A study of the *Autographa californica* multiple nucleopolyhedrovirus ODV envelope protein p74 using a GFP tag

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The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) protein p74 is associated with the occlusion-derived virus (ODV) envelope. p74 is essential for oral infectivity of ODV and has been proposed to play a role in midgut attachment and/or fusion. In this study, p74 protein was expressed in-frame with green fluorescent protein (GFP) to create a p74–GFP chimera. The C-terminal GFP portion of the chimera facilitated visualization of the trafficking of p74 in baculovirus-infected *Spodoptera frugiperda* (Sf-9) cells. p74–GFP chimeric proteins localized in the intranuclear ring zone of the nucleus and were found to co-precipitate with the microvesicle fraction of cell lysates. A series of truncations of p74 was expressed as p74–GFP chimeras in recombinant baculoviruses. When C-terminal region S580–F645 was deleted from p74, p74–GFP chimera localization became non-specific and chimeras became soluble. p74 region S580–F645 directed GFP to the intranuclear ring zone in a similar pattern to full-length p74. The hydrophobic C terminus of p74 plays a role in protein localization and possibly in transmembrane anchoring and insertion.

Introduction

Baculoviruses are distinct from other virus families in that there are two viral phenotypes: budded virus (BV) and occlusion-derived virus (ODV). BVs disseminate viral infection throughout the tissues of the host and ODVs transmit baculovirus between insect hosts. ODVs form in the peristromal space or intranuclear ring zone of the nucleus (Stoltz *et al*., 1973; Summers & Volkman, 1976) and are occluded in paracrystalline protein matrices called occlusion bodies (OBs). When an insect dies from baculovirus infection, its tissues liquefy due to the synergy of the viral chitinase, ChiA (Hawtin *et al*., 1997), and the viral proteinase, V-CATH (Slack *et al*., 1995). Liquefying tissues release OBs into the environment. When OBs enter the alkaline midgut of a new host, they dissolve and liberate ODVs. ODVs infect columnar epithelial cells by attaching and fusing viral envelopes with cell membranes (Granados & Lawler, 1981).

ODV attachment to and fusion with midgut cells are critical steps in baculovirus infection. Little is known of the mechanisms by which these steps occur. There is evidence that ODV attachment to midgut cells requires the interaction of virion surface proteins and midgut cell surface proteins (Horton & Burand, 1993). ODV envelopes contain a number of proteins including ODV-E18, ODV-EC27, ODV-E35 (Braunagel *et al*., 1996a), ODV-E25 (Russell & Rohrmann, 1993), ODV-E56 (ODVP-6E) (Braunagel *et al*., 1996a; Theilmann *et al*., 1996), ODV-E66 (Hong *et al*., 1994) and p74 (Kuzio *et al*., 1989). The specific functions of these proteins have not been characterized. These proteins may individually or co-operatively be involved in ODV attachment and/or fusion with midgut cell membranes.

ODV envelope protein p74 is essential to ODV infectivity (Faulkner *et al*., 1997; Kuzio *et al*., 1989). Insertional inactivation of the p74 gene resulted in loss of ODV infectivity and retention of BV infectivity. The p74 protein is transcribed from a late viral transcript between 16 and 20 h post-infection (p.i.) (Kuzio *et al*., 1989). Detergent and proteinase studies suggest that p74 protein is an ODV envelope protein that is exposed on the virion surface (Faulkner *et al*., 1997). Exposure of p74 on the surface of ODVs suggests the possibility that p74 plays a role in ODV attachment and/or fusion with midgut cell membranes.
Full-length p74 gene homologues have been identified in at least 11 other baculovirus species. When we aligned the predicted amino acid sequences of p74 gene homologues (see Methods), conservation with AcMNPV p74 ranged from 91 to 31% identity.

