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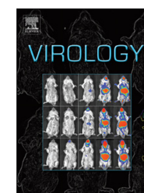

Fine structure of the vaccinia virion determined by controlled degradation and immunolocalization

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ABSTRACT

The vaccinia virion is a membraned, slightly flattened, barrel-shaped particle, with a complex internal structure featuring a biconcave core flanked by lateral bodies. Although the architecture of the purified mature virion has been intensely characterized by electron microscopy, the distribution of the proteins within the virion has been examined primarily using biochemical procedures. Thus, it has been shown that non-ionic and ionic detergents combined or not with a sulfhydryl reagent can be used to disrupt virions and, to a limited degree, separate the constituent proteins in different fractions. Applying a controlled degradation technique to virions adsorbed on EM grids, we were able to immuno-localize viral proteins within the virion particle. Our results show after NP40 and DTT treatment, membrane proteins are removed from the virion surface revealing proteins that are associated with the lateral bodies and the outer layer of the core wall. Combined treatment using high salt and high DTT removed lateral body proteins and exposed proteins of the internal core wall. Cores treated with proteases could be disrupted and the internal components were exposed. Cts8, a mutant in the A3 protein, produces aberrant virus that, when treated with NP-40 and DTT, releases to the exterior the virus DNA associated with other internal core proteins. With these results, we are able to propose a model for the structure the vaccinia virion.

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Introduction

Poxviridae comprise a family of viruses characterized by the presence of a large dsDNA genome and a complex morphology (Condit et al., 2006; Moss, 2013). Poxviruses encode a complete transcription apparatus and thus are able to replicate in the cytoplasm of infected cells. Vaccinia virus (VACV), the prototype member of this family, encodes more than 200 proteins and the role of many virus proteins during the virus replicative cycle has been determined (Goebel et al., 1990; Moss, 2013). The protein composition of purified mature virions has been determined by mass spectrometry and at least 70 virus proteins have been identified (Chung et al., 2006; Matson et al., 2014; Resch et al., 2007; Yoder et al., 2006). Although the proteomic analysis has been important for the identification of the total protein content of the mature particle, the fine localization of a significant fraction of the virion proteins is still unknown. Membrane proteins and enzymes involved in early transcription have been assigned positions in the particle, but the location of the other proteins still needs to be determined.

Electron microscopy is the preferred method for studying the morphology of the VACV particle and various electron microscopic techniques have been applied in the visualization of the virus structure (Cyrklaff et al., 2005; Dales and Siminovith, 1961; Easterbrook, 1966; Harris and Westwood, 1964; Ichihashi et al., 1984; Muller and Peters, 1963; Naginton and Horne, 1962; Peters and Muller, 1963; Westwood et al., 1964; Wilton et al., 1995). Overall, poxvirus virions have an ellipsoidal, barrel or brick shaped appearance. Analysis of VACV on a whole mount preparation using negative staining of the particles revealed the presence of a membrane that enclosed two distinct virus sub-domains: the lateral bodies and the core (Dales, 1962; Harris and Westwood, 1964; Muller and Peters, 1963; Peters and Muller, 1963; Westwood et al., 1964). The lateral bodies, which flank the core, are amorphous structures composed of proteins of unknown function. The core is comprised of a proteinaceous wall that encloses a nucleocapsid (Condit et al., 2006). Analysis of VACV by cryo-microscopy and reconstruction using electron tomography revealed pore-like structures spanning the core wall (Cyrklaff et al., 2005). No function has been determined for this structure although it could be involved in the extrusion from the core of the viral mRNA during early transcription.

Using negative staining electron microscopy, the surface of the mature virion presents two different morphological forms that are directly related to the integrity of the particle. The predominant

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form in a fresh virion preparation contains on its surface rodlet-like structures called surface tubule elements, creating a mulberry-like appearance (Harris and Westwood, 1964; Muller and Peters, 1963; Naginton and Horne, 1962; Westwood et al., 1964; Wilton et al., 1995). Under various conditions, the negative stain can penetrate through the virus membrane so that the surface tubule elements are no longer apparent and the virus now exhibits a capsule-like form. When virions are exposed to high pH, the lateral bodies, core wall, and the nucleocapsid can be visualized (Muller and Peters, 1963).

Analysis of VACV by atomic force microscopy has permitted a more accurate determination of the dimensions of the virus particle, because measurements are obtained with fully hydrated virions (Malkin et al., 2003). Using this approach, the virus dimensions vary between 320 and 380 nm in the major axis and 260 and 340 nm in the minor axis, similar to measurements described by other methods (Cyrklaff et al., 2005; Griffiths et al., 2001; Malkin et al., 2003; Roos et al., 1996; Sodeik and Krijnse-Locker, 2002). However, the height of the hydrated virion varies between 240 and 290 nm, a measurement significantly higher than what is observed with dried sample but similar from the cryo-electron microscopy results.

Treatment of purified virions with a non-ionic detergent and a reducing agent solubilizes the proteolipid membrane complex exposing the core and the lateral bodies (Easterbrook, 1966; Ichihashi et al., 1984). The soluble and insoluble components of the virions can be separated by centrifugation and the protein content of each fraction can be identified by various techniques (Boyd et al., 2010; Chiu and Chang, 2002; Ichihashi et al., 1984; Kato et al., 2007; Nichols et al., 2008; Resch and Moss, 2005; Unger et al., 2008). Using this approach, proteins associated with the membrane fractionate into the soluble fraction whereas the insoluble fraction contains proteins from both the lateral bodies and core. Purification of the lateral bodies away from the core has not been achieved using the current methodologies.

