

Evidence for proteolytic cleavage of the baculovirus occlusion-derived virion envelope protein P74

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Baculovirus occlusion-derived virions (ODVs) are released from occlusion bodies by the alkaline environment of the insect midgut. The ODV envelope protein P74 is required for oral infectivity. A soluble form of the *Autographa californica multiple nucleopolyhedrovirus* P74 protein, P74^{sol}, was engineered as part of a chimeric protein with jellyfish green fluorescent protein (GFP). P74^{sol}-GFP was overproduced by the baculovirus expression system and purified away from the wild-type P74. Brush border membrane vesicles (BBMVs) were prepared from the midguts of third-instar *Helicoverpa zea* larvae. When P74^{sol}-GFP was incubated under alkaline conditions with BBMVs, a P74^{sol}-GFP product with a smaller molecular mass was produced. Immunoblots indicated that the smaller product was generated by N-terminal cleavage of P74. This cleavage was prevented by soybean trypsin inhibitor. Analysis of the peptide sequences of P74 homologues identified a conserved trypsin cleavage site that could generate the observed P74^{sol}-GFP BBMV-specific cleavage product.

INTRODUCTION

Baculoviruses are pathogens of insect species that include Lepidoptera (moths and butterflies), Diptera (mosquitoes) and Hymenoptera (sawflies). There are two baculovirus phenotypes: occlusion-derived virus (ODV) and budded virus (BV). ODVs are required for the oral transmission of virus between insect hosts, while BVs transmit virus infection within insect host tissues. BVs attach to and enter insect cells by receptor-mediated endocytosis and acid-triggered membrane fusion of the BV envelope with the endosomal membrane. This process is mediated either by the fusion protein GP64 (Monsma *et al.*, 1996; Hefferon *et al.*, 1999) or another unrelated protein called F protein (Lung *et al.*, 2002; Westenberg *et al.*, 2004). Detailed knowledge of how ODVs establish infection in the complex and often harsh environment of the insect midgut, however, is not known.

ODVs are occluded in a protein matrix of either polyhedrin (polyhedroviruses) or granulins (granuloviruses), and the alkaline environment of the larval midgut releases ODVs to infect the midgut cells. ODVs attach to midgut cell receptor proteins and enter by direct fusion of the viral envelope with the host cell membrane (Horton & Burand, 1993). ODV envelopes contain a number of viral proteins including

ODV-E66, ODV-E25, ODV-E56, ODV-E18/E35, ODVP-6E and P74 (Braunagel *et al.*, 1996a, b; Theilmann *et al.*, 1996; Faulkner *et al.*, 1997). Several ODV proteins have been identified as essential for oral infection. These include P74 (Kuzio *et al.*, 1989) and the more recently described group of proteins called *per os* infectivity factors or PIFs (Kikhno *et al.*, 2002; Pijlman *et al.*, 2003).

In a GenBank search, we identified 26 P74 homologues. The P74 amino acid sequence is well conserved among baculoviruses with the notable exception of the P74 homologue of *Heliothis zea* virus 1 (Cheng *et al.*, 2002), which has only 15% identity with *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) P74 and little relatedness to other P74 homologues. In the context of AcMNPV infection, the *p74* gene is a weakly transcribed late transcript that is expressed between 16 and 20 h post-infection (Kuzio *et al.*, 1989). The low abundance of P74 protein has made it difficult to study in the wild-type virus. P74 most likely plays a role in ODV attachment and/or ODV envelope fusion with midgut cells. Recently, it was shown that P74 has binding affinity for midgut tissues (Haas-Stapleton *et al.*, 2004; Yao *et al.*, 2004). These studies provide new emphasis for characterizing P74 and elucidating the essential role that P74 plays in oral infection by ODVs.

P74 is C-terminally anchored by a transmembrane (TM) domain in the ODV envelope and has its N terminus exposed on the virion surface (Faulkner *et al.*, 1997; Slack *et al.*, 2001). P74 is an unusual virus envelope protein as it

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lacks an N-terminal membrane localization signal sequence and its C terminus contains a conserved double TM hairpin motif. We have proposed that this TM hairpin permits the direct insertion of P74 into the ODV envelope (Slack *et al.*, 2001). This is supported by the fact that P74 in suspension can rescue the oral infectivity of a *p74*-null virus (Yao *et al.*, 2004). Removal of the double TM domain (residues S580–F645) of AcMNPV P74 produces a soluble form of P74 (Slack *et al.*, 2001), which we call here P74^{sol}.

