BACULOVIRUS SUGGESTS A COMMON BUT MULTIFACETED PATHWAY FOR SORTING PROTEINS TO THE INNER NUCLEAR MEMBRANE

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ABSTRACT

Multiple unique protein markers sorted to the INM from the Autographa californica nucleopolyhedrovirus occlusion-derived viral envelope were used to decipher common elements of the sorting pathway of integral membrane proteins from their site of insertion into the membrane of the endoplasmic reticulum (ER) through their transit to the inner nuclear membrane (INM). The data show that during viral infection, the viral protein FP25K is a partner for all known ODV envelope proteins and BV/ODV-E26 (E26) is a partner for some, but not all such proteins. The association with the ER membrane of FP25K, E26 and the cellular INM-sorting protein, importin-α-16 is not static, but rather these sorting proteins are actively recruited to the ER membrane based upon requirements of the proteins in transit to the INM. Co-localization analysis using an ODV envelope protein and importin-α-16 shows that during viral infection, importin-α-16 translocates across the pore membrane to the INM and is then incorporated into the virus-induced intranuclear membranes. Thus, the association of importin-α-16 and INM-directed proteins appears to remain at least through protein translocation across the pore membrane to the INM. Overall, the data suggests that multiple levels of regulation facilitate INM-directed protein trafficking and that proteins participating in this sorting pathway have a dynamic relationship with each other and the membrane of the ER.
**INTRODUCTION**

Baculovirus infection results in a synchronous, amplified pulse of synthesis of integral membrane proteins that integrate into the membrane of the endoplasmic reticulum (ER) and utilize the membranes of the nuclear envelope during transit to intranuclear membrane vesicles. These virus-induced intranuclear membrane vesicles ultimately become the envelope of the occlusion-derived virus [ODV (6)]. The N-terminal 33 amino acids of the viral envelope protein ODV-E66 (E66) are sufficient to target this protein and fusion proteins thereof, to the ODV envelope (14); and in the absence of infection, target proteins to the inner nuclear membrane [INM (15)]. Thus, this sequence has been termed an inner nuclear membrane-sorting motif [INM-SM (8)]. Knowledge of the INM-SM and the ability to generate fusion and mutant constructs provide unique tools to study the molecular mechanisms that regulate trafficking of integral membrane proteins from their site of membrane insertion in the ER to the membranes of the nuclear envelope (8, 15).

The E66-derived INM-SM sequence functions as an N-terminal signal anchor and has two major targeting features. The first feature is a hydrophobic domain of approximately eighteen amino acids that during translation is precisely positioned within the central channel of the ER translocon within close proximity to both Sec61α and TRAM (21). The second feature is a positively charged amino acid within four to eight amino acids from the end of the hydrophobic sequence that is positioned on the cytoplasmic face of the ER membrane (8). Chemical crosslinking studies show that when the nascent chain of the INM-directed protein resides within the central channel of the translocon, the positively charged amino acid of the INM-SM sequence is in close proximity to both Sec61α and TRAM (21).
proximity with the cellular protein importin-\(\alpha\)-16, and importin-\(\alpha\)-16 remains associated
with this sequence after release from the translocon and ER membrane integration (22).

In infected cells, after the INM-directed protein has been released from the translocon
and integrated into the ER membrane, the positively charged amino acid of the INM-SM
sequence is proximal to the viral proteins BV/ODV-E26 (E26) or FP25K (8, 21). The
sequence of molecular events that occur after association of the INM-SM with cellular
importin-\(\alpha\)-16 and subsequent association of the same region of the INM-SM with the
viral proteins E26 or FP25K are unknown, but clearly these data suggest that sorting of
INM-directed proteins begins during protein translation and continues after protein
integration into the ER membrane.