In this work, our goal was to develop a method to locate VACV proteins in viral sub-domains. For this propose, we combined whole mount preparation of virions with immunogold labeling of the proteins. Virions attached to grids were subjected to various treatments in order to expose viral proteins located in the different sub-domains and permit their identification by immunogold labeling. Our results show after NP40 and DTT treatment, membrane proteins such as A14 and A27 are removed from the virion surface revealing proteins that are associated with the lateral bodies and the outer layer of the core wall. Combined treatment using high salt and high DTT removed lateral body proteins and exposed proteins of the internal core wall. Cores treated with proteases could be disrupted and the internal components were exposed. Cts8, a mutant in the A3 protein, produces aberrant virus that, when treated with NP-40 and DTT, releases to the exterior the virus DNA associated with other internal core proteins. With these results, we are able to propose a model for the structure the vaccinia virion.

Results

Negative staining of vaccinia virus

The establishment of a standardized procedure to visualize purified virions is an important step in the understanding viral structure (De Carlo and Harris, 2011). Poxviruses were one of the first mammalian viruses to be visualized using electron microscopic techniques. The structural analyses of purified VACV in whole mount preparations revealed a unique pattern that has been confirmed by various procedures (Griffiths et al., 2001; Harris and Westwood, 1964; Heuser, 2005; Malkin et al., 2003; Muller and Peters, 1963; Westwood et al., 1964; Wilton et al., 1995). To analyze the structure of purified VACV we used five different negative staining conditions (Fig. 1). Similar to previous work (Harris and Westwood, 1964; Muller and Peters, 1963; Peters and Muller, 1963), when virions were negative-stained with phosphotungstic acid (PTA) at pH 7.0 particles could be visualized in two different forms, the mulberry-like form and the capsule (Fig. 1A). While the mulberry form shows a rodlet-like structure surrounding the particle, the capsule form presents a defined frame that surrounds the internal structure of the virion. When the pH of the PTA solution was elevated to pH 10.5, only the capsule form was observed, presumably because the integrity of the virion membrane is compromised (Fig. 1B). Under these conditions, a tubular-like internal structure of the virion was revealed that represents the virus nucleocapsid (Muller and Peters, 1963). Virions in a whole mount preparation were also negative-stained with ammonium molybdate (AmMo), uranyl acetate (UA) and methylamine tungstate (NanoW) (Fig. 1C–E). Under all these conditions, particles with a mulberry appearance were the major form observed. When whole mount preparations of VACV were treated with the neutral detergent NP40 before negative staining, a different appearance of the particle was observed (Fig. 1F–J). In all preparations, because the lipid constituent of the virion membrane is removed allowing stain to penetrate the particles, only the capsule forms were observed and the core internal structure became apparent. It is noteworthy that after this treatment the nucleocapsid appears amorphous, different from what was observed when virions were stained with PTA at pH 10.5 (Fig. 1G). The nucleocapsid is surrounded by a thick, well-defined boundary layer and the presence of the lateral bodies could be observed (Fig. 1H). In some images, this boundary layer appears to be formed by two thinner layers (Fig. 1F, H, and J). The outermost layer is composed of a protein array that comprises proteins involved in virion attachment, the entry-fusion complex and structural membrane proteins. The internal layer includes the spike-like "palisade layer" and additional core wall proteins (Condit et al., 2006; Cyrklaff et al., 2005; Roos et al., 1996). The majority of the proteins present in the outermost (membrane) layer cannot be removed from the particle by NP40 treatment alone (Chiu and Chang, 2002; Nichols et al., 2008). With this treatment, the lipid component of the membrane is extracted and virions lose their infectivity (Ichihashi and Oie, 1983; Laliberte and Moss, 2009). Remarkably, the infectivity can be restored if the lipid is replenished. After treatment of virus on the grids with NP40 and DTT, the virion membrane, including the lipid and outer protein array, is removed completely from the virion surface, so that the boundary surrounding the core is reduced in thickness or even not apparent in some of the electron micrographs (Fig. 1K–O).

Electron dense structures can be observed on the top of cores stained with UA (Fig. 2A). Upon carbon–platinum shadowing of these same samples, these structures present themselves in a globular form and are presumed to be the lateral bodies (Fig. 2B). Close inspection of particles treated with NP40+DTT and stained with NanoW revealed pore-like structures with an average diameter of 11.71 ($\pm$1.25) on the surface of the core (Fig. 2C). The presence of pore-like structures traversing the core wall has been described, but the nature and function of these structures have yet to be determined (Cyrklaff et al., 2005).

Importantly, the NP40-treated particles resemble virions in the capsule form. Furthermore, both the capsule form and NP40 treated viruses are larger than the mulberry form of the particle. Analysis of the two largest dimensions of the mulberry-like viruses ($n=67$) yielded 302.30 nm ($\pm$22.92) for the long axis and 240.90 nm ($\pm$22.18) for the short axis. By contrast, the dimensions of the capsule-like particles ($n=65$) were 319.36 nm

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