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Three-dimensional structure of Rubella virus factories

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ABSTRACT

Viral factories are complex structures in the infected cell where viruses compartmentalize their life cycle. Rubella virus (RUBV) assembles factories by recruitment of rough endoplasmic reticulum (RER), mitochondria and Golgi around modified lysosomes known as cytopathic vacuoles or CPVs. These organelles contain active replication complexes that transfer replicated RNA to assembly sites in Golgi membranes. We have studied the structure of RUBV factory in three dimensions by electron tomography and freeze-fracture. CPVs contain stacked membranes, rigid sheets, small vesicles and large vacuoles. These membranes are interconnected and in communication with the endocytic pathway since they incorporate endocytosed BSA-gold. RER and CPVs are coupled through protein bridges and closely apposed membranes. Golgi vesicles attach to the CPVs but no tight contacts with mitochondria were detected. Immunogold labelling confirmed that the mitochondrial protein p32 is an abundant component around and inside CPVs where it could play important roles in factory activities.

Introduction

The replication of many viruses is associated with specific intracellular compartments called virus factories. These are thought to provide a physical scaffold to concentrate viral components for genome replication and morphogenesis (Netherton et al., 2007; Novoa et al., 2005). The formation of virus factories often results in rearrangement of cellular membranes, reorganization of the cytoskeleton, and recruitment of mitochondria. One of the early events in factory formation is the assembly of replication complexes (RCs). RNA viruses modify specific membranes of the factory to concentrate viral replicases and necessary co-factors. Their RCs include the viral-RNA-dependent RNA polymerase (RdRp), further accessory non-structural proteins, viral RNA and host cell factors (Miller and Krijnse-Locker, 2008). Studies of individual non-structural proteins have revealed that the replication complexes are targeted to the respective organelle by the non-structural proteins rather than RNA (Salonen et al., 2005). These complexes could have a sophisticated organization for a more efficient replication of the viral genome (Dye et al., 2005; Fontana et al., 2007; Lyle et al., 2002; Mackenzie, 2005; Spagnolo et al., 2010; Wang et al., 2002). Why different viruses choose different subcellular membranes for their replication, how the viral proteins are targeted to those membranes and the host factors involved are currently under intensive investigations (Barajas et al., 2009; Goff, 2008; Nagy 2008; Ortin and Parra, 2006; Sessions et al., 2009).

Rubella virus (RUBV) is an important human teratogenic virus and the only member of the genus Rubivirus in the family *Togaviridae* (Frey, 1994). RUBV anchors its RNA synthesis in membranes of a cell organelle known as the "cytopathic vacuole" or CPV that derives from modified endosomes and lysosomes (Fontana et al., 2007; Lee et al., 1994; Magliano et al., 1998). This is a very unique feature of RUBV shared with alphaviruses, the other genus of the family (Froshauer et al., 1988; Kujala et al., 2001). RER cisternae, mitochondria and Golgi stacks are recruited around CPVs to build RUBV factories. Such structures are likely to help the virus to evade host cell defense responses as well as connecting viral replication with assembly and

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maturation of new viral particles in recruited Golgi membranes (Risco et al., 2003).

The RUBV genome is a single-stranded, "plus-sense" RNA of ~10 kb in length. The genome RNA contains two long open reading frames (ORFs): a 5' proximal or non-structural (NS)-ORF encodes the two non-structural proteins, P150 and P90, involved in viral RNA replication and a 3' proximal ORF or structural protein (SP)-ORF encodes the structural proteins (capsid or C, and two envelope glycoproteins, E1 and E2). The nonstructural proteins (NSPs) are translated from the genome RNA while the SP-ORF is translated from a subgenomic (SG) RNA that is co-terminal with the 3' end of the genomic RNA. From a RUBV infectious cDNA clone, a replicon derivative was generated in which the 3' proximal ORF was replaced with reporter genes and drug resistance markers. In transfected cells, replicons create CPVs and factories ultra-structurally identical to those formed during viral infection and thus the P150 and P90 replicase proteins are sufficient to generate these structures (Fontana et al., 2007). Cell signaling pathways involved in RUBV factory construction, as well as the nature of contacts between organelles within the structure, are basically unknown.

