer was 10.5. In some experimental groups, BBMV were either preheated to 95 °C for 5 min or type II SBTI (Sigma-Aldrich) was added to a final concentration of 500 μg ml⁻¹. BBMV were further diluted in BI buffer to desired concentrations of BBMV proteins and 55 μl volumes of the vesicles were added to the ODV pellets. Control groups just received BI buffer. ODV pellets were suspended by brief vortexing and incubated for 20 min at 28 °C and then placed on ice. This was followed by the addition of 55 μl 2 × Laemmli SDS-PAGE disruption buffer and heating at 95 °C for 10 min.

Western blots. Proteins were fractionated under reducing conditions in 8.75% w/v acrylamide:bis (37:1) SDS-PAGE gels and transferred to nitrocellulose. Western blotting was done using an enhanced chemifluorescence (ECF) Western blotting kit (Amersham Biosciences). Primary antibodies were either the GP4-specific monoclonal antibody, AcV5 (Hohmann & Faulkner, 1983) or a EGFP-specific monoclonal antibody JL-8 (Clontech). Blots were blocked overnight at 4 °C with 10% w/v powdered milk in PBS (pH 7.4) and were probed for 2 h at room temperature with either AcV5 or JL-8 diluted 1:500 in PBS (pH 7.4), 0.05% v/v tween-20 (PBS-T). Blots were probed for 2 h with secondary anti-mouse, fluorescein conjugated antibody at 1:500 dilution in PBS-T and then probed for 2 h with tertiary anti-fluorescein, alkaline phosphatase (AP) conjugated antibody at 1:2000 dilution in PBS-T. After tertiary antibody probing, the blots were washed briefly with 1 mM MgCl₂, 10 mM Tris-OH (pH 9.5) and then reacted with 1.25 μg ml⁻¹ dimethylaminophenyl phosphate (Molecular Probes) fluorescent substrate. Blots were scanned using a Typhoon Trio+ laser bed scanner (GE Healthcare Life Sciences) (635 nm excitation, 670 nm BP 30 nm emission).

Protein sequence analysis and sequence sources. Alignment of the amino acid sequences of the P74 homologues was done using MEGALIGN 5.08 (DNASTAR). The CLUSTAL w method (Thompson et al., 1994) and Gonnet 250 protein weight matrix were used in alignments (gap penalty 10, gap length penalty 0.2).

Hydropathy profile (15 aa window) was determined using the program Protein 5.08 (DNASTAR). For transmembrane (TM) domain prediction, the site http://www.cbs.dtu.dk/services from the Center for Biological Sequence Analysis at the Technical University of Denmark was accessed and the program TMHMM 2.0 (Krogh et al., 2001) was used.

RESULTS

Role of trypsin in the infectivity of lepidopteran baculoviruses

P74 is essential for per os infection and is cleaved at a specific location by lepidopteran midgut trypsin. As a model system for other baculoviruses, we wanted to ascertain the role of indigenous midgut trypsin in per os infectivity. The experiments involved feeding T. ni larvae with parental AcMNPV OBs along with either SBTI or trypsin. Varying amounts of OBs were fed to groups of insects such that lethal concentration for 50% (LC₅₀) values could be estimated. It was observed that, relative to control groups of insects that were fed only OBs, the SBTI increased the LC₅₀ threefold and the presence of trypsin decreased the LC₅₀ fivefold (Fig. 1). Differences in the LC₅₀ in the presence of either additives clearly demonstrate that trypsin plays an important role in virus infectivity.