Faulkner *et al.* (1997) demonstrated that P74 is susceptible to proteolytic digestion and in the present study we examined the effect of brush border membrane vesicle (BBMV)-specific proteolytic activity on P74 in the context of a P74^{sol}-green fluorescent protein (GFP) chimera. Our results suggested that, under alkaline conditions, P74 is cleaved by BBMV-specific trypsins. Our analysis of the P74 peptide sequence revealed several candidate tryptic cleavage sites, one of which is highly conserved among other P74 homologues. In addition, our analysis suggested that the alkaline midgut environment may prime P74 for cleavage.

METHODS

Cells lines and viruses. *Spodoptera frugiperda* (Sf9) cells were used to propagate recombinant AcMNPV-based viruses. Cell lines were cultured at 27 °C in TNM-FH medium (Hink, 1970) containing 10% (v/v) fetal bovine serum.

Constructs and recombinant baculoviruses. Recombinant baculoviruses were based on the BacPAK6 baculovirus expression vector system (Kitts & Possee, 1993) and have been described previously (Slack *et al.*, 2001). The viruses BAC-GFP and BAC-p74(M1–S580)-GFP were used in the current study. As described previously, both BAC-GFP and BAC-p74(M1–S580)-GFP viruses produce a product that has a C-terminal poly-His tag. In the current study, we refer to BAC-p74(M1–S580)-GFP as BAC-P74^{sol}-GFP.

Purification of P74^{sol}-GFP and GFP. Spinner flasks (150 ml) of Sf9 cells (1×10^6 cells ml⁻¹) were infected at an m.o.i. of 0.1 infectious units per cell with the recombinant baculovirus BAC-GFP or BAC-P74^{sol}-GFP. GFP or P74^{sol}-GFP produced by these viruses is His-tagged. At 5 days post-infection at 28 °C, suspended cells were collected by centrifugation at 2500 g for 5 min at 4 °C. Cell pellets were suspended in 40 ml PBS (pH 7.4) and frozen at –20 °C. Cell suspensions were thawed and 1% (v/v) NP-40 and 10 mM imidazole were added. This mixture was forced through a 20-gauge needle followed by centrifugation at 15 000 g for 1 h at 4 °C. The soluble protein lysate supernatant was filtered through a #1 Whatman paper filter followed by filtration through a 0.45 µm filter.

His-tag purification columns (Pierce) were washed with 10 ml water (14 column vols) before being charged with 0.7 ml 0.1 M NiSO₄. Excess Ni was removed by elution with 10 ml water. Columns were then equilibrated with 10 ml binding buffer (1% NP-40, 10 mM imidazole, 500 mM NaCl, 20 mM Na₂HPO₄, pH 7.4). Soluble protein lysates (35 ml vols) were eluted through Ni-charged columns followed by washing with 10 ml binding buffer. Bound proteins were eluted using 5 ml elution buffer (500 mM imidazole, 500 mM NaCl, 20 mM Na₂HPO₄, pH 7.4). Protein elution was monitored on a UV spectrophotometer (OD₂₈₀) and 150 µl fractions were collected. Protein fractions were examined by SDS-PAGE and immunoblotting, and desired fractions were pooled.

Imidazole was removed by applying pooled fractions to PD-10 desalting columns (Amersham Biosciences) that had been equilibrated in 10 ml PBS. Desalted protein fractions were pooled and concentrated using YM-10 Centriprep centrifugation columns (Millipore). Concentrated protein fractions were combined with glycerol at 40% (v/v) and stored at –20 °C. Final protein concentrations of p74^{sol}-GFP and GFP purifications were 2 and 55 ng µl⁻¹, respectively.