The discovery using baculovirus of *Spodoptera frugiperda* (Sf) importin-\(\alpha\)-16 (and
human KPNA4-16) was hailed as the discovery of a “missing link” to understanding the
molecular pathway of integral membrane proteins directed to the INM (19). However,
the discovery of importin-\(\alpha\)-16 and its association with INM-directed proteins had greater
implications. The identification of importin-\(\alpha\)-16 led to the discovery of other isoforms
of importin-\(\alpha\): KPNA4-26; KPNA1-12; KPNA2-12, although nothing is known of their
function (7). It is widely held that importin-\(\alpha\) is a soluble protein, however several
reports now show that a subset of importin-\(\alpha\) associates with cellular membranes (1, 12,
13). While little is known of the function of full-length, membrane-associated importin-
\(\alpha\), we know that the truncated isoform importin-\(\alpha\)-16 not only associates with the ER
membrane but is positioned at the translocon such that it can optimally survey translating
nascent chains, recognize INM-SM sequences and associate with them. However,
importin-\(\alpha\)-16 and KPNA4-16 can also recognize INM-SM sequences and associate with
them post-translationally. As such, importin-α-16 may serve to direct INM-SM containing proteins into a common and specific INM-directed trafficking pathway regardless of their method of membrane integration. Until the identification of Sf importin-α-16 and its human homologue KPNA4-16, it was widely held that INM-directed proteins were not specifically targeted to the INM, but rather diffused freely through the contiguous membranes of the ER and nuclear envelope, and were only enriched at the INM after binding with nucleoplasmic components. Clearly, not only is our knowledge of the well-described adaptor protein importin-α incomplete, but the previous doctrine of the trafficking pathway of integral proteins to the INM is challenged by current data.

Data suggests that during baculovirus infection, cellular importin-α-16 and the viral proteins FP25K and E26 all function to sort and traffic ODV-envelope proteins to the INM and viral envelope. Yet, understanding of how these proteins interact with each other, or associate with the ER is unknown. Full-length importin-α is predominantly soluble and it is likely the ER membrane/translocon association of importin-α-16 is also transient, although its membrane association is resistant to salt and alkali extraction (22). A transient membrane association is predicted for the viral proteins FP25K and E26; FP25K is predominantly a soluble protein and the factors regulating its membrane association are unknown, while E26 membrane association is apparently regulated by a reversible palmitoylation event (9). The idea of proteins transiently associating with the molecular machinery of the translocon and providing a unique molecular activity is not new; signal peptidase, oligosaccharyltransferase, signal-recognition particle and BiP are all well-described examples of such proteins (16). Based upon other known pathways
involving the ER, it is reasonable to postulate that the cell has evolved mechanisms to keep such accessory proteins at optimal stoichiometric levels relative to the demand of molecular events occurring at the ER membrane. Thus, such accessory proteins may be recruited or displaced from the ER membrane as needed. Such dynamic interactions are difficult to detect and study in intact cells or by using routine *in vitro* assays; however, study of amplified pathways of protein transport, like that produced by baculovirus infection, substantially increases the potential to detect such transient and dynamic interactions of known proteins.

The goals of this manuscript are threefold: 1) use multiple viral ODV-envelope proteins with INM-SM like sequences to determine if the molecular events identified for the E66-derived INM-SM sequence are also general features of the molecular sorting pathways for other INM-directed proteins; 2) use various recombinant viruses which amplify the quantity of INM-directed proteins or control the levels of expression of known sorting proteins (FP25K, E26 and importin-α-16), to determine if altered expression affects the quantity of sorting proteins present at the ER membrane; and 3) use a recombinant baculovirus to determine if importin-α-16 transits to the INM.

**MATERIAL AND METHODS**

*Insect Cell Lines, Virus and Preparation of Microsomal Membranes.* *Spodoptera frugiperda* IPLB-Sf21-AE clonal isolate 9 (Sf9) cells were cultured in suspension at 27°C in TNMFH medium (23) supplemented with 10% fetal bovine serum. Cells were infected at a multiplicity of infection of 10 with time zero set at the time of virus addition. Viruses used were: AcMNPV (Strain E2), ΔFP25K (2, 20), and the recombinant viruses
importin-α-16-T7\(_{\text{polh}}\), E66-His\(_{\text{polh}}\), E66-SM\(_{\text{polh}}\). Sf9 microsomes were prepared from 33 h p.i. infected cells using techniques described in (24) and (18).

**Generation of crosslinking cassette sequences for the multiple orfs.** The sequence of the lysine free portion of the crosslinking cassette containing the T7 epitope and His\(_7\) sequence was previously reported (8). The sequence encoding the linking amino acid sequence GANA includes a unique Nar\(_1\) site and this site was used to insert annealed, complementary synthetic oligonucleotides encoding the N-terminal INM-SM sequence of each of the open reading frames into the cassette located in the in vitro translation vector pGEM4Z. All gene constructs were sequence confirmed.

**SDS-PAGE and Western Blot Analyses.** Protein concentrations of microsomal membranes were determined by the method of Bradford (4) and all lanes were loaded at equal protein concentrations. SDS-PAGE was performed according to Laemmli (17) using a 4% stack and 15% separating gel. Samples were incubated in 1.5% SDS, 0.5% β-mercaptoethanol, 25 mM Tris-HCl (pH 6.8) and 7% glycerol for 15 min at 65°C.