It is our long-term objective to discover the functional role of p74. In the current study, we looked for regions of p74 that are important in trafficking and localization of p74 in the context of baculovirus infection. This was done by C-terminally tagging p74 with green fluorescent protein (GFP). We found that p74-GFP chimeras localized to discrete regions of the intranuclear ring zone. Several deletion mutants of p74 were then fused to GFP. A region of p74 was identified that is involved in localization within the nucleus. Our data suggest that this region may be involved in membrane association. In this study we also discovered a near full-length soluble form of p74 that we anticipate will be useful in functional assays.

Methods

- Cells and antisera. Spodoptera frugiperda (Sf-9) cell lines (Vaughn et al., 1977) were maintained at 28 °C in Grace’s TMN-FH insect medium (Hink, 1970) supplemented with 10% (v/v) foetal bovine serum. Ac5 (Hohmann & Faulkner, 1983) anti-gp64 and N25 (Faulkner et al., 1997) anti-p74 monoclonal antisera were provided as a gift by G. W. Blissard of Boyce Thompson Institute (Ithaca, NY, USA). Anti-GFP polyclonal antiserum was obtained from Clontech.

- Constructs. The open reading frame (ORF) of enhanced green fluorescent protein (EGFP) (Clontech) was amplified by PCR from plasmid — 16eEGFP (Chang et al., 1999). In this study we refer to ‘EGFP’ simply as ‘GFP’. PCR primers GFP-LP-Ncol/PstI (5’ ACCGGCCATGGGGGTCTGAGTTGACAGCGAGGAG 3’) and GFP-RL-HindIII (5’ GCCGCTACGTCGTAACGGCAACGGCAAGGGCAGG 3’) and GFP-RL-HindIII (5’ GCCGCTACGTCGTAACGGCAACGGCAAGGGCAGG 3’) were used to engineer 5’ Ncol/PstI sites and a 3’ HindIII site onto the ends of the GFP gene. Primer GFP-LP-Ncol/PstI modified the GFP gene such that the peptide sequence ‘Met-Gly-Ala-Ala-Gly-Val-Val’ would be translated on the N terminus of GFP in place of the original initiator methionine. The GFP gene PCR product was cloned into the Ncol/HindIII sites of pBAC-5 (Novagen). The resulting construct, pBAC-5-GFP, included an in-frame fusion of a polyhistidine tag on the 3’ end of the GFP gene.

The p74 gene was amplified by PCR from an AcMNPV Hr3 DNA template (Faulkner et al., 1982). Primers p74-LP-Ncol (5’ ATATA-CCATGCGGGTTTTAACAGCCGCTG 3’) and p74-RP-Xbal/PstI (5’ GTAAACTGCAGCTGTCGTAACAAATACAAATCAATTG 3’) were used to engineer a 5’ Ncol site and 3’ Xbal/PstI sites onto the p74 gene. The p74 PCR product was cloned as above into the Ncol/Xbal sites of pBAC-5-GFP to create pBAC5-p74-GFP (see Fig. 1a, ii). Cloning was such that the p74 ORF was upstream and in-frame with the GFP ORF. A series of 5 deletions of the p74 gene (see Fig. 2a) was accomplished by PCR with primer p74-RP-Xbal/PstI and primers that engineered a 5’ Ncol site onto each deletion. 5’ deletion primers were as follows: p74-S68-LP-Ncol (5’ GTCAAAACATGGCCACACAGCAGGCGGCTGTAACAC 3’); p74-S213-LP-Ncol (5’ TTTAGATCTAACGAGTCCGATAGGATCTAGGCATGCTG 3’); p74-S335-LP-Ncol (5’ GAGCCGCCATGGCCATTCAACAGGCGAGGTCGTCGTCCTG 3’); p74-S580-LP-Ncol (5’ CAAAACATGGCCACACAGCAGGCGGCTGTAACAC 3’); p74 gene regions were subcloned into pBAC-5-p74-GFP in place of the full-length p74 ORF by cloning into the Ncol/Xbal sites. A 3’ deletion of the p74 gene (Fig. 2a) was amplified by PCR with primer p74-LP-Ncol and primer p74-S580-RP-Xbal (5’ AGCGACGTCTAGAATGTGTGTTTTCGCT 3’). The resulting PCR product was cloned to make construct pBAC-5-p74 (M1-S580) (see Fig. 2a, iii). All PCR amplified regions were cloned and sequenced prior to use.

