A soluble form of P74 can act as a per os infectivity factor to the *Autographa californica* multiple nucleopolyhedrovirus

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The baculovirus occlusion-derived virion (ODV) is required to spread virus infection among insect hosts via the per os route. The *Autographa californica* multicapsid nucleopolyhedrovirus P74 protein is an ODV envelope protein that is essential for ODVs to be infectious. P74 is anchored in the ODV envelope by a C-terminal transmembrane anchor domain and is N-terminally exposed on the ODV surface. In the present study, a series of N-terminal and C-terminal truncation mutants of P74 were evaluated for their ability to rescue per os infectivity of the P74-null virus, AcLP4. It was discovered that a P74 truncation mutant lacking the C-terminal transmembrane anchor domain of P74 was able to rescue per os infection. This result shows that a soluble form of P74 retains per os infectivity factor function and suggests that P74 may be complexed with other proteins in the ODV envelope.

Baculoviruses are a group of insect viruses that are used as biological insecticides and as foreign gene expression vectors. They have a biphasic replication cycle and produce two virion phenotypes: the early-phase budded virus (BV) and the late-phase occlusion-derived virus (ODV) (for a review see Slack & Arif, 2007). The BV spreads viral infection within host tissues and the ODV spreads viruses between hosts. The BV is produced when nucleocapsids are translocated from the nucleus to the cell surface, where they bud out from the cell membrane, simultaneously acquiring viral envelopes and envelope proteins. The ODV forms in the nucleus, where nucleocapsids are enveloped by nuclear membranes which contain ODV envelope proteins (Braunagel & Summers, 1994). ODVs are subsequently occluded in the nucleus in protein crystals called occlusion bodies (OBs). OBs are released by cell lysis after the death of the host. OBs preserve ODVs until they are ingested by a new host insect larva. The alkaline environment of the host gut triggers OBs to disintegrate and to liberate infectious ODVs.

ODVs attach to host midgut epithelial cells and fuse their envelopes directly with the cell membrane (Granados, 1978; Horton & Burand, 1993). The ODV envelope proteins that are essential to oral infection are called per os infectivity factors (PIFs); they include P74 (Kuzio et al., 1989), PIF1 (Kikhno et al., 2002), PIF2 (Pijlman et al., 2003) and PIF3 (Ohkawa et al., 2005). P74, PIF1 and PIF2 are implicated in ODV attachment to midgut cells (Haas-Stapleton et al., 2004; Ohkawa et al., 2005; Yao et al., 2004). P74 may also interact with P26 and P10 proteins to direct packaging of ODVs into OBs (Wang et al., 2009).

We have been interested in studying the P74 protein on a structural and functional level. The *Autographa californica* multicapsid nucleopolyhedrovirus (*AcMNPV*) P74 protein is a 645 amino acid integral membrane protein with N-terminal exposure on the ODV envelope surface (Faulkner et al., 1997; Slack et al., 2001). P74 lacks an N-terminal membrane insertion signal peptide, and the C-terminal transmembrane (TM) domain of the protein is sufficient to direct the translocation and insertion of P74 into the ODV envelope (Slack et al., 2001). Proteolytic cleavage of the N-terminus of P74 by host trypsin proteases is also required to functionally activate P74 on the ODV (Slack & Lawrence, 2005; Slack et al., 2008).

In past studies to characterize the TM domain of P74, a series of N-terminal and C-terminal truncations of P74 were engineered to be produced as N-terminal fusion proteins with GFP in recombinant baculoviruses (Slack et al., 2001). These P74–GFP fusion proteins enabled observation of how different truncated regions of P74 directed GFP translocation in the context of virus-infected cells. P74–GFP fusion proteins were intended only for observing protein localization in cells and it was initially assumed that these proteins would not be functional like native P74 in promoting per os infection. Recombinant P74–GFP baculoviruses had also been made in a baculovirus expression vector that had an intact p74 gene, thus
making it impossible to carry out functional assays of P74–GFP fusion proteins.

In our most recently published study of P74 (Slack et al., 2008), a transfection-based plasmid technique was developed to rescue per os infectivity of the P74-null virus, AcLP4 (Kuzio et al., 1989). The P74–GFP fusion protein was discovered to be functional like native P74 with respect to promoting per os infectivity of the ODV. The rescue plasmid was pBAC-5-p74-GFP (Slack et al., 2001), which contains the early AcMNPV gp64 gene promoter driving expression of a p74 ORF fused at the 3′ end in-frame with a GFP ORF. In the Slack et al. (2008) study, a series of site-directed mutants were made of the pBAC-5-p74-GFP plasmid and an essential trypsin cleavage site was mapped on the N-terminus of P74.

In the present study, the same rescue plasmid and P74-null virus were used to investigate N-terminal and C-terminal truncations of P74 for their ability to rescue per os infectivity of a P74-null virus. This study made use of the N-terminal and C-terminal P74–GFP truncation series that was used earlier to characterize the TM domain of P74. As described previously (Slack et al., 2001), the N-terminal and C-terminal truncation series was made by replacing the full-length p74 ORF (encoding M1–F645) in pBAC-5-p74-GFP with p74 ORF regions encoding S580–F645, S438–F645, S335–F645, M1–S335 and M1–S580. The P74 truncation P74(M1–S236)–GFP was not described previously. The corresponding M1–S268-encoding region of the p74 ORF was amplified by PCR using primers p74-S335-RP-XbaI (5′-ACCTACCTAGAACCGTGAGACCTCGAA-3′) and p74-LP-NcoI (5′-ATATACCATGGCGGTTTTAACAGCCGTCG-3′). The restriction endonucleases NcoI and XbaI were used to excise the p74 ORF from pBAC-5-p74-GFP and to clone in the M1–S236 PCR product.