Following electrophoresis, the gels were transferred onto Immobilon-P (Millipore, Bedford, MA). The membranes were blocked with TTBS-BLOTTO (150 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 8.0, supplemented with 1% nonfat dry milk,). Antibody was bound overnight (4°C), blots were washed twice with TBS and horseradish peroxidase-linked IgG (1:10,000) was bound for 1 hr at room temperature. Blots were washed three times with TTBS, reacted for 1 min with ECL (Amersham, Arlington Heights, IL) chemiluminescence reagent and exposed to X-ray film. The following antibodies and dilutions were used: anti-FP25K, No. 2804, (1:5000); anti-T7 (Novagen, No. 69522; 1:5000); anti-E26 No. 7554, [1:10,000 (9)].
**Immunofluorescence confocal microscopy.** Cells were prepared for microscopy as adapted from (10). Cells were collected, resuspended in Graces, and 2.8 x 10^5 cells were transferred to a 1 well cytofuge container (Statspin Technologies, Norwood, MA.). After allowing attachment at room temperature for five minutes, the cells were fixed with 3.7% paraformaldehyde (made in PBS; room temperature) for 10 minutes. The cells were washed 3 times with PBS, incubated with methanol for 10 minutes, washed, incubated with Triton X-100 (0.5%; made in PBS) for 10 minutes at RT and washed. The cells were incubated in blocking solution (1% porcine serum, 3% bovine serum albumin in PBS) for 1 hour at RT. Primary antibodies were diluted in blocking solution and the incubated at 4°C. The cells were washed with PBS and incubated with secondary antibody (Alexa Fluor conjugates, Molecular Probes, Inc., Eugene, OR) diluted at 1:2000 in blocking solution for 2 hours at RT. The cells were washed again 3 times. When DNA staining was required, the cells were incubated with DAPI (4’, 6’ diamidino-2-phenylindole) diluted at 0.1 µg/ml in PBS for 5 seconds and washed 3 times in PBS. Ten microliters of DAKO Fluorescent Mounting Medium (DAKO Corporation, Carpinteria, CA) was added and covered with a cover slip (No. 1.5). Slides were viewed using a Zeiss Axiovert 135 (Carl Zeiss MicroImaging, Inc. Thornwood, NY) with CARV confocal module (Atto Bioscience, Rockville, MD). After viewing at least 20 fields, representative cells/fields were collected using either the CARVer software (Atto Bioscience), or Zeiss Axiovision v. 3.1 (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Confocal sections were collected at 0.75 µm intervals. The following antibodies and dilutions were used: E26, No. 7554 (1:5000) and anti-T7, (Novagen, No. 69522; 1:5000).
**Chemical Crosslinking and Immunoprecipitation.** *In vitro* translations were performed in the presence of appropriate mRNA, nuclease treated rabbit reticulocyte lysate (Promega), amino acid mixture minus methionine (Promega), RNasin (Promega), approximately 8 equivalents of microsomes and [³⁵S] Met (8; 24). Following translation, samples were pooled and then subsequently re-aliquoted so that all treatments were performed on identical samples. Membranes were sedimented through a 0.5M sucrose cushion in a Beckman Coulter Optima TLA ultracentrifuge at 4°C for 3 mins at 100,000 rpm in a TLA 100 rotor. The resulting membrane pellet was resuspended in 50µl of crosslinking buffer (25mM Na-phosphate, 150mM NaCl, pH 7.0), one sample representing the non-crosslinked control was put aside while the remaining samples were treated with the crosslinking reagent Bis (sulfosuccinimidyl) suberate, (BS³; 2.5 mM; Pierce). Samples were incubated at RT for 30 mins. **TALON Purification:** The crosslinked samples were solubilized [4M urea, 0.5% (w/v) SDS; 30 min, 37°C], and incubated with 20µl of Dynabeads TALON per manufacturers instructions (Dynal Biotech). The bound material was eluted using SDS-sample buffer and resolved using 15% SDS-PAGE. **Immunoprecipitation:** The crosslinked samples were solubilized with RIPA buffer (50 mM Tris, pH 8.0; 100 mM NaCl; 1% NP-40; 1% desoxycholate; 0.1% SDS) and incubated with primary antibody overnight (4°C) followed by incubation with protein A/G agarose (Sigma; 2 hrs, 4°C). The protein A/G-bound complexes were recovered by sedimentation, washed, separated using SDS-PAGE (15%), and visualized using x-ray film.