- Recombinant viruses and infections. Recombinant baculoviruses were made in the polyhedrin (polh) locus (polh gene deleted) of the baculovirus BacPAK6 (Clontech). Recombinant baculoviruses were based on the transfer vector pBAC-5 in which the polh promoter is replaced with the early/late — 166 AcMNPV gp64 promoter (Whitford et al., 1989) (Fig. 1a, i). All recombinant viruses used in this study retained the wild-type (wt) p74 gene locus. One µg of BsaI-linearized BacPAK6 viral DNA was co-transfected with 5 µg of transfer plasmid DNA into 1.5 × 10⁶ Sf-9 cells. Transfections were performed by CaCl₂ precipitation, which has been described previously (Slack & Blissard, 1997). Recombinant viruses were named Bac-5, Bac-GFP, Bac-p74(M1–F645)-GFP, Bac-p74(M1–S580)-GFP, Bac-p74(S68–F645)-GFP, Bac-p74(S213–F645)-GFP, Bac-p74(S335–F645)-GFP and Bac-p74(S580–F645)-GFP. Viruses were screened through three rounds of plaque purification (Hink & Vail, 1973). With the exception of virus Bac-5, recombinant viruses were screened by looking for fluorescent plaques using an Olympus SXZ12 fluorescent dissecting microscope (470 nm excitation, 525 nm emission). Viral stocks were generated by propagating viruses in Sf-9 monolayers (175 cm² flasks). Initial inoculums were diluted to an m.o.i. of 0.1 p.f.u. for all viruses.

- Fluorescence imaging. Fluorescence images were made from either living or paraformaldehyde-fixed Sf-9 cells. Living cells were suspended in medium (3 × 10⁶ cells/ml) and then deposited onto ice-chilled glass slides (30 µl per sample). Prior to microscopic examination, cells were stored on ice. For paraformaldehyde fixation, Sf-9 cells were seeded onto ice-chilled glass slides. Medium was aspirated away and replaced with 2% (w/v) paraformaldehyde, 125 mM NaCl, 2.5 mM KCl and 10 mM NaH₂PO₄, pH 6.2. Cells were fixed for 10 min and then washed twice with PBS (125 mM NaCl, 2.5 mM KCl and 10 mM NaH₂PO₄, pH 6.2). Paraformaldehyde fixation was followed by staining with the DNA stain 4-6-diamidine-2-phenylindole dihydrochloride (DAPI). A 200 ng/ml DAPI, 0.5% (v/v) Tween 20, PBS solution was applied to cells for 10 min followed by two washes in PBS. Sf-9 cells were visualized with a Nikon Eclipse E600 compound microscope and a Plan Fluor 100 × /1.30 oil, Ph3 DLL objective. All images were digitally recorded using a Fujifilm LAS-1000 CCD camera system. GFP-specific fluorescence was visualized using a Chroma endow GFP band-pass emission filter (HQ 470/40 nm excitation; HQ 495LP BS, HQ 525/50 nm EM), DAPI-specific fluorescence was visualized using Nikon epifluorescence filter UV-2A (355/25 nm EX, 400 nm DM, 420 nm BA).