Development of a transient expression-based assay for characterization of P74

A simple method to evaluate P74 mutants without constructing new recombinant viruses was developed. It involved transfecting cells with plasmids that transiently express P74 in the presence of a co-infecting P74Δ virus. This was similar to a successful approach involving p74'-expressing insect cell lines complementing a P74Δ virus (Wilson, 1997). We used pBAC-5-based plasmids to transiently express the p74 ORF fused in-frame with the 5’ end of the EGFP ORF (Fig. 2a). These plasmids were
previously used to study P74 localization in infected cells (Slack et al., 2001). Though designed as baculovirus shuttle vectors, pBAC-5-based plasmids are excellent for transient gene expression in insect cell culture (Ogay et al., 2006), due to the presence of an AcMNPV gp64 early/late promoter (Whitford et al., 1989; Blissard & Rohrmann, 1991). Homologous recombination of plasmids such as pBAC-5 with circularized viral DNA is an infrequent event (less than 1%) (Kitts et al., 1990) and thus would not be significant for these transient experiments. Also, pBAC-5 plasmids are designed to eliminate the polh gene after recombination and any incidental recombinant viruses would not produce the polyhedra that were used to perform experiments. The C-terminal tagging of P74 with EGFP enabled monitoring of transfections and also provided an epitope tag for immunodetection (Fig. 2b).

The OBs produced from P74Δ virus/P74–EGFP plasmid co-transfections appeared by microscopic examination to be similar to parental virus OBs. Western blotting confirmed that P74–EGFP proteins co-purified with ODVs (Fig. 2b, ODV lane). More importantly, the P74Δ virus was rescued for per os infectivity by P74–EGFP, but not by EGFP (Table 1).

**Trypsin cleavage site mutants**

The previous hypothesis of midgut-specific P74 cleavage at R156 (Slack & Lawrence, 2005) was based on the estimated size of tryptic digest fragments in Western blots and on conservation of the R156 trypsin cleavage site among P74 homologues. It was not possible to obtain enough material to do N-terminal sequencing of P74 tryptic digest fragments.

![Fig. 1. Effects of trypsin and SBTI on susceptibility of T. ni larvae to AcMNPV infection. Second instar T. ni larvae were fed dosages of AcMNPV OBs in the presence of trypsin or SBTI and compared to OBs alone (control). Test animals received 1 mg/diet well porcine parvovirus tested (1:250) trypsin or 1 mg/diet well type II SBTI. (a) Cumulative % mortality is plotted against OB amounts that were added to diet wells. (b) The LC50 of each group is graphed and was determined by probit analysis using the program PoloPlus 1.0 (LeOra Software). Numerical bioassay data are provided in Supplementary Table S2.](http://vir.sgmjournals.org)

![Fig. 2. Control co-transfection experiments. Initial experiments involved co-transfection of the P74Δ virus DNA with plasmid DNA expressing either EGFP or p74–EGFP. (a) The EGFP and P74–EGFP ORFs are schematically illustrated along with the predicted molecular masses of their products. (b) We confirmed the synthesis of EGFP and P74–EGFP by Western blotting with an anti-EGFP antibody. Lane M is mock-infected Sf9 cells, lane 1 is cells transfected with P74Δ virus DNA only, lane 2 is cells transfected with pBAC-5-EGFP and P74Δ virus DNA, lane 3 is cells transfected with pBAC-5-p74-EGFP and P74Δ virus DNA. The lane labelled ‘ODV’ is purified ODVs from cells that had been co-transfected with pBAC-5-p74-EGFP and P74Δ virus DNA. The positions and molecular masses in kDa of markers are indicated on the sides.](http://vir.sgmjournals.org)

**Table 1.** Mortality of 2nd instar T. ni larvae 7 days post feeding OBs (100 000 OBs per well) from P74Δ virus, P74Δ virus/plasmid co-transfections or wt AcMNPV

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plasmid</th>
<th>Mortality (dead/total)</th>
</tr>
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<tbody>
<tr>
<td>P74ΔAcMNPV</td>
<td>–</td>
<td>0/16</td>
</tr>
<tr>
<td>P74ΔAcMNPV</td>
<td>EGFP</td>
<td>0/16</td>
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<td>P74ΔAcMNPV</td>
<td>p74–EGFP</td>
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<tr>
<td>Mock</td>
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fragments and site-directed mutagenesis was chosen to verify if R156 or nearby trypsin cleavage sites are required for P74 function. In the vicinity of R156, there are 11 consensus trypsin cleavage site motifs (R^X, K^X except R^P, K^P) (Fig. 3) and all are present in AcMNPV P74 at R114, R119, R132, K138, K147, R156, R184, K186, R195, R196 and R199.