**RESULTS**
Experimental Rationale and Design: An advantage of using viral models to study cellular events is that viruses often provide multiple unique protein markers sorted to the same destination. A comparison of the molecular events that control the sorting of these viral proteins destined for the same cellular location can collectively reveal general features of a common trafficking pathway. In addition, the use of baculovirus and recombinant viruses thereof, provides the opportunity to selectively increase the quantity of proteins targeted to the INM and intranuclear membranes vesicles derived from the INM. In this manner, the stoichiometric ratios of INM-directed proteins can be increased relative to the quantity of sorting factors present in the cell (or vice versa). Considering that microsomal membranes are easily prepared from infected or uninfected Sf9 cells, the effects that such alterations in protein expression have on other proteins associated with the ER membrane can be detected. Finally, the use of recombinant baculoviruses can reveal cellular destination(s) of uncharacterized proteins. Considering that much is known about the destination of ODV envelope proteins and intermediates states of ODV envelopment (6), we can compare the cellular destination of known ODV envelope proteins with that of the less well characterized protein, importin-α-16. In this way, we can predict if importin-α-16 disassociates from the ODV protein on the cytoplasmic face of the nuclear envelope and is released into the cytoplasm, or continues through the pore complex to the INM.

Proximity with the INM-SM-like sequences and FP25k or E26 is not consistent:

Perusal of the AcMNPV genome reveals eight genes that encode proteins containing N-terminal domains similar to the targeting sequence identified from E66 (Fig. 1; highlighted). With the exception of orf 91 (which is still uncharacterized), all of these
orf’s encode ODV envelope proteins (6). To test if these sequences utilize molecular events similar to those described for E66, constructs were made that allowed chemical crosslinking analyses similar to that previously performed for the E66-derived INM-SM sequence. Each of the viral orf sequences had the appropriate positively charged amino acid in the N-terminal region replaced with a lysine, and this sequence was fused to a cassette that contained a T7 epitope, a lysine-free sequence of amino acids and a C-terminal His\textsubscript{7} tag [Fig. 1; the cassette sequence is fully detailed in (8)]. Thus, each fusion protein has an approximate mass of 15-18 kDa and the lysine(s) flanking the hydrophobic sequence are the only amino acid(s) available as a substrate for the chemical crosslinking reagent BS\textsuperscript{3} (11.4-Å linking arm; lysine-lysine crosslinker).

To determine if these N-terminal INM-SM-like sequences crosslinked with any protein, each construct was translated and radiolabeled \textit{in vitro} in the presence of microsomal membranes prepared from AcMNPV infected cells and exposed to the chemical covalent crosslinking reagent BS\textsuperscript{3}. To enrich the INM-SM containing bait protein and potential crosslinked protein complexes, the samples were bound to His-binding TALON beads (shown schematically in Fig. 2a), and the bound protein or covalently linked protein complex was separated using SDS-PAGE. When the samples were analyzed, crosslinked adducts with similar molecular mass were detected for each of the fusion proteins except that of orf 91 (Fig. 2b; lanes 2,4,6,8,10,12,14,16; •; orf 22 is difficult to observe in this experiment and the crosslinked adduct is detected more clearly in Fig. 3g). Repeated trials testing the ability of orf91 to crosslink with another protein failed to detect a crosslinked adduct and since orf91 is uncharacterized, it was not pursued further.
To determine if the identity of the crosslinked protein was either E26 or FP25K, immuno-precipitation experiments using antiserum to these proteins were performed (Fig. 3a; ii and iii). For E26, two well-characterized antibodies are available. One of these (#7554) only recognizes viral E26 and does not cross-react with the cellular protein importin-α-16 (9). This antibody was used throughout. As a control, enrichment of the crosslinked adduct using TALON beads was repeated (Fig. 2a) along with the immunoprecipitation protocol using antibody to the T7 epitope (Fig.3a; i). A control sample (not exposed to BS$_3^3$) was bound to TALON beads to determine the background binding of non-crosslinked proteins and normal rabbit serum was used to determine background reactivity for the immunoprecipitation experiments. We note that when the fusion-cassette is exposed to BS$_3^3$, some portion of it non-specifically precipitates with normal rabbit serum. So in these lanes, instead of seeing a blank lane, it is routine to see some amount of bait protein and if the bait has crosslinked with an adduct, a trace amount of the adduct may also be visible. We have no explanation for why the chemically treated cassette-bait sequence has a tendency to precipitate with random IgG’s, but this result has been reported previously using multiple fusion proteins exposed to chemical crosslinking reagents (7, 22).