- Fractionation by centrifuge. Spinner flasks (150 ml) were infected at an m.o.i. of 0.1 p.f.u. At 4 days p.i. cells were harvested and pelleted by a 10 min, 1000 g centrifugation, 5 × 10⁶ cells were suspended in 35 ml of PBS, 1 × protease inhibitor cocktail (Sigma). Cells were then disrupted by sonication and freeze–thaw. Sonication was done using a Heat Systems Microson Ultrasonic Cell Disruptor XL. Cells were pelleted (1 min, 1000 g) in 50 ml plastic tubes. Tubes were placed on ice and pellets were suspended by pulling for 30 s at the maximum setting. Centrifugation and pulsing were repeated five times. Complete cell disruption was ensured by microscopic inspection. After sonication, lysates were stored at −20 °C. Lysates were thawed on ice and then centrifuged at increasing rates in a Sorvall SW-28 rotor. Centrifugation
rates were 10 min at 2000 g, 60 min at 15 000 g and 60 min at 110 000 g. Samples (200 μl) of supernatant were collected after each centrifugation. Samples were combined with equal volumes of 2 × disruption buffer (125 mM Tris–HCl, 1%, w/v, SDS, 2.5%, v/v, mercaptoethanol, 10% glycerol, 0.2%, w/v, bromophenol blue).

**SDS-PAGE and immunoblots.** For SDS-PAGE analysis Sf-9 cells were washed twice with PBS (pH 6.2) and suspended in PBS (pH 6.2) at a concentration of 1 × 10^6 cells/μl. Samples were then boiled for 5 min with an equal volume of 2 × disruption buffer. Proteins were fractionated in 15 lane (1.5 × 10^6 cells per lane) 10% (w/v) acrylamide–bisacrylamide (37:5:1) SDS-PAGE minigels (Bio-Rad). Gels were blotted onto nitrocellulose. Blots were blocked overnight in PBS (pH 7.4) and 5% (w/v) powdered milk. Blots were probed for 1 h in primary antibody (diluted 1:50) followed by 1 h in secondary antibody (goat anti-rabbit or goat anti-mouse) (diluted 1:5000) that had been conjugated to horseradish peroxidase (HRP). All antibodies were diluted in PBS (pH 7.4), 0.5% (v/v) Tween 20 (PBS-T). Blots were washed in PBS-T and then bound HRP was visualized using the Pierce SuperSignal West Pico Enhanced Chemiluminescence (ECL) kit and X-ray film. Protein sizes were determined using Bio-Rad precision pre-marked standards.

**Protein analysis and structural predictions.** Only full-length p74 homologue sequences were used in analysis and predictions. Protein sequences analysed in this study were obtained from GenBank at the National Center for Biotechnology Information (NCBI). Baculovirus sources and GenBank protein identification numbers for p74 homologues are as follows: AcMNPV, NP 054168.1; BomNPV, AAC63805.1; CunNPV, AAA46730.1; OpMNPV, NP 046290.1; SeMNPV, AAE33660.1; SpliNPV, CAA67755.1; SpliNPV, CAA09849.1; LbMNPV, NP 075089.1; XenoGV, NP 059225.1; PLoGV, NP 068268.1. The ConsMNPV p74 homologue was translated from nucleotides 64–2019 of GenBank DNA sequence accession number AF274288 in accordance with Moser et al. (2001). Protein multiple alignments were performed using the program Megalign (DNASTAR). The Clustal V alignment algorithm (Higgins & Sharp, 1989) was used with GAP penalty and length penalty set to 10.

TM helix predictions were made using the program TMHMM via the internet site: http://genome.cbs.dtu.dk/services/TMHMM/. The TMHMM program applied the hidden Markov model (HMM) for predicting TM helices. This program has a greater than 95% accuracy in predicting TM domains in sample data sets of 160 proteins and 216 free parameters are examined for each prediction (for details refer to Sonnhammer et al., 1998).

**Results**

**Detection of p74–GFP and GFP**

For this study we initially made three recombinant baculoviruses; BAC-GFP and BAC-p74-GFP and BAC-5 (Fig. 1a). In these constructs, an early/late gp64 promoter drove the expression of GFP or p74–GFP. Recombinant insertions were made into the polyh locus and the native p74 locus was left intact. Protein products were confirmed by immunoblotting and fluorescence. A polyclonal antibody that was specific to GFP was used to probe for GFP and p74–GFP proteins at 36 h p.i. A cross-reacting protein was identified in protein lysates of the GFP virus (Fig. 1b, i) that correlated with the predicted molecular mass of 29 kDa for GFP. A cross-reacting protein was identified in protein lysates of the p74–GFP virus (Fig. 1b, ii). The protein migrated at close to the expected molecular mass of 103 kDa for p74–GFP. The same sized band was identified when protein lysates of the p74–GFP virus were probed with anti-p74 monoclonal antibody. This antibody also detected wt p74 expression in all virus constructs (Fig. 1c, i and iii).

