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Three-dimensional visualization of the *Autographa californica* multiple nucleopolyhedrovirus occlusion-derived virion envelopment process gives new clues as to its mechanism

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ABSTRACT

Baculoviruses produce two virion phenotypes, occlusion-derived virion (ODV) and budded virion (BV). ODV envelopment occurs in the nucleus. Morphogenesis of the ODV has been studied extensively; however, the mechanisms underlying microvesicle formation and ODV envelopment in nuclei remain unclear. In this study, we used electron tomography (ET) together with the conventional electron microscopy to study the envelopment of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) ODV. Our results demonstrate that not only the inner but also the outer nuclear membrane can invaginate and vesiculate into microvesicles and that intranuclear microvesicles are the direct source of the ODV membrane. Five main events in the ODV envelopment process are summarized, from which we propose a model to explain this process.

Introduction

Baculoviruses are enveloped, rod-shaped, large, closed circular DNA viruses, with nucleocapsids of 250–300 nm in length and 30–60 nm in diameter (Jehle et al., 2006). The hosts in which the baculoviruses are commonly found are the orders *Diptera*, *Hymenoptera* and *Lepidoptera*. During the baculovirus life cycle, two virion phenotypes are produced, namely, budded virion (BV) and occlusion-derived virion (ODV). In these two phenotypes, genetic information and the nucleocapsid proteins are almost identical, and both DNA replication and nucleocapsid assembly of the two phenotypes occur in the virogenic stroma (VS) (Fraser, 1986; Young et al., 1993). However, the origin of envelopes and the envelope composition differ between the two phenotypes (Braunagel and Summers, 1994; Hou et al., 2013). At about 12 h post-infection (p.i.) (Slack and Arif, 2007), nucleocapsids shuttle out of the nucleus and bud through the plasma membrane and, in this way, BVs are enveloped (MacKinnon et al., 1974; Summers and Volkman, 1976). After approximately 20 h p.i. (Slack and Arif, 2007), ODVs form as nucleocapsids are transported to the region between the nuclear membrane and the VS, termed the ring zone (RZ), enveloped, and embedded within a proteinaceous crystal matrix, forming polyhedra or occlusion bodies (Williams and Faulkner, 1997). Based on the number of nucleocapsids within the ODV envelope, baculoviruses can be categorized as single nucleopolyhedroviruses (SNPVs) or multiple nucleopolyhedroviruses (MNPVs). Notably, there is only one nucleocapsid in the BVs of both SNPVs and MNPVs. Once eaten by larvae, ODVs will be released from the occlusion bodies in the alkaline environment of the larval midgut and initiate infection in the midgut epithelium. On the other hand, BVs can bud from infected cells without cell disintegration and initiate the systemic infection of other susceptible tissues (Engelhard et al., 1994; Tanada and Hess, 1976).

The process of BV envelopment at cell surface is similar to other viral envelopment processes (Welsch et al., 2007); however ODV envelopment in nuclei is common only in baculovirus. In early morphogenesis studies of ODV envelopment by transmission electron microscopy (TEM), viral-induced intranuclear microvesicles and membrane fragments were found in nuclei, and nucleocapsids were bound to them (Fraser, 1986; Kawamoto et al., 1977; Stoltz et al., 1973). Moreover, the inner nuclear membranes (INMs) of infected cells bleb fold in and vesiculate (Tanada and Hess, 1976). Fluorescence and immuno-gold labeling of ODV envelope proteins have

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been performed using confocal microscopy and immuno-electron microscopy, respectively. This identified ODV envelope proteins including ODV-E66 (Hong et al., 1994), ODV-E56 (Braunagel et al., 1996), ODV/BV-E26 (Beniya et al., 1998), and Ac76 (Hu et al., 2010; Wei et al., 2014) on the nuclear membranes of infected cells, microvesicles or nucleocapsids associated with membrane fragments, and ODV envelopes. Although phospholipid analysis found distinct differences between ODV envelopes and uninfected Sf9 nuclear membranes (Braunagel and Summers, 1994), a semi-permeabilization assay demonstrated that the cholesterol concentration increased in infected Sf9 cell nuclear envelopes and approached that in the ODV envelope (Braunagel and Summers, 2007). This is considered as that baculovirus infection induces lipid remolding of the nuclear envelope. Taken together, the aforementioned protein positioning and lipid analyses imply that nuclear membranes are the source of ODV envelopes.

However, no direct observation or evidence of a relationship among the nuclear envelope, virus-induced intranuclear microvesicles, and the ODV envelope has been reported. Furthermore, no systematic model has been proposed to explain how the microvesicles envelope nucleocapsids.

In this study, we performed electron tomography (ET) combined with sectioning of plastic-embedded samples to visualize microvesicle formation and ODV envelopment in *Autographa californica* MNPV (AcMNPV), the most widely studied Baculoviridae species, in three dimensions (3D). Based on two-dimensional (2D) observations and visualization of specific areas of interest in 3D, we summarize ODV envelopment and provide a model to explain how the numerous nucleocapsids are enveloped. Moreover, based on our observation, we propose an optional way for microvesicles formation that microvesicles can form via large-scale invagination of double nuclear membranes and vesiculation into single-membrane vesicles.

Results and discussion

We examined more than 300 cells at 24, 36, 48, and 72 h p.i. by conventional TEM and ET. Based on these data, we deduced a model (Fig. 1) to explain how microvesicles form and ODVs are enveloped. Microvesicles are from the invaginated nuclear membrane and the vesiculated nuclear membranes then interact with and envelop nucleocapsids. Thereafter, membranes invaginate and nucleocapsids are separated into different ODVs such that all nucleocapsids in a given ODV are oriented parallel to each other. The various steps of this process are described below. None of these features were observed in non-infected cells.