BBMV preparation. BBMV tissue preparations were carried out on ice. Our methodology was an adaptation of the procedure described by Wolfersburger *et al.* (1987). Midguts were dissected from third-instar *Helicoverpa zea* larvae. Gut contents including peritrophic membranes were removed and guts were washed in buffer A (300 mM D-sorbitol, 5 mM EGTA, 17 mM Tris/HCl, pH 7.5). Midguts were suspended in buffer A at 10% (w/v). Approximately 4.5 g midgut tissue was produced from 110 animals. Midgut suspensions were combined with equal volumes of 24 mM MgCl₂ and incubated for 15 min on ice. Midgut suspensions were homogenized using a 10 ml glass tube and a Teflon pestle. Volumes of 5 ml were homogenized with nine strokes of the pestle while spinning at 3000 r.p.m. Homogenates were centrifuged at 2500 g for 15 min at 4 °C. Supernatants were collected and centrifuged at 30 000 g for 30 min at 4 °C. BBMV pellets were suspended in buffer B (150 mM D-sorbitol, 2.5 mM EGTA, 8.5 mM Tris/HCl, 12 mM MgCl₂, pH 7.5). BBMV pellets were resuspended with one stroke of the pestle while spinning at 3000 r.p.m. The suspension volume was diluted in buffer B to a total protein concentration of 400 ng µl⁻¹. Suspended BBMVs were aliquoted into Eppendorf tubes, frozen in liquid nitrogen and stored at –80 °C.

Alkaline phosphatase assays. Midgut tissue suspensions were diluted in 96-well plates in buffer B (150 µl, 1/2 dilution series). 1-Step NBT/BCIP reagent (Pierce) was added to each well (50 µl) and OD₅₇₀ was determined on an MRX Revelation microtitre plate absorbance reader (Dynex Lab systems). The NBT/BCIP reagent used in detection is normally used for alkaline phosphatase-based immunoblots.

Azocasein proteinase assays. Buffers were made with either phosphate (20 mM NaH₂PO₄, 150 mM NaCl, pH 6.0 and pH 7.2) or carbonate (100 mM Na₂CO₃, 500 mM KCl, pH 9.5 and pH 10.5). In a 96-well plate, 15 µl BBMV suspension (2 µg total protein) was combined with 200 µl of each pH buffer. A 100 µl 1/2 dilution series was made and 50 µl 0.5% (w/v) azocasein solution was added to each BBMV dilution. Samples were incubated for 3.5 h at 37 °C. Fifty microlitres of 20% (w/v) trichloroacetic acid was added and samples were centrifuged at 1000 g for 10 min. Supernatants were transferred to new wells and OD₄₀₅ was determined. Proteinase inhibitor experiments were done as above using carbonate buffer that had been adjusted to pH 10.5. Inhibitors were added to azocasein solutions prior to incubation.

Cleavage experiments. BBMVs were incubated with p74^{sol}-GFP or GFP. BBMVs were diluted to varying concentrations in buffer B. While on ice, 60 µl BBMV was combined with 30 µl containing either 60 ng p74^{sol}-GFP or 330 ng GFP. Carbonate buffer (100 mM Na₂CO₃, 500 mM KCl, pH 10.5) was then added in volumes of 50 µl. Mixtures were incubated at 20 °C for 20 min. This was followed by the addition of 100 µl Laemmli disruption buffer (Laemmli, 1970) and immediate boiling for 5 min.

Immunoblotting. For SDS-PAGE analysis, proteins were fractionated in 15-lane 10 or 15% (w/v) acrylamide:bisacrylamide (37.5:1) SDS-PAGE minigels (Bio-Rad). Gels were blotted on to nitrocellulose. Blots were blocked overnight in PBS (pH 6.8) with 5% (w/v) powdered milk. Blots were probed for 2.5 h with primary 1:20-diluted anti-P74 mAb N25 8c (Faulkner *et al.*, 1997) or 1:20-diluted anti-GFP polyclonal antiserum (Clontech). This was

followed by incubation for 2 h in 1:5000-diluted secondary goat anti-rabbit or goat anti-mouse antiserum (Pierce) that had been conjugated to horseradish peroxidase (HRP). All antisera were diluted in PBS (pH 7.4) with 0.5% (v/v) Tween 20 (PBS-T). Blots were washed in PBS-T and bound HRP was visualized using the SuperSignal West Pico Enhanced Chemiluminescence kit (Pierce) and X-ray film. Protein sizes were determined using Bio-Rad Precision pre-marked standards.