Insect cells that had been infected with viruses BAC-5, BAC-GFP or BAC-p74-GFP were examined for GFP-specific fluorescence (Fig. 1d). We observed that fluorescence in cells infected with BAC-p74-GFP (Fig. 1d, ii) was distinct from
fluorescence in BAC-GFP-infected cells (Fig. 1d,i). Fluorescence was observed throughout the cytosol and the nucleus of BAC-GFP virus-infected Sf-9 cells. In contrast, fluorescence localized discretely in the intranuclear ring zone of BAC-p74-GFP-infected cells. When phase contrast light images were compared with fluorescence images it could be observed that fluorescence corresponded to dark regions within the intranuclear ring zone (Fig. 1d,ii). Cells infected with BAC-5 virus and uninfected Sf-9 cells did not have any GFP-specific fluorescence (Fig. 1d,iii and M). The observed intranuclear localization of p74–GFP changed over time. After 24 h p.i., fluorescent proteins accumulated into ‘vesicles’ in the intranuclear ring zone. By 72 h p.i., p74–GFP-specific fluorescence became condensed in the centre of the nucleus (data not available).
shown). A similar pattern of localization was reported for GFP when a portion of the ODV envelope protein ODV-E66 was expressed on the N terminus of GFP (Hong et al., 1997).

**Dissection of p74**

A series of viruses was made that expressed N-terminal truncations of p74 in-frame with C-terminal GFP tags (Fig. 2a). With the exception of baculovirus homologues, p74 has little similarity with other proteins. N-terminal truncations were arbitrarily made with consideration to the predicted hydrophobicity and charge profile of p74. Truncations were also made at serine residues for convenience of cloning. In addition, serine residues are predicted to permit conformational mobility between p74 peptide regions and GFP.

Recombinant baculoviruses were screened by PCR, immunoblotting and fluorescence. ImmunobLOTS were used to compare the molecular masses of virus-synthesized p74–GFP proteins with their expected sizes (Fig. 2b). It was notable that many of the constructs migrated about 5 kDa faster than expected. We also probed with anti-p74 monoclonal antibody N25 8c (Faulkner et al., 1997). With this antibody we were able to detect *wt* p74 and the p74–GFP constructs containing regions S68–F645 and M1–F645 (Fig. 2c). It was reported previously (Faulkner et al., 1997) that antibody N25 8c was specific to the N-terminal 194 amino acids of *wt* p74. Our detection of p74(S68–F645)–GFP using N25 8c, when combined with data from Faulkner et al. (1997), suggests that the N25 8c antigen is between S68 and S194 in p74. In some blots

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**Fig. 4.** Examination of the hydrophobic C terminus of p74. (a) p74–GFP constructs p74(S580–F645)–GFP (i) and p74(M1–F645)–GFP (ii) are illustrated. These two constructs contain a common hydrophobic region that is predicted to contain two TM domains. Construct p74(M1–S580)–GFP (iii) was designed to investigate the effects of loss of this hydrophobic region from p74. (b) The C-terminal regions of p74 homologues from 12 different baculoviruses are shown. Hydrophobic amino acids with a hydropathic index greater than 0 (Kyte & Doolittle, 1982) are indicated by white letters on a black background. The boxed regions are putative TM domains that are predicted by the Hidden Markov model (see Methods). The abbreviations are as follows: CMNPV, *Choristoneura fumiferana* MNPV; OpmNPV, *Orygia pseudotsugata* MNPV; SpliNPV, *Spodoptera littoralis* NPV; SpltNPV, *Spodoptera litura* NPV; BmNPV, *Bombyx mori* NPV; LdMNPV, *Lymantria dispar* MNPV; SeNPV, *Spodoptera exigua* NPV; HearNPV, *Heliocoverpa armigera* single NPV; CuniNPV, *Culex nigripalpus* NPV; XecnGV, *Xestia c-nigrum* granulovirus; PlxyGV, *Plutella xylostella* GV.
we observed that wt p74 migrated at an apparent molecular mass lower than 74 kDa. It is notable that accelerated SDS–PAGE migration was reported for the ODV envelope protein ODV-E66 (Hong et al., 1994). In that study it was suggested that membrane proteins migrate faster because they absorb more SDS.