The results from orf’s 96, 119, 83 and 115 (Figs. 3b, 3c, 3d and 3e, respectively) showed similar results. Each of the fusion cassette sequences crosslinked with a protein that was enriched on the His-binding TALON beads and precipitated using T7 antibody (Figs. 3b, 3c, 3d and 3e; lanes 2 and lane 6; ●). The crosslinked adduct for each of these proteins was easily detected when precipitated using either E26 or FP25K antibodies (Fig. 3b, 3c, 3d and 3e; lanes 4 and 5; ★). These data suggest that the INM-SM cassette
sequences of orf’s 96, 119, 83 and 116 independently associates with both of the viral proteins FP25K and E26 while they are located in the ER membrane of the infected cell.

The data obtained from the fusion cassette sequences of orf’s 150 and 22 also showed that these proteins crosslinked with a protein adduct (Fig. 3f and 3g; lanes 2 and 6 respectively; •) and antibody to FP25K convincingly precipitated the crosslinked adduct (Fig. 3f and 3g; lane 4; ⭐). However, E26 antibody precipitated a crosslinked adduct that was discernable but barely increased relative to the control lane using normal rabbit IgG (Figs. 3f and 3g; compare lanes 3 and 5; ⭐). This result was consistent and reproducible. So while the crosslinking data shows that orf’s 150 and 22 are in close proximity to FP25K, an interaction with E26 is less certain.

**Quantity of FP25K, E26 and importin-α-16 in the ER membrane is dynamic:** It is possible that the abundance of either E26 or FP25K associated with the ER membrane is determined by the quantity of ODV envelope protein in transit (i.e., E26 and FP25K are recruited to the ER membrane when demand for sorting of INM-directed proteins is increased). Considering that cellular importin-α-16 is clearly interacting in the pathway of INM-directed protein trafficking, its concentration in the ER membrane may also affect, or be affected by events of virus infection. To test this, microsomal membranes were prepared from recombinant virus infected cells that either had increased amounts of INM-directed proteins expressed or that had altered expression of the sorting factors importin-α-16 or FP25K. For comparison, control microsomal membranes were prepared from uninfected or AcMNPV infected Sf9 cells. Two recombinant viruses were used that abundantly expressed INM-directed proteins; one of these had increased quantities of the viral ODV envelope protein E66 [tagged with a C-terminal His7]
sequence (E66HIS)], while the second increased the quantity of the E66 derived INM-SM cassette [E66SM (Fig. 4a)]. Several recombinant viruses were used that altered the expression levels of the sorting factors, FP25K and importin-α-16. Recombinant virus was generated that abundantly expressed a T7-epitope tagged version of Sf9 importin-α-16 (Fig. 4a), while the affects of deleting the sorting factor FP25K was studied using a ΔFP25K deletion recombinant virus (2, 20). While it would be desirable to include a virus with $E26$ deleted, such is not possible because $E26$ is essential (9). Microsomal membranes were prepared from cells infected at equivalent multiplicities of infection and harvested at 33 h p.i. Approximately equal quantities of microsomal membranes were analyzed using SDS-PAGE (Fig. 4b). The microsomal membranes were western blotted and reacted against various antisera.

When the membranes were tested using T7 antibody, tagged importin-α-16 was detected in total cell lysates and microsomal membranes prepared from virus expressing importin-α-16-T7 (Fig. 4c; lanes a and 4, respectively). Using the T7-epitope specific antibody, no non-specific bands were detected in any of the microsomal membrane preparations (Fig. 4c; lanes 1-3 and 5-6). When the microsomes were tested for presence of FP25K, FP25K was clearly detected in total cell lysate and in microsomal membranes prepared from AcMNPV infected cells (Fig. 4d; lanes a and 2 respectively). As expected, FP25K was not detected in microsomes prepared from ΔFP recombinant virus infected cells (Fig. 4d; lane 3). The relative amount of FP25K was increased in microsomes prepared from cells infected with viruses in which the quantity of the INM-directed proteins E66 or the E66 derived INM-SM-fusion cassette was increased (Fig. 4d; compare lane 2 with lanes 5 and 6). When the membranes prepared from cells infected
with the recombinant virus expressing increased quantity of Sf9 importin-α-16 were analyzed, the amount of FP25K decreased to undetectable levels (Fig. 4d; lane 4), suggesting that importin-α-16 could be: i) displacing FP25K or nullifying the need for FP25K within the microsomal membranes by providing an equivalent function; or ii) interfering with another protein interaction responsible for FP25K membrane association.