Protein sequence analysis. The 26 P74 homologues used in analysis for conserved trypsin cleavage sites are listed as follows with their GenBank protein identification number: AcMNPV, AAA66768; *Rachiplusia* ou MNPV, AAN28023; *Helicoverpa zea* single NPV, AAL56164; *Heliothis zea* virus 1, AAM45758; *Helicoverpa armigera* single NPV, AAK96273; *Lymantria dispar* MNPV, AAC70212; *Orgyia pseudotsugata* MNPV, AAC59133; *Epiphyas postvittana* NPV, AAK85685; *Choristoneura fumiferana* defective NPV, AAQ91644; *Choristoneura fumiferana* MNPV, AAP29905; *Spodoptera exigua* MNPV, AAF33660; *Mamestra configurata* NPV-A, AAQ11179; *M. configurata* NPV-B, AAM95145; *Culex nigripalpus* NPV, AAK94152; *Spodoptera litura* NPV, AAL01707; *Spodoptera littoralis* NPV, CAA67755; *Adoxophyes honmai* NPV, BAC67278; *Neodiprion lecontei* NPV, AAQ99067; *Cryptophlebia leucotreta granulovirus* (GV), AAQ21653; *Adoxophyes orana* GV, AAP85690; *Cydia pomonella* GV, AAK70720; *Agrotis segetum* GV, AAS82682; *Choristoneura fumiferana* GV, AAL13071; *Xestia c-nigrum* GV, AAF05191; *Plutella xylostella* GV, AAG27346; *Phthorimaea operculella* GV, AAM70253. Amino acid alignments were performed using the DNASTar program MEGALIGN 4.05 (1993–2000) using the CLUSTAL V algorithm (gap penalty 10, gap length penalty 10, PAM250).

Estimated amino acid charge profiles for AcMNPV P74 were calculated using the DNASTar program, Protein 4.05 (1990–1999). Later versions of this software do not permit specification of pH in charge prediction. Charges were predicted over a 15-residue mean. Positive- and negative-region minimal charges were set at 0.1 and -0.1, respectively.

RESULTS AND DISCUSSION

To begin this study, we prepared BBMV from the midguts of third-instar *Helicoverpa zea* larvae. To ensure the purity of our BBMV preparation, we measured alkaline phosphatase activity compared with total midgut (Fig. 1a). Alkaline phosphatase is a marker enzyme for BBMVs of lepidopteran larvae (Wolfersberger *et al.*, 1987). We also detected BBMV-specific alkaline protease activity (Fig. 1b) that could be inhibited by soybean trypsin inhibitor (SBTI) and the serine proteinase inhibitor, aprotinin (Fig. 1c). SBTI has been shown to inhibit noctuid alkaline proteinase activity (Johnston *et al.*, 1991). These observations suggested successful purification of enzymically active BBMVs.

For this study, we worked with P74^{sol}-GFP, a soluble form of P74 that had its C-terminal TM domain replaced with the GFP (Slack *et al.*, 2001). This protein chimera consisted of amino acid residues M1–S580 of P74 (66.1 kDa) and a 256-residue GFP that also had a C-terminal His tag (28.7 kDa). The resultant 94.7 kDa P74^{sol}-GFP fusion protein was produced using recombinant baculoviruses and was purified away from membrane-bound wild-type P74 using ultracentrifugation followed by nickel His-tag affinity columns. We confirmed the absence of wild-type P74 from our preparations by immunoblotting (Fig. 2).