**Localization of p74–GFP constructs**

When we fused different portions of p74 to GFP we observed differing patterns of cellular localization (Fig. 3). The p74 region S580–F645 directed GFP-specific fluorescence into intranuclear vesicles that later became highly condensed within the nucleus at times past 48 h p.i. (data not shown). This pattern of localization was notably similar to that of full-length p74 when fused to GFP. The p74 regions S438–F645 and S335–F645 did not direct GFP fluorescence into the intranuclear ring zone. Instead, fluorescent proteins accumulated abundantly along the outer periphery of the nucleus and nuclear import appeared to be inhibited. These p74 constructs may simply have been too large to enter the nucleus passively and they may lack the appropriate p74 region to promote nuclear import.

p74 regions S213–F645 and S68–F645 directed GFP-specific fluorescence to strands within the cytosol. We did not investigate the origin of these strands.

**p74 C-terminal region S580–F645**

Only the smallest C-terminal portion of p74 (S580–F645) localized GFP in a similar pattern to the full-length p74 (Fig. 3, second panel). The C-terminal region S580–F645 is the most hydrophobic region of p74. The observation that this hydrophobic region alone could localize GFP in a similar manner to the full-length p74 protein prompted further investigation. A p74–EGFP-expressing construct was made that lacked the S580–F645 hydrophobic region of p74 (Fig. 4a, iii). The construct, p74(M1–S580)–GFP, was then expressed in a recombinant baculovirus and compared to full-length p74–GFP. When we examined GFP-specific fluorescence, we observed that the p74(M1–S580)–GFP fluorescence looked similar to the fluorescence of GFP alone (Fig. 5). Removal of the hydrophobic region, S580–F645, from p74 resulted in complete loss of specific localization. This correlated well with the hydrophobic domain alone directing GFP to localize to specific regions in the nucleus.

It was speculated from this observation that the p74 region S580–F645 may be membrane associated. Membrane-associated proteins can be separated from soluble proteins by differential centrifugation (Dignam, 1990). In this study we disrupted virus-infected cells by sonication. Resulting cell lysates were sequentially centrifuged at 2000 g, 15 000 g and 110 000 g (see Methods). Supernatant samples were then collected after each centrifugation and proteins were fractionated by SDS–PAGE and blotted onto nitrocellulose (Fig. 6a). Blots were probed separately with the antibodies anti-gp64 (AcV5) (Hohmann & Faulkner, 1983), anti-p74 (N25 8c) and anti-GFP. Results showed that the relative amounts of gp64 and p74 proteins in supernatants diminished as the centrifugation rate increased. This was to be expected, as both of these proteins are membrane associated. Results also showed that the amounts of full-length p74–GFP (Fig. 6a, iii) and p74(S580–F645)–GFP (Fig. 6a, ii) proteins diminished as the centrifugation rate increased. We were able to solubilize these proteins using NP-40 detergent (data not shown). In contrast, the amounts of GFP (Fig. 6a, i) and p74(M1–S580)–GFP (Fig. 6a, ii) proteins in supernatants remained relatively unchanged as the centrifugation rate increased.