RUBV has genomic coding and replication strategies similar to alphaviruses, but the two genera are only distantly related, sharing no sequence homology at either the nucleotide or amino acid level. However, CPVs assembled by RUBV and alphaviruses are very similar: they have been described as structures of 600–2000 nm in size with a surface consisting of small vesicular invaginations or spherules (the sites of viral RNA replication) that have a diameter of approximately 50 nm and line the vacuole membrane at regular intervals (Froshauer et al., 1988; Griemley et al., 1972; Lee et al., 1994; Magliano et al., 1998). The recent use of improved specimen preparation techniques for electron microscopy of cells, such as freeze-substitution before embedding, showed that CPVs are indeed filled with electron-dense material, small and large vesicles and elongated "straight elements" which had not been observed previously (Fontana et al., 2007). In cells transfected with RUBV replicons, these structures were shown to contain P150, P90 and dsRNA, and small amounts of the mitochondrial p32 protein, as shown by immunoelectron microscopy. In the present work we have gone one step further in preservation by applying high-pressure freezing on live cells and subsequently avoiding chemical treatments before fast freezing and freeze-substitution. High-pressure freezing is considered the best method of cryo-immobilization for obtaining the greatest depth of optimal ultra-structural cell preservation for electron microscopy (Studer et al., 2008). With highly preserved cells processed by high-pressure freezing and freeze-substitution we have then applied methods for imaging in three dimensions (3D). Random thin-sectioning and electron microscopy (EM) has missed many of the fine features in RUBV factories such as connections between recruited organelles and the relationships between different membranous compartments inside CPVs and with the surrounding cytosol. The main technical options for 3D characterization of cells include reconstruction from serial sections, which is particularly useful for very large structures such as whole eukaryotic cells (Fiala, 2005; Fontana et al., 2008; Romao et al., 2008), metal replication after freeze-fracture or freeze-etching that provides planar views of the internal organization of membranes (Cabezas and Risco, 2006; Risco and Pinto da Silva, 1998; Severs, 2007; Severs and Robenek, 2008), and electron tomography (ET), a process involving rotation of the specimen in the electron beam, capturing images at incremental rotations, and using the images for 3D reconstruction. Cellular electron tomography (ET) is a powerful technique that can provide molecular resolution of cell ultra-structure if combined with "close-to-native" preservation of biological samples (Lucić et al., 2005). Nowadays, ET is becoming a mainstream research tool in cell biology and structural virology (Cyrklaff et al., 2005; McIntosh et al., 2005; Miller and Krijnse-Locker, 2008; Steven and Aebi, 2003).

Here we present the structural characterization of RUBV factories by electron tomography and metal replication after freeze-fracture followed by freeze-etching. We have found that recruited organelles (RER, mitochondria and Golgi) exhibit a variety of contacts with the CPVs, whose internal membranes are interconnected and in communication with the endocytic pathway. Immunogold labelling showed the presence of significant amounts of the mitochondrial protein p32 inside and around the CPVs, which suggests a role for this protein in the assembly and activities of the viral factory.

Results

2D organization of highly preserved RUBV factories

Thin-section electron microscopy shows the basic features of Rubella virus factories built around modified lysosomes or CPVs (Fig. 1). Both lysosomes and CPVs are round-shaped organelles filled with lamellar membranes and vesicles but as described in previous work from our lab CPVs have a more complex content with different types of vesicles, one or several larger vacuoles and rigid sheets (Fontana et al., 2007). RER cisternae, mitochondria and sometimes one Golgi stack surround the CPV as assembled in RUBV-infected cells and in cells transfected with RUBV replicons. These general features are appreciated in thin-sections of cells transfected with the RUBrep/GFP/neo RUBV replicon and conventionally dehydrated and embedded in an epoxy-resin before TEM (Fontana et al., 2007) (Fig. 1A). For three-dimensional studies at higher resolution a superior preservation of the structure is needed and for this purpose we processed our samples by high-pressure freezing. Structures associated with RUBV factories are better preserved after high-pressure freezing of live cells followed by freeze-substitution and thin-sectioning (Fig. 1B). For example, RER cisternae are thicker and closer to CPVs, mitochondrial membranes are smooth, and the interior of CPVs exhibits a variety of spherical vesicles and vacuoles. Semi-thick sections (~250–300 nm) were then obtained to encompass a larger proportion of the viral factories. High contrast was obtained by testing the most adequate substitution medium and resin (see Materials and methods). Specimen stability in the electron beam was optimized by collecting the sections on holey carbon grids (Fig. 1C and D). CPVs were easily identified on the holes at low magnification (Fig. 1C, arrows) which helped in selection of representative factories (CPVs with associated organelles) for electron tomography (Fig. 1D).

Three-dimensional analysis of RUBV factories: General organization and contacts with recruited organelles

Freeze-fractured RUBV factories were submitted to short etching and replicated with Ta/W for higher resolution (see Materials and methods). TEM of these metal replicas shows particular details of the factories, such as crowding of organelles around CPVs that are completely surrounded by mitochondria (Fig. 2A). Fine details of the CPV are also seen, such as the variety of sizes for the internal vesicles and the heterogeneity in the protein content of these membranes, visualized as small particles corresponding to integral membrane proteins. These images also reveal the need for electron tomography to study the structure with higher resolution: it is not possible to appreciate if organelles contact or how they attach to the CPV's surface in images from metal replicas (arrows in Fig. 2A, mainfield and inset). Electron tomography has solved this dilemma by showing where real contacts between organelles are established. Fig. 2B–G show low magnification (B) and high magnification (C to G) views of several areas from a selected computational tomographic slice corresponding to one of the eleven reconstructions obtained by ET and image processing.

This analysis showed two types of contacts between RER and CPVs: closely apposed membranes without evident fusion (Fig. 2C and D) together with protein bridges (Fig. 2E and F). The macromolecular