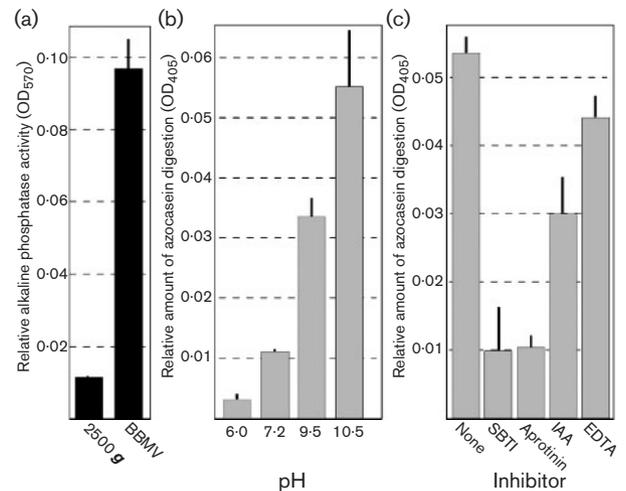


Fig. 1. BBMV purification from third-instar *Helicoverpa zea* larvae midguts. Midguts from *Helicoverpa zea* larvae were dissected, homogenized and processed to produce BBMVs. (a) The quality of the BBMV preparation was determined by comparing the relative amount of alkaline phosphatase activity of the crude midgut homogenate centrifuged at 2500 *g* with that of the final BBMV preparation. The bar graph indicates the change in OD₅₇₀ as a result of NBT/BCIP substrate reaction. (b) BBMV-specific proteolytic activity was determined with an azocasein substrate at various pH values. The bar graph shows the relative OD₄₀₅ of solubilized azocasein. (c) The effect of proteinase inhibitors on BBMV proteolytic activity was determined. Inhibitor concentrations were 10 mg SBTI ml⁻¹, 10 mg aprotinin ml⁻¹, 10 mg iodoacetamide (IAA) ml⁻¹ and 500 mM EDTA. The bars show the relative OD₄₀₅ of solubilized azocasein. Error bars in all bar graphs represent the standard deviation of at least four data points.

We observed that, after purification, P74^{sol}-GFP precipitated and was only soluble at a pH above pH 8.5. This did not concern us greatly as the alkaline environment better resembles the physiology of the lepidopteran midgut (Terra & Ferreira, 1994). When we incubated P74^{sol}-GFP with BBMV preparations at alkaline pH, we lost the ability to detect P74^{sol}-GFP on immunoblots using anti-P74 mAb N25 8c. In an earlier study (Slack *et al.*, 2001), we determined that mAb N25 8c recognizes an epitope somewhere at the N terminus of P74 between residues S68 and R195. Faulkner *et al.* (1997) reported that P74 was susceptible to proteinase K digestion while C-terminally anchored in ODV envelopes. They also reported that proteinase K-treated P74 could not be detected with mAb N25 8c. We suspected that in our study P74^{sol}-GFP was susceptible to BBMV-specific proteinase activity.

To investigate this, we incubated P74^{sol}-GFP with varying amounts of BBMVs. We also incubated P74^{sol}-GFP with BBMVs that had been boiled or been mixed with SBTI. These incubations were immunoblotted and probed with either anti-GFP polyclonal antiserum (Fig. 3a) or

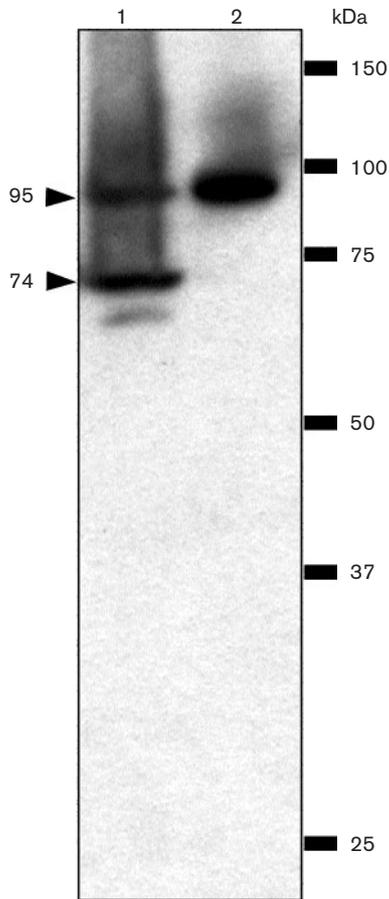


Fig. 2. Purification of His-tagged P74^{sol}-GFP. P74^{sol}-GFP was produced by recombinant baculoviruses in Sf9 cells. We purified P74^{sol}-GFP away from the wild-type P74 protein using a His-tag-based methodology. This purification was confirmed by immunoblotting with P74-specific mAb N25 8c. Lane 1, original crude cell lysate; lane 2, resultant purified lysate. The upper and lower arrows indicate P74^{sol}-GFP and P74, respectively.