All of the recombinant baculoviruses used in this study were polh minus. To investigate what occurred in the presence of OBs we co-infected wt AcMNPV with the recombinant baculoviruses BAC-p74(M1–S580)–GFP, BAC-p74(S580–F645)–GFP, BAC-p74-GFP, and BAC-GFP (Fig. 6b). OBs retained a fluorescent green glow in virus combinations that expressed p74(M1–F645) or p74(S580–F645). In contrast GFP and p74(M1–S580) were excluded from OBs. These observations imply that the p74 region S580–F645 may be directing GFP into ODV envelopes within OBs.
Fig. 6. Effects of p74 region S580–F645 on solubility and OB localization. (a) Sf-9 cells were infected with recombinant baculoviruses expressing GFP (i), p74(M1–S580)–GFP (ii), p74(M1–F645)–GFP (iii) or p74(S580–F645)–GFP (iv). Infected cells were lysed by sonication. 2S, 15S and 110S represent proteins that remained in supernatants after 2000 g, 15000 g and 110000 g centrifugation. Proteins were immunoblotted with either AcV5 monoclonal (anti-gp64), N25 8c monoclonal (anti-p74) or anti-GFP polyclonal antisera. Anti-gp64 and anti-p74 columns are control blots and detect the baculovirus membrane proteins gp64 and p74. The anti-GFP panel is specific to GFP or p74–GFP proteins that were expressed by the different viral constructs. (b) Sf-9 cells were co-infected at an m.o.i. of 1 p.f.u. with wt AcMNPV and recombinant baculoviruses that expressed p74–GFP or GFP. At 48 h p.i., cells were examined for GFP-specific fluorescence. Horizontal bars in the lower right-hand corner represent 5 µm.

**Discussion**

The C-terminal tagging of p74 with GFP has permitted the observation of p74-specific trafficking in baculovirus-infected insect cells. p74–GFP chimeras localized into the intranuclear ring zone at late times post-infection. This localization contrasted to the non-specific localization of wt GFP. p74 is therefore directing the transport and localization of p74–GFP chimeras. The formation of ODV occurs within the intranuclear ring zone of the nucleus (Summers & Volkman, 1976). Localization of p74–GFP in the same region coincides with what would be anticipated for an ODV-specific protein. Differential centrifugation of cell lysates indicates that p74–GFP chimera proteins pellet in the microsomal membrane fraction. That fraction would presumably also include ODV and intranuclear microvesicles. ODV envelope proteins ODV-E66, ODV-E25, ODV-E56 and ODV E18/E35 are all translocated to intranuclear microvesicles in the nucleus (Braunagel et al., 1996 b). Intranuclear microvesicles are the proposed source of ODV envelopes (Hong et al., 1997). Others have demonstrated that p74 is an ODV envelope protein (Faulkner et al., 1997). It is therefore likely that pelleting of p74–GFP in the microsomal fraction is reflecting microvesicle or ODV association. Furthermore, the observation of p74–GFP localizing in polyedra (Fig. 6 b) suggests that p74–GFP is associating with ODV as would be expected for wt p74.

p74(M1–S580) was soluble despite the presence of a highly conserved internal predicted TM domain (Fig. 4 a, domain I). Putative TM domain I (T423–L452) may be buried within the p74 protein as it is found within a large hydrophobic region of p74 (I344–M465) (Fig. 2 a). p74–GFP deletion constructs p74(S438–F645) and p74(S335–F645) produced intense condensation along the periphery of the nucleus (Fig. 3). An alternative explanation to loss of a nuclear import signal is that these N-terminally truncated p74 proteins exposed all or portions of TM domain I (T423–L452) to interact non-specifically with membranes in the cytosol.

As a whole, the fluorescence patterns of the N-terminal deletion series were difficult to interpret. Interpretation is complicated by the fact that wt p74 was co-expressed with p74–GFP constructs. We believe that this may not have been a problem as non-reducing SDS–PAGE immunoblots did not reveal any evidence of p74 oligomerization (data not shown).