A series of site-directed mutants were constructed by substituting R or K residues with glutamine (Q) residues. Glutamine was chosen because its side chain size is similar to R or K residues. Glutamine is a polar amino acid residue and R and K are positively charged residues. However, in the highly alkaline conditions of the insect gut where P74 is to function, K and R residues would also become polar residues. Also, this Q substitution occurs naturally in some P74 homologues at K138 and R186 (Fig. 3). In the first group of mutants, each of the residues R114, R119, R132, K138, K147 and R156 were changed to Q residues. It was initially opted to make multiple mutations where trypsin cleavage sites were clustered close to each other (R184/K186 and R195/R196/R199).

Site-directed mutants were made in P74–EGFP plasmids that were then co-transfected with P74Δ virus DNA into insect cells. It was confirmed by Western blot with EGFP-specific monoclonal antibody that similar amounts of P74–EGFP were being translated from each mutant P74–EGFP plasmid (Fig. 4a). As an internal control to virus replication, Westerns were also probed with anti-GP64 monoclonal antibody, AcV5 (Hohmann & Faulkner, 1983; Monsma & Blissard, 1995).

OBs from each co-transfection group were fed to 2nd instar T. ni larvae at several dosages. The bioassay results of the first group of P74 mutants are plotted as cumulative mortality (Fig. 4c). It was evident from this experiment and others that most of the P74–EGFP mutants rescued per os infection of the P74A virus as effectively as the native P74–EGFP protein. R156 was not required for P74’s function in oral infection. One clear and interesting observation is that cells producing the multiple site P74 mutant R195Q/R196Q/R199Q resulted in OBs that were 1000-fold less infectious than OBs derived from the other groups and warranted further investigation.

![Fig. 3. Alignment of predicted trypsin cleavage sites. A portion of the N terminus of AcMNPV P74 was aligned with other P74 homologues. The consensus sequence (Con) is shown at the top. The R and K residues of predicted trypsin cleavage sites are highlighted. P74 homologues are arranged into group I alphabaculoviruses (I), group II alphabaculoviruses (II), betabaculoviruses (B), hymenopteran-specific gammabaculoviruses (G), the dipteran-specific deltabaculovirus (D), Cuni and Hz-1. The group is indicated on the right side. The numbers along the top correspond to amino acid positions of AcMNPV P74. For this alignment, short form virus source names are indicated on the left side. Ac/Px, C/Op, Hear/Hz, and Co/CiGv are baculovirus species with P74 homologues that are identical in the region shown and are represented by single sequences. Also Spil(2) and Si(2) indicates there are two virus isolates with P74 homologues that are identical in the region shown. P74 homologue source baculovirus full names and GenBank protein accession numbers are listed in Supplementary Table S5.](image-url)
P74 site-directed mutants in region R195/R196/R199

The multiple mutations in this region of the protein could have caused conformational changes that precluded the ability of the mutant to rescue infectivity. Also, the P74Δ virus used in these experiments contained a p74–EGFP plasmid or p74–EGFP plasmids with mutations in the P74 ORF. Numbers refer to the amino acid position where R or K residues were changed to Q residues. The control group was just P74Δ virus DNA. (a and b) Western blotting was done with two different antibodies to confirm the presence of P74–EGFP proteins or to detect the virus-specific protein, GP64. The blots were probed with either anti-GFP monoclonal antibody (α-GFP) or with the AcV5 anti-GP64 monoclonal antibody (α-AcV5). Antibody reacting portions of blots are shown in upper two panels (102.6 kDa P74–EGFP and 64 kDa GP64). Also in the lower panel of (a) and (b), SDS-PAGE gels were stained with Coomassie brilliant blue and the dominant staining 29 kDa protein which is likely to be polyhedrin is shown (Polh). (c and d) In two separate bioassays, purified OBs from mutant groups were fed to 2nd instar T. ni larvae at varying dosages. The cumulative mortality curve is plotted. Most P74–EGFP mutants rescued per os infectivity of the P74Δ virus like wt P74–EGFP except for mutants P74(R195Q/R196Q/R199Q)–EGFP (c and d) and P74(R196Q/R199Q)–EGFP (d). Numerical bioassay data are provided in Supplementary Tables S3 and S4.