anti-P74 mAb N25 8c (Fig. 3b). The anti-GFP antibody revealed the presence of two major cross-reactive proteins at estimated molecular masses of 95 and 75 kDa. The largest protein corresponds to the 94.7 kDa predicted molecular mass of P74^{sol}-GFP. The 75 kDa protein was not detected in the absence of BBMV or when BBMV had been boiled (Fig. 3b, lane B) prior to incubation with P74^{sol}-GFP. The addition of SBTI to BBMV also prevented the appearance of the 75 kDa cross-reactive protein (Fig. 3b, lane I). We deduced from this that the 75 kDa protein is a BBMV-specific proteolytic cleavage product of P74^{sol}-GFP (Fig. 4a, cleavage site I). At the highest BBMV concentrations, we detected a 28 kDa cross-reactive protein (Fig. 3a). This was interpreted as the 28 kDa GFP being proteolytically cleaved from P74^{sol}-GFP (Fig. 4a, cleavage site II). The junction between P74^{sol} and GFP is most likely a second trypsin-sensitive region due to the unnatural state of the fusion of

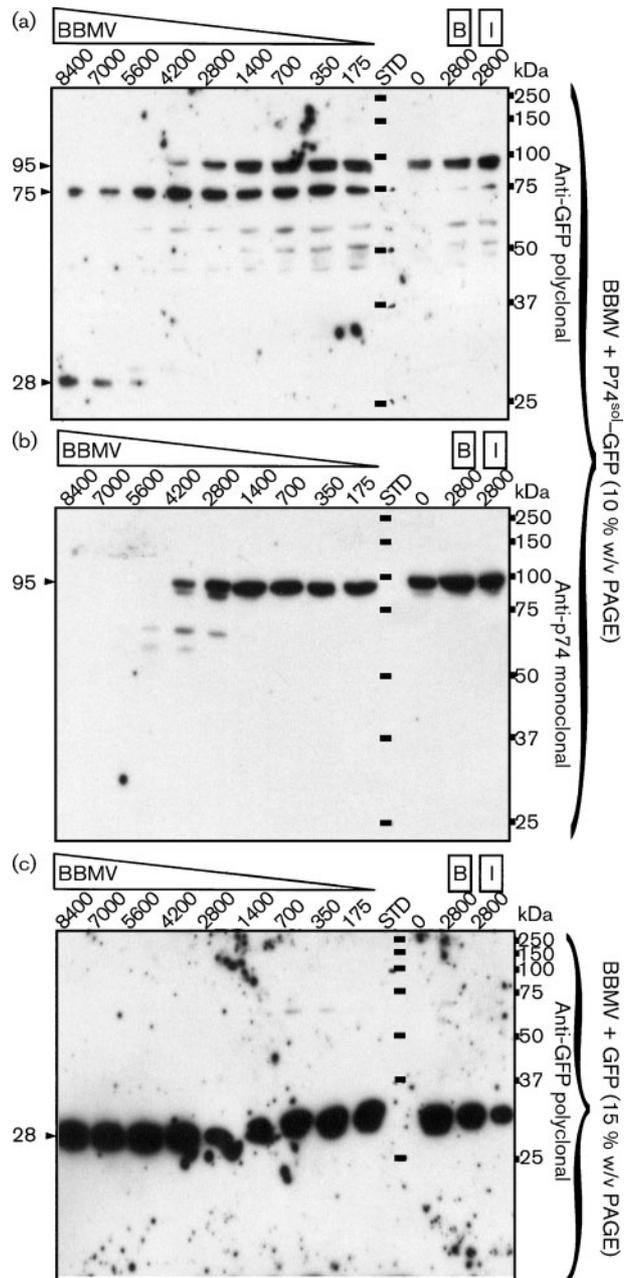


Fig. 3. Immunoblot evidence for proteolytic cleavage of P74 by BBMV proteases. BBMV preparations were mixed and incubated with either His-tag-purified p74^{sol}-GFP (a, b) or His-tag-purified GFP (c). Mixtures were fractionated by denaturing SDS-PAGE, transferred to nitrocellulose and probed with either anti-GFP polyclonal antiserum (a, c) or anti-P74 mAb N25 8c (b). For all blots, the amount of BBMV protein (ng) is indicated at the top of each lane. Lane B, BBMV boiled prior to incubation; lane I, addition of 50 µg SBTI prior to incubation. The positions of protein standards as they appeared on the blot are indicated in the STD lane. The estimated molecular masses (kDa) of immunodetected proteins of interest are indicated by arrowheads on the left of each blot.