A previous study has suggested that the N terminus of p74 is exposed on the ODV surface and the C terminus of p74 acts as a TM anchor (Faulkner et al., 1997). The C terminus of p74 is highly hydrophobic (Fig. 2 a). Hidden Markov analysis (see Methods) predicts two TM domains at the C terminus of p74 (Fig. 4 a). Despite low sequence conservation, there is remarkable conservation of the double TM domain motif among ten of twelve p74 homologues (Fig. 4 b). SpliNPV has only one predicted C-terminal TM domain and the PlxyGV p74 homologue (Yoshifumi et al., 2000) completely lacks a predicted C-terminal TM domain. Downstream of the PlxyGV
p74 gene is an ‘orphaned’ ORF region that has similarity to the C terminus of the XecnGV p74 homologue (Hayakawa et al., 1999). Lack of a hydrophobic C terminus may reflect a recent evolutionary change in the PlasyGV p74 homologue. For AcMNPV, the hydrophobic C terminus of p74 is essential for this protein’s function (Kuzio et al., 1989).

In our study, deletion of 65 amino acids from the C terminus of p74 resulted in a soluble form of p74–GFP (Fig. 6). From this and TM prediction we propose that the C-terminal hydrophobic region of p74 contains a C-terminal TM anchor. The only other baculovirus protein that has been reported to have a C-terminal TM anchor is the BV envelope protein, gp64. Deletion of the gp64 TM anchor resulted in the solubilization of that protein (Oomens & Blissard, 1999). Kuzio et al. (1989) reported that deletion of 25 amino acids from the C terminus of p74 (I621–F645) produced non-infectious ODV. The loss of ODV infectivity in the Kuzio study likely resulted from disruption of the C-terminal TM anchoring domain of p74 and consequential loss of p74 from ODV envelopes.

The hydrophobic C-terminal region, S580–F645, was sufficient to direct GFP into the insoluble microsomal membrane fraction (Fig. 6a). It is possible that the C-terminal hydrophobic region of p74 can act as membrane insertion sequence in addition to a TM anchor. ODV-E66 and ODV-E25 contain hydrophobic domains that induce post-translational membrane insertion (Hong et al., 1997). A class of proteins has been described that post-translationally insert into membranes via hydrophobic C-terminal membrane insertion sequences (Kutay et al., 1993). The vaccinia virus H3L envelope protein is an example of this class of protein (Fonseca et al., 2000). p74 has a similar hydrophobicity profile to H3L. Hidden Markov analysis predicts that AcMNPV p74 TM domains II and III form a hairpin or loop in the membrane. This may explain why C-terminal fusion of GFP to p74 did not abrogate p74 membrane insertion.

ODV envelope proteins ODV-E66, ODV-E25, ODV-E56 and ODV E18/E35 also contain putative hydrophobic domains. The N-terminal hydrophobic domains of ODV-E66 and ODV-E25 were sufficient to target GFP and β-Gal reporters to intranuclear microvesicles (Hong et al., 1997). This coincides well with our data showing that hydrophobic p74 region S580–F645 could target GFP to the intranuclear ring zone. A notable difference is that larger p74 fragments containing the C-terminal hydrophobic domain were not transported into the nucleus. The smaller p74(S580–F645)–GFP protein is predicted to passively enter the nucleus as its molecular mass is less than 50 kDa (Talcott & Moore, 1999). The N terminus of p74 may be required for nuclear import and the C terminus may be necessary for intranuclear localization.

Our data are consistent with the premise that p74 is an ODV envelope protein and that p74 is translocated to intranuclear microvesicles in the intranuclear ring zone. We have provided evidence that the hydrophobic C-terminal region of p74 is involved in that translocation. The solubility of p74(M1–S580)–GFP suggests that p74 region S580–F645 is involved in C-terminal anchoring of p74 into membranes. Studies examining the mechanisms of p74 membrane insertion and the possible functional activities of soluble p74 are currently under way.

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