E26 was easily detected in the total cell lysate and microsomal membranes prepared from AcMNPV infected cells (Fig. 4e; lanes a and 2). The relative amount of E26 within the membranes does not appear to be greatly influenced by the presence or absence of FP25K; in ∆FP25K microsomes, E26 was present in quantities approximately equal to AcMNPV infected cells (Fig. 4b and 4e; compare lanes 2 and 3) and when FP25K itself was expressed at high levels, the levels of FP25K or E26 did not substantially increase in the microsomal membranes (this experiment was performed as an independent data set and is not shown here). An increase in the quantity of E26 was detected in microsomes prepared from the recombinant virus abundantly expressing the E66-SM cassette, but a much larger increase was detected in membranes prepared from cells generating abundant copies of full-length E66 (Fig. 4e; lanes 6 and 5 respectively). The quantity of E26 was also increased in membranes prepared from cells infected with virus expressing abundant quantities of Sf9 importin-α-16 (Fig. 4e; lane 4).

**E26 and importin-α-16 are targeted to the same cellular destination during infection:** While we know that cellular importin-α-16 associates with the INM-directed sequence at the time of translation and remains with the protein after membrane integration, we have no insights into the time of dissociation of importin-α-16 with the INM-directed protein. If it disassociates from its cargo prior to translocation across the
pore membrane, then one would expect to detect it within the cytoplasm. A very strong
case can be made for the conclusion that the INM serves as a precursor for the virus
induced intranuclear membranes (6). So, if importin-alpha-16 is detected within the foci
of intranuclear microvesicles, then it must have utilized the INM during its transit to
these membranes. ODV-envelope proteins are known to be enriched within the virus
induced intranuclear membranes, so use of a recombinant virus expressing a epitope
tagged version of importin-α-16 and co-localization studies using ODV-envelope marker
proteins provides a straight forward assay to determine if importin-α-16 passes the pore
membrane to the INM. E26 was chosen as the ODV-envelope protein marker for the
intranuclear membranes (3) and recombinant virus expressing importin-α-16-T7 was
used for infection and confocal analysis. Perusal of many cells showed that E26 and
importin-α-16 were targeted to the same regions within the infected cells (Fig. 5a, b).
These sites include membranes at the periphery of the nucleus, and foci of microvesicles
that accumulate within the infected cell and are predicted to serve as precursors of the
ODV envelope (6). The trafficking of importin-α-16 to the intranuclear membranes is
not random, when the mammalian INM protein lamin B receptor [N-terminal region
through transmembrane sequence 1-fused to GFP-described in (11)] was abundantly
expressed in recombinant virus infected Sf9 cells, lamin B receptor remained at the
periphery of the nucleus and only minor amount of protein can be detected in the virus-
induced intranuclear membranes (Fig. 5c, d). This pattern of localization is clearly
different from the ER/ONM localization that is detected for another LBR construct that
has the INM-SM sequence deleted [Fig. 5e-f; construct is described in (7)]. These results
suggest that during infection, importin-α-16 remains with the ODV envelope protein
from its time of membrane integration in the ER membrane, throughout its transit to the INM and the virus-induced intranuclear microvesicles.

**DISCUSSION**

Previous studies deciphering the molecular mechanisms of sorting and trafficking of INM-directed proteins predict a common pathway. For both viral and cellular INM-directed proteins, this pathway is mediated in part by a cellular protein belonging to the importin-α family (Sf importin-α-16 or human KPNA4-16). In AcMNPV infected cells, two viral proteins, E26 and FP25K, participate in this pathway and both of these proteins function in association with the INM-SM sequence after the INM-directed protein has been integrated into the ER membrane and released from the translocon (21). The data set used to decipher this molecular pathway is still relatively small and includes several viral ODV envelope proteins (E66, E25, INM-SM sequence fusion proteins) and the cellular proteins lamin B receptor and nurim (7). An important goal of this study was to determine critical features of this common pathway by expanding the data set to include additional INM-directed proteins. Since addressing this question using mammalian cells or in vitro approaches is difficult, we turned once again to the baculovirus to provide the necessary insights. As a first approach, multiple ODV envelope proteins, each containing a characteristic INM-SM like sequence were tested for their ability to crosslink with the viral sorting factors FP25K and E26. All the known ODV envelope proteins containing an N-terminal, INM-SM-like sequence crosslinked with FP25K, suggesting that FP25K provides a very important activity during this sorting pathway.