Fig. 4. Bioassay of P74 site-directed mutants. Sf9 cells were co-transfected with P74Δ virus DNA and the p74–EGFP plasmid or p74–EGFP plasmids with mutations in the P74 ORF. Numbers refer to the amino acid position where R or K residues were changed to Q residues. The control group was just P74Δ virus DNA. (a and b) Western blotting was done with two different antibodies to confirm the presence of P74–EGFP proteins or to detect the virus-specific protein, GP64. The blots were probed with either anti-GFP monoclonal antibody (α-GFP) or with the AcV5 anti-GP64 monoclonal antibody (α-AcV5). Antibody reacting portions of blots are shown in upper two panels (102.6 kDa P74–EGFP and 64 kDa GP64). Also in the lower panel of (a) and (b), SDS-PAGE gels were stained with Coomassie brilliant blue and the dominant staining 29 kDa protein which is likely to be polyhedrin is shown (Polh). (c and d) In two separate bioassays, purified OBs from mutant groups were fed to 2nd instar T. ni larvae at varying dosages. The cumulative mortality curve is plotted. Most P74–EGFP mutants rescued per os infectivity of the P74Δ virus like wt P74–EGFP except for mutants P74(R195Q/R196Q/R199Q)–EGFP (c and d) and P74(R196Q/R199Q)–EGFP (d). Numerical bioassay data are provided in Supplementary Tables S3 and S4.
R196 and R199 may be functionally redundant for the per os infectivity. None of the mutants compromised P74 function as much as mutant R195Q/R196Q/R199Q, indicating that the three R residues may work in concert.

**BBMV-specific cleavage of P74**

BBMV from *T. ni* larvae were used to evaluate the effects of insect trypsins on P74. By using azocasein assays (Slack & Lawrence, 2005) and different proteinase inhibitors, it was determined that BBMV from *T. ni* contained almost entirely trypsin protease activity (data not shown). In experiments, P74–EGFP or mutant P74(R195Q/R196Q/R199Q)–EGFP-containing ODVs were purified and incubated with BBMV, followed by fractionating the proteins on SDS-PAGE. P74–EGFP cleavage products were detected by Western blot using an anti-EGFP antibody (Fig. 5). BBMV-specific trypsin cleavage of native P74–EGFP was compared with that of the mutant P74(R195Q/R196Q/R199Q)–EGFP. After incubation with BBMV, the 102.6 kDa native P74–EGFP protein produced a cleavage product of 80 kDa (Fig. 5, lanes 9 and 11) whereas the mutant P74–EGFP protein did not produce this product (Fig. 5, lanes 10 and 12). The presence of SBTI inhibited the cleavage of P74–EGFP by the BBMV (Fig. 5, lanes 7 and 8). Although our ODV preparations were partially purified, these experiments suggest that P74–EGFP cleavage occurs in the context of the ODV and that the cleavage takes place at residues R195, R196 and R199.

**DISCUSSION**

The data presented here clearly suggest that trypsin activates the per os infectivity of baculovirus ODVs by cleaving P74 at the R195/R196/R199 vicinity. Tryptic activation has been documented for other viruses including coronaviruses (Frana et al., 1985), rotaviruses (Vonderfecht et al., 1988), Sendai virus (Muramatsu & Homma, 1980) and poxviruses (Ichihashi & Oie, 1982). The lepidopteran midgut is rich in trypsins (Johnston et al., 1991; Terra & Ferreira, 1994; Oliveira et al., 2005; Pereira et al., 2005) and it is probable that baculoviruses have evolved to exploit this environment. Trypsin is a serine protease and, in past studies, serine proteases have been found associated with insect-derived baculovirus OBs (Eppstein & Thoma, 1975; Eppstein et al., 1975; Langridge & Balter, 1981; Maeda et al., 1983).