All P74–GFP plasmid constructs were separately co-transfected with P74-null virus DNA onto Sf9 insect cells. Production of different truncated P74–GFP proteins in the context of P74-null virus infection was confirmed using Western blotting and an anti-GFP monoclonal antibody (Fig. 1). All of the expected protein sizes were observed. An unexpected 39.5 kDa protein was detected in addition to the expected 102.6 kDa P74(M1–F645)–GFP protein. We are uncertain of the identity of this smaller protein. It is not a product from translational initiation at an internal methionine. This small protein was also weakly detected in other samples, including P74(S580–F645)–GFP. The expected and observed size of P74(S580–F645)–GFP protein was 36.5 kDa. It is not possible for an internal methionine initiation to create a larger 39.5 kDa secondary product. This cross-reactive protein is not required for P74 function based on its absence in P74(M1–S580)–GFP samples and the ability of this construct to rescue per os infection of the P74-null virus (Fig. 2).

Rescue of per os infectivity of the P74-null virus by plasmids was assessed by purifying OBs from co-transfected insect cells and feeding them to Trichoplusia ni larvae as described previously (Slack et al., 2008). The only OBs that were orally infectious were the ones obtained from cells that had been co-transfected with P74-null virus DNA and either the plasmid encoding P74(M1–F645)–GFP or the plasmid encoding P74(M1–S580)–GFP (Fig. 2).
GFP includes the complete P74 protein and it is associated with ODVs (Slack et al., 2001, 2008). P74(M1–S580)–GFP was previously shown to be soluble (Slack et al., 2001) because it lacks the primary TM anchor domain of P74 (Faulkner et al., 1997; Slack et al., 2001). The soluble P74(M1–S580)–GFP protein was not expected to rescue per os infection of the P74-null virus. We were unable to detect this soluble P74(M1–S580)–GFP protein associating with ODVs but association with ODVs in abundance below the level of Western blot detection is possible.

The P74(M1–S580)–GFP protein is abundant and it may have co-occluded with ODVs during OB formation. Fluorescence images of virus-infected cells showed P74(M1–S580)–GFP protein localized throughout the cell, including the nucleus (Fig. 3). Our data support a study showing that detergent-solubilized, full-length P74–GFP fusion protein could rescue per os infectivity of a recombinant P74-null Heliothis armigera single nucleocapsid nucleopolyhedrovirus (Yao et al., 2004). We commented on this study in an earlier publication and suggested that the double TM domain of P74 may spontaneously insert itself into the ODV envelope (Slack & Lawrence, 2005). Our present data would suggest that ODV envelope insertion is not a prerequisite for P74 to facilitate per os infectivity of the ODV.

It is possible that some of the soluble P74(M1–S580)–GFP complexes with other ODV envelope proteins such as PIF1 and PIF2. Such complexes would hold the TM anchorless P74(M1–S580)–GFP protein on the ODV surface. This phenomenon has been observed in experiments with other virus systems. The herpesvirus envelope protein gD is an attachment protein that forms a complex with other viral envelope proteins involved in virus fusion (Gianni et al., 2009). When the TM anchor of gD is experimentally deleted, its interaction with the other viral envelope proteins maintains its association and function in the complex (Cocchi et al., 2004). We attempted non-reducing Western blots but found no evidence of P74 being involved in disulfide-bonded protein complexes (data not shown). However, there are other types of potential protein–protein interactions such as hydrophobic interactions.

The P74 protein has a central highly hydrophobic region that contains a predicted TM domain from T423 to L452 (Fig. 2b). This domain does not participate in membrane insertion given that P74(M1–S580)–GFP is soluble. However, when the central hydrophobic region of P74 is exposed on the N-terminus of P74 such as in the case of truncation mutants P74(S335–F645)–GFP and P74(S438–F645)–GFP, fusion proteins accumulate around the nuclear membrane and are inhibited from entering the nucleus (Slack et al., 2001). The central hydrophobic region may normally interact with other viral envelope proteins and disruption of P74 structure in the truncation mutants may lead to inappropriate interaction of the hydrophobic region with the nuclear membrane. Much work remains to be done to understand the structure of P74 and its possible association with other proteins. Presently the P74 core functional domain can be defined as lying between the trypsin cleavage site at R199 and the TM domain at S580 (Fig. 2b).

Fig. 2. Bioassay of P74 N-terminal and C-terminal truncation mutants. Sf9 cells were co-transfected with P74-null virus DNA and a series of plasmids which encoded N-terminally or C-terminally truncated P74 proteins fused to GFP. (a) Purified OBs from co-transfection groups were fed to second-instar Trichoplusia ni larvae. Larvae (16 per dose) received doses of 10^2, 10^3, 10^4 or 10^5 OBs. The cumulative mortality curve is plotted. The control group consisted of insects that were fed only the P74-null virus OBs. (b) Schematic illustration of P74 truncations that were evaluated as P74–GFP fusion proteins in the bioassay. The P74 regions that rescued per os infection are shaded. Above the schematic is a TM prediction profile of P74 produced using the program TMHMM server v.2.0 from http://www.cbs.dtu.dk/services (Krogh et al., 2001). The position of a functionally essential trypsin cleavage (Slack et al., 2008) site is also indicated.
**Fig. 3.** Fluorescent localization of P74–GFP chimeras in virus-infected cells. Sf9 cells were transfected with just P74-null virus DNA (ctrl) or with virus DNA and plasmids encoding either P74(M1–F645)–GFP or P74(M1–S580)–GFP proteins. At 60 h post-transfection, cells were visualized for GFP-specific fluorescence under a compound fluorescence microscope (Olympus BX50). Visible light and fluorescent images (excitation 450–480 nm band pass, emission 515 nm long pass) are shown of representative cells. Scale bars, 10 μm.

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**References**