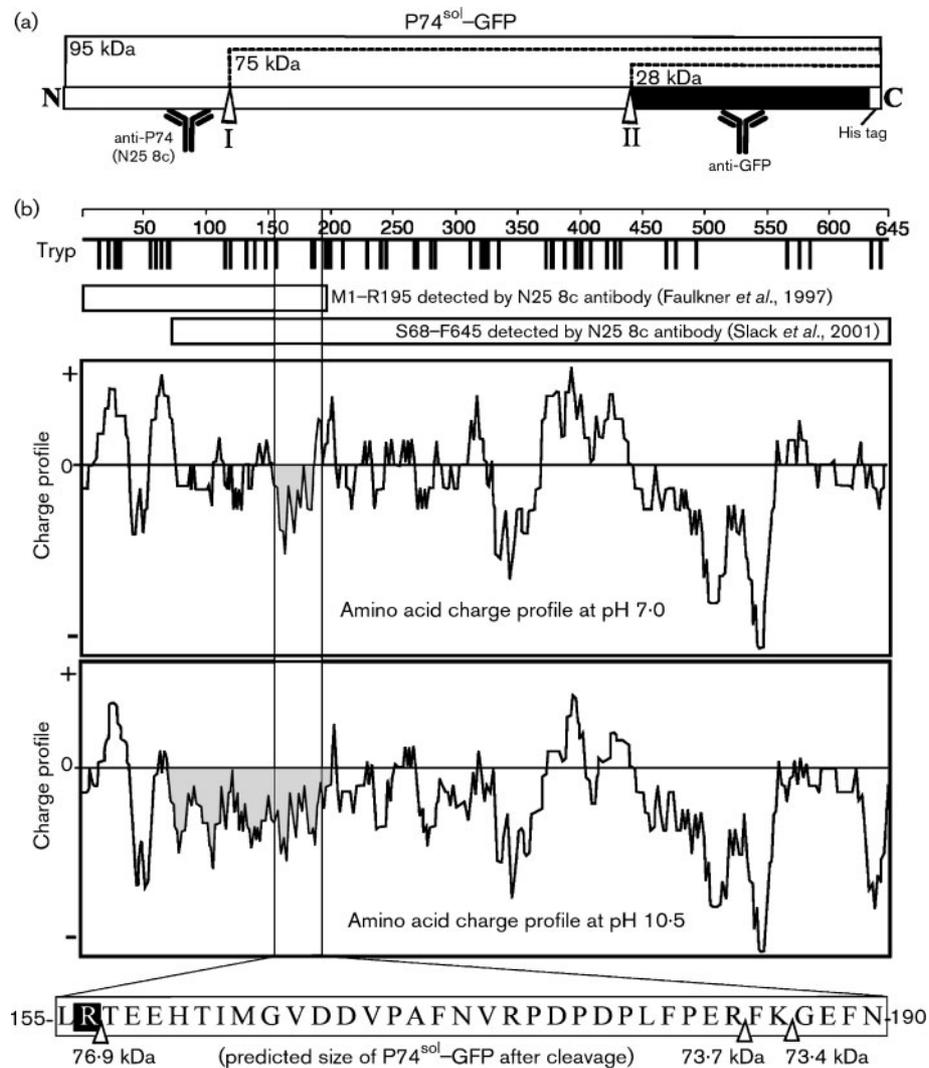


Fig. 4. Schematic of P74^{sol}-GFP cleavage and analysis of the P74 protein for tryptic cleavage sites and amino acid charge profiles. (a) The chimeric P74^{sol}-GFP is illustrated with P74^{sol}-GFP (M1-S580) in white and the 256-residue GFP-His protein in black. The predicted locations of BBMV-specific proteolytic cleavage of P74^{sol}-GFP are indicated by triangles. The molecular masses of cleavage products as detected by immunoblot are indicated. (b) The predicted tryptic cleavage sites for AcMNPV P74 are indicated below the amino acid residue scale bar for P74 (labelled Tryp). The epitope boundary of anti-P74 mAb N25 8c is defined by the P74 regions that were detected by Faulkner *et al.* (1997) and our previous publication (Slack *et al.*, 2001). The 15-residue mean predicted amino acid charge profile is plotted at pH 7.0 and 10.5. The shaded region on the charge plots is an area of interest where the predicted charge profile was dramatically affected by pH. A P74 region of interest is also boxed in the figure and expanded into the amino acid sequence. Arrows indicate potential tryptic cleavage sites and the solid, filled amino acid, R156, is conserved by position among 26 P74 homologues. This was the only such conserved trypsin cleavage site.