Site-directed mutagenesis data and BBMV cleavage data suggest that P74 is a target of midgut trypsins and point to R196 and R199 being alternate primary trypsin cleavage sites on P74. So long as R196 or R199 are present, the full P74 function is retained. Residues R196 and R199 are present among P74 homologues of alphabaculoviruses, betabaculoviruses and gammabaculoviruses. The only exception is one isolate of *Spodoptera frugipera* multiple nucleopolyhedrovirus which has a P74 homologue lacking R196 (SF-CV, Fig. 3). There was insufficient material for N-terminal sequencing of BBMV-specific P74–EGFP cleavage products, but the molecular masses of the products correspond well with cleavage in the R195/R196/R199 region. This is supported by lack of a specific cleavage product in the R195Q/R196Q/R199Q mutant.

Two studies (Haas-Stapleton et al., 2004; Yao et al., 2004) have concluded that P74 is a viral attachment protein. Haas-Stapleton et al. (2004) showed that the P74Δ virus could not compete with wt virus ODVs for binding to the midgut and Yao et al. (2004) identified a 30 kDa midgut receptor protein for P74. P74 cleavage by midgut proteases may expose a midgut receptor binding domain on P74. However P74 binds to BBMV in the presence of serine protease inhibitors (Yao et al., 2004) and thus trypsin cleavage of P74 is not prerequisite to virus attachment.

P74 has characteristics of proteolytically activated virus envelope proteins such as influenza virus haemagglutinin (HA) protein and paramyxovirus F protein. Like P74, these proteins are C-terminally anchored in the virion envelope and have a large surface-exposed N-terminal domains. When influenza virus HA proteins and paramyxovirus F proteins are cleaved by host trypsins, a hydrophobic membrane insertion N-terminal domain is exposed...
(Collins et al., 1995; Lamb & Krug, 1995). Analysis of P74 (Fig. 6) shows that cleavage at R199 would result in an exposed hydrophobic N-terminal domain (region L214–F235) with a probability of membrane insertion based on hidden Markov model transmembrane prediction software (Krogh et al., 2001). Perhaps this exposed hydrophobic portion of P74 enables tighter attachment to the cell membrane by interaction with host receptor or other ODV envelope protein.

Further analysis of the region R195/R196/R199 reveal it to be homologous to a furin or protein convertase cleavage motif (R/K-Xn-R/K, n=0,2,4,6) (Duckert et al., 2004). This observation coincides with baculovirus BV envelope F proteins that require furin cleavage for function (Westenberg et al., 2002). We however found that the furin inhibitor, Dec-RVKR-CMK, did not affect per os infection of AcMNPV to T. ni larvae (data not shown). R195/R196 is also a preferred cleavage site motif for the baculovirus protease, V-CATH (Bromme & Okamoto, 1995). V-CATH is produced at the end of infection and aids release of OBs (Ohkawa et al., 1994; Slack et al., 1995). ODV-bound P74 would have limited exposure to V-CATH due to occlusion. However, much of the orally infectious virions in insect tissues do not become occluded in OBs and are referred to as preoccluded virions (POVs) (Hughes & Wood, 1996). POVs are more virulent per os than ODVs (Hughes & Wood, 1996) and their P74 peptides would be exposed to V-CATH proteolysis at cell lysis. Perhaps proteolytic activities of V-CATH on POV-bound P74 are contributing to increased POV virulence. Experiments remain to be done to link V-CATH with POV infectivity or with P74 cleavage.

In conclusion, we show that midgut trypsins play an important role in P74-mediated per os infectivity of AcMNPV to T. ni. This is likely not a host-specific phenomena, as in an earlier study we showed trypsin cleavage of AcMNPV P74 by midgut enzymes of the semi-permissive host Helicoverpa zea (Slack & Lawrence, 2005). We have identified R196 and R199 to be important trypsin cleavage site residues for function of AcMNPV P74. The conservation of R196 and R199 among P74 homologues suggest trypsin cleavage may be required for per os infection of alphabaculoviruses, betabaculoviruses and gammabaculoviruses. P74 is at least one PIF that has specific trypsin sites that are essential for its function. P74 is a large and a complex protein coordinated with an array of PIFs to facilitate per os infectivity of the ODV. Much work remains to be done on the structure and function of P74, including possible interactions of P74 with other PIFs.

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