So what is known of the function of FP25K in the INM-directed protein sorting process? Like cellular importin-α, most of the FP25K protein in the cell is soluble and...
only a very small proportion of the total protein associates with cellular membranes. The function of the soluble FP25K is unknown, however membrane associated FP25K has been implicated as a protein that facilitates the transit of ODV envelope proteins from the outer to inner nuclear membrane. When FP25K is absent, instead of the rapid transit of E66 to intranuclear membrane vesicles, E66 accumulates in punctate regions in the ONM and is not detected at the INM. Only after prolonged infection can E66 be detected within the intranuclear membranes (5, 20). When another ODV envelope protein, E25 is studied in ΔFP25K infected cells, its trafficking to intranuclear membranes is also delayed and E25 slowly accumulates in the INM, where it aggregates in punctate clusters (20). Thus the efficiency and rate of trafficking of the envelope proteins E66 and E25 are substantially affected in the absence of FP25K; and the transit across the pore membrane to the INM appears to be the step most impaired. However, the observation that a small amount of these proteins eventually transit to the intranuclear membrane vesicles in the absence of FP25K suggest that other proteins may mediate this process and the activity of these other proteins are sufficient to retain production of viable virus. As such, other proteins (cellular or viral) may provide redundant activity to that normally provided in optimal levels by FP25K. Analysis of the dynamic interactions of sorting factors within microsomal membranes show that when importin-α-16 is abundantly present in ER membranes, FP25K is no longer recruited to these membranes. This result suggests that when additional copies of importin-α-16 are present in the ER membrane, FP25K may no longer be required. Importin-α-16 then, may be a reasonable candidate to provide the redundant function for FP25K. If that is true, and FP25K facilitates the transit of INM-directed proteins across the pore membrane to the INM, then one would predict that...
importin-α-16 might also perform such a function. Co-localization analysis using the known ODV-envelope protein E26 and importin-α-16 show that importin-α-16 is trafficked to the nuclear envelope and the virus-induced intranuclear membrane during viral infection. As such, importin-α-16 is appropriately positioned to have functional activity during protein translocation across the pore membrane to the INM.

The data suggest that multiple levels of regulation facilitate INM-directed protein trafficking and that proteins participating in this sorting pathway have a dynamic relationship with each other. The association of FP25K, E26 and cellular importin-α-16 with the ER membrane is not static, but rather their presence at the ER membrane appears to be actively recruited and based upon requirements of the INM-directed protein in transit. While presence of increased amounts of importin-α-16 in the ER membrane results in the displacement of FP25K, the opposite relationship is observed between importin-α-16 and E26. When levels of importin-α-16 are increased in the ER membrane, there is a concomitant increase in the levels of E26. While these results suggest a synergistic relationship between E26 and importin-α-16, they do not provide substantial new insights into the functional activity of E26 in the infected cell. The complexity of these observations suggest that while there are common mechanisms within the pathway of directing proteins to the INM, individual INM-directed proteins may have specific requirements, and multiple proteins facilitate and direct each stage of integral membrane protein movement through their passage from the ER membrane to the INM.

There is always the impulse to conclude a study of a molecular sequence of events by drawing a simple diagram to explain them. However, repeatedly such models expand
until each pathway becomes a complex series of precisely positioned and interacting proteins that are not only responsive to the elements in the main pathway but also sensitive to the environment and global requirements of the cell. It appears that the molecular pathway of INM-directed proteins will ultimately prove to be such a complicated and multi-faceted pathway. Current data suggest that sorting of INM-directed proteins begins during translation and interaction of the nascent chain with the translocon; and translocon or ER-associated proteins are sensitive to the cellular requirements for such sorting. Sorting continues once the protein has been integrated into the ER and released from the translocon; and studies using baculovirus suggest that multiple sorting factors facilitate this process. As the INM-directed protein translocates across the pore membrane to the INM additional sorting events occur and in baculovirus infected cells, FP25K has a functional role during this event. Considering that viruses are regional experts in the molecular pathways of their host cell, and that insights derived from the study of baculovirus infected cells thus far can be correlated with similar events in human cells, one can only expect that continuing insights derived by study of the baculovirus will provide substantial steps forward to our understanding of the molecular events that occur during sorting and trafficking of INM-directed proteins to the INM in mammalian cells.