Formation and maturation of the microvesicles

Among all our data at different time points post-infection, we seldom captured the clear images of invaginating INMs, which is a widely accepted model to explain the origin of microvesicles (Braunagel and Summers, 2007) (labeled as steps A2 and B2 in Fig. 1). However, surprisingly, at 24 and 36 h p.i., not only the INM but also the outer nuclear membrane (ONM) invaginate into the nuclei of infected cells (Fig. 2A–C). Some parts of the inward-folded nuclear membrane form vesicle-like structures (boxed in Fig. 2B). The ONM (indicated by black arrows in Fig. 2B) is noticeably prone to form the vesicle-like structures compared to the INM (indicated by red arrows in Fig. 2B). This process is labeled step A1 in Fig. 1. Moreover, large pieces of intranuclear membranes (Fig. 2D–F) are found within the nuclei in 24 and 36 h p.i. This is labeled steps B1 and C1 in Fig. 1. These intranuclear membranes exhibit vesiculation and are double membranes reminiscent of the invaginating nuclear membrane. This suggests that these intranuclear membranes are derived from the invaginated nuclear membranes. Furthermore, it is

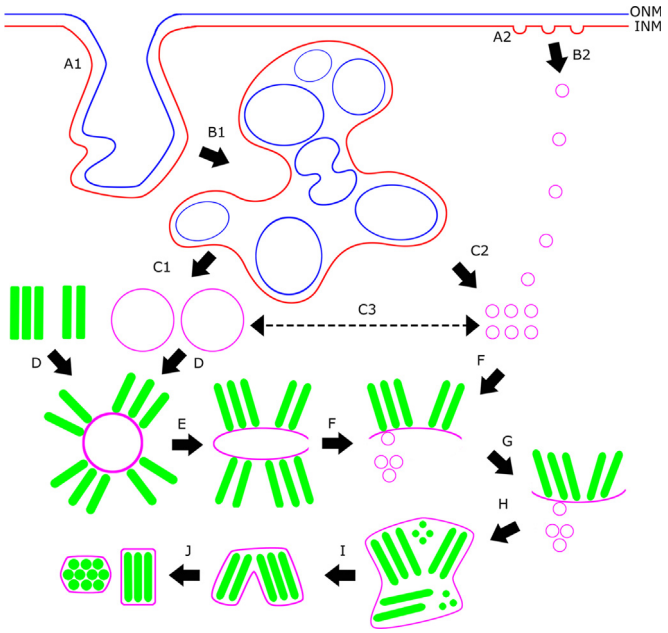

Fig. 1. Schematic model of microvesicle formation and occlusion-derived virion (ODV) envelopment. The outer nuclear membrane (ONM) is in blue, the inner nuclear membrane (INM) in red, large microvesicles, small microvesicles, and ODV membranes in purple, and nucleocapsids in green. (A1) The nuclear membranes (both ONM and INM) fold inwards. Part of the invaginating nuclear membrane sags, especially the ONM. (A2) Only the INM is invaginating. (B1) The invaginated intranuclear membrane vesiculates, and vesicles appear earlier from the ONM than from the INM. (B2) Small microvesicles are formed by invaginating INM. A1 and B1 are the steps based on our data. A2 and B2 are the widely accepted microvesicle formation pathway (Braunagel and Summers, 2007). (C) After a series of complicated steps, intranuclear double membranes can form several single-membrane vesicles that can be categorized as large (C1) and small (C2) microvesicles. They may transform to one another (C3) by fusion and fission. (In the data of our research, we could not validate this process, therefore, we use dashed line to show the C3 step.) (D) Several nucleocapsid bundles adhere to large microvesicles (C1). (E) As more nucleocapsids adhere, the microvesicle deformed and ruptured. Thereafter, the expanding gaps cause the membranes of microvesicles with nucleocapsids attach to become arch-shaped. (F, G) Small microvesicles (C2) continuously fuse with these arch-shaped membranes such that they become large enough to accommodate all the nucleocapsids. (H) The initially formed ODVs contain several nucleocapsid bundles that are not oriented parallel to each other. Thereafter, the membrane invaginates (I) and these bundles separate to form new ODVs (J).

easy to find single-membrane vesicle-like structures (indicated by red arrows in Fig. 2F) are observed around vesiculating intranuclear membranes (indicated by black arrows in Fig. 2F). The diameters, electron densities of the these single-membrane vesicles, together with the thickness of membrane of these vesicles are similar to those of the microvesicles, which are abundant in infected Sf9 cells and are generally accepted to be the origin of ODV membranes. From these results, we infer that microvesicles are derived not only from the INM but also from the ONM. We have summarized the process in a model, comprising steps A1, B1, C1 and C2 (Fig.1). We do not find any of these patterns in non-infected cells.

In previous studies using immuno-gold labeling, ODV membrane proteins were detected on both the INM and ONM (Braunagel et al., 1996; Hong et al., 1997), which does not conflict with our finding that the membranes of microvesicles are derived from both the INM and ONM. Therefore, our model (steps A1 and B1 in Fig. 1) can be an alternative pathway to the previously proposed INM invagination model (steps A2 and B2 in Fig. 1).

Several studies have identified ODV envelopment-related genes. *ac142* (McCarthy et al., 2008) and *ac103* (Yuan et al., 2008) are related to this envelopment, but do not affect nucleocapsid bundling. *ac76* (Hu et al., 2010; Wei et al., 2014) and *ac93* (Yuan et al., 2011) are related to intranuclear microvesicle formation and subsequent ODV