P74 with GFP. Our results, however, showed that N-terminal cleavage of P74 was much more sensitive to BBMV proteinases than the junction with GFP. GFP was remarkably resistant to BBMV alkaline protease activity. When we incubated GFP with BBMVs, we saw no change in protein size (Fig. 3c).

When we probed BBMV/P74^{sol}-GFP immunoblots with anti-P74 mAb N25 8c, we detected a 95 kDa protein, which

is consistent with the higher molecular mass protein detected by the anti-GFP antibody (Fig. 3b). However, mAb N25 8c, specific for the N-terminal region of P74, did not detect the 75 kDa protein that had been detected by the anti-GFP antiserum. These results suggested that the 75 kDa protein resulted from cleavage of the N terminus of P74^{sol}-GFP by BBMV-specific alkaline proteases (see Fig. 4a).

When Faulkner *et al.* (1997) overproduced P74 or an

N-terminal region of P74 called NT25 in the baculovirus expression system, a minor ~20 kDa peptide was detected by mAb N25 8c in addition to the expected 74 and 25 kDa peptides. Some of the abundantly produced P74 or NT25 proteins may have been susceptible to cleavage by background proteinase activity present in insect cells. We attempted to identify a 20 kDa BBMV-specific N-terminal P74 cleavage product using higher-percentage acrylamide immunoblots, but were unsuccessful (data not shown). In the N25 8c-specific immunoblot in Fig. 3(b), we detected a protein that was slightly smaller than 95 kDa using 4200 and 2800 ng of BBMVs. This may be evidence that BBMV proteinases are degrading the cleaved N-terminal P74 peptide fragment.

Examination of the amino acid sequence of AcMNPV P74 revealed a large number of predicted trypsin cleavage sites (Fig. 4b, top). When we aligned the peptide sequences of 25 other P74 homologues with AcMNPV P74, only one cleavage site was conserved by position. This conserved cleavage site at R156 would produce a predicted P74^{sol}-GFP product of 76·9 kDa (Fig. 4b, bottom). Other nearby, non-conserved trypsin cleavage sites at R184 and K186 would produce predicted P74^{sol}-GFP cleavage products of 73·7 and 73·4 kDa, respectively. It is difficult to make solid conclusions without N-terminal sequence data. We could not isolate enough material for this.

When we compared the predicted amino acid charge profiles of P74 at pH 7·0 and 10·5 (Fig. 4b), we discovered a possible mechanism that would make P74 susceptible to N-terminal cleavage at alkaline pH. At pH 7·0, the N-terminal region preceding our predicted P74 cleavage site is neutral in charge and hydrophobic. This region becomes very negatively charged when the pH is shifted to pH 10·5 (Fig. 4b, highlighted regions on the amino acid charge plots). We propose that alkaline conditions prime P74 for N-terminal cleavage by favouring a peptide region to become more hydrophilic and thus more exposed on the protein surface. Whether this cleavage is significant or not for P74 function remains to be determined.

There is precedence for proteolytic activation of baculovirus virion envelope proteins. Westenberg *et al.* (2004) recently showed that the group II nucleopolyhedrovirus BV envelope F proteins require proteolytic cleavage. Trypsin cleavage is important for other entomopathogens. Midgut tryptic cleavage is required for activation of the *Bacillus thuringiensis* Cry delta-endotoxin (Rukmini *et al.* 2000). P74 is a highly conserved ODV protein and is essential for ODV infection. Yao *et al.* (2004) recently reported that the P74 protein from *Heliothis armigera* single NPV binds to BBMV, but they did not report cleavage of P74. However, they modified the BBMV preparation protocol of Wolfersberger *et al.* (1987) by adding the trypsin inhibitor PMFS, which would have reduced or prevented such cleavage. Encountering active trypsins by P74 would be unavoidable in natural infection by ODVs. Our study points to a new direction of research on

P74. We hope to characterize P74 cleavage further and to evaluate its significance in baculovirus infection.

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