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REFERENCES:


21. **Saksena, S., Y. Shao, S. C. Braunagel, M. D. Summers and A. E. Johnson.**


22. **Saksena, S., M. D. Summers, J. K. Burks, A. E. Johnson and S. C. Braunagel.**


**Figure Legends:**

**Figure 1.** *INM-SM cassettes used for crosslinking experiments.* The N-terminal amino acids of each orf encoding an ODV-envelope protein and containing the features of the INM-SM sequence are shown. The characterized INM-SM sequence derived from E66 is shown for reference (highlighted). Each N-terminal sequence was fused to the same cassette as that shown for E66 containing the amino acids GANA as linker sequence followed by a T7 epitope, lysine free sequence and C-terminal His\(^7\) tag. The hydrophobic sequences are highlighted in yellow, and the associated positively charged amino acid(s) mutated to a lysine to serve as bait for the BS\(^3\) crosslinking reagent is noted above the sequence in dark red. All of the resultant fusion proteins have a molecular mass of 15-18 kDa.

**Figure 2.** *Crosslinking of ODV-envelope protein cassettes to proteins within microsomal membranes.* **a.** Schematic of the experimental protocol utilized for chemical crosslinking assays. After *in vitro* translation and generation of a radiolabeled protein, the sample was treated with BS\(^3\), the microsomal membranes were denatured and the bait cassette or covalently linked associated complex was enriched using His-binding TALON beads. The enriched samples were separated using SDS-PAGE. (O) represents uncrosslinked control; (X) represents enriched crosslinked samples; (●) notes the crosslinked adducts. No detectable crosslinked adduct was detected for orf 91 but the expected site of an adduct comparable to that detected for the other orf’s is noted with a question mark.

**Figure 3.** *Crosslinking of ODV-envelope protein cassettes to FP25K and E26.*
**Figure 4. Association of E26, FP25K and importin-α-16 with enriched ER membranes.**

a. Schematic of recombinant viruses used for infection and preparation of *in vitro* translation competent microsomal membranes. In every case, the recombinant gene was inserted into the polyhedrin gene locus under the control of the polyhedrin promoter.  

b. Coomassie blue stained SDS-PAGE gel showing that the microsomal preparations were loaded at approximately equal concentration.  

c-e. The SDS-PAGE separated gels were blotted and analyzed using various antisera [c- antibody to T7 epitope; d- FP25K antibody; e- E26 antibody]. In c-e, lane a shows the control from a total cell lysate obtained from cells infected with the relevant virus.  

Lanes 1-6 represent microsomal membranes prepared from (1) control Sf9 cells, cells
infected with (2) wild type AcMNPV, (3) ΔFP25K virus, (4) importin-α-16-T7 virus, (5) E66HIS virus, (6) E66-SM virus.

**Figure 5.** *Confocal microscopy showing co-localization of E26 and importin-α-16 in infected Sf9 cells.* a-b. Cells were infected with the recombinant virus expressing importin-α-16-T7 (as described in Fig. 4a). At 33 hours post infection cells were harvested and prepared for confocal microscopy. E26 was visualized using primary antibody to E26, importin-α-16-T7 was detected using antibody specific for the T7 epitope, and DNA (thus overall location of the nucleus) was visualized using DAPI. Single Z-sections representing 0.75μm slices are shown. c-d. Cells were infected with recombinant virus expressing a lamin B receptor (LBR) construct consisting of the N-terminal region of LBR through transmembrane sequence 1 fused to GFP (11). Cells were harvested at 33 hours post infection and prepared for confocal microscopy. The nuclear region was detected using DAPI and LBR was detected using GFP autofluorescence. LBR remains at the periphery of the nucleus. e-f. Cells were infected with recombinant virus expressing a lamin B receptor (LBR) construct consisting of the LBR transmembrane sequence 1 with the INM-SM-like sequence deleted, fused to GFP (7). Cells were harvested at 33 hours post infection and prepared for confocal microscopy. The nuclear region was detected using DAPI and LBR was detected using GFP autofluorescence. When the INM-SM sequence of LBR is deleted, the protein orients in the membrane incorrectly (7) and now locates throughout the ER.
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<th>ODV-E66 (79 kDa)</th>
<th>MSIVLIIVVIFLICFLYLNSNNSNNKDNANKNNAFIGANA</th>
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a. Recombinant virus microsome preparations

E66-His

ODV-E66

His

d

polh

E66-SM

E66-SM

Lysine free cassette

His

d

polh

Sf9-α-16-T7

Sf9 importin-α-16

T7

d

polh

b. microsomes
c. microsomes
d. microsomes
e. microsomes