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Marburg virus inclusions: A virus-induced microcompartment and interface to multivesicular bodies and the late endosomal compartment

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ABSTRACT

Filovirus infection of target cells leads to the formation of virally induced cytoplasmic inclusions that contain viral nucleocapsids at different stages of maturation. While the role of the inclusions has been unclear since the identification of Marburg and Ebola viruses, it recently became clear that the inclusions are the sites of viral replication, nucleocapsid formation and maturation. Live cell imaging analyses revealed that mature nucleocapsids are transported from inclusions to the filopodia, which represent the major budding sites. Moreover, inclusions recruit cellular proteins that have been shown to support the transport of nucleocapsids. For example, the tumor susceptibility gene 101 protein (Tsg101) interacts with a late domain motif in the nucleocapsid protein NP and recruits the actin-nucleation factor IQGAP1. Complexes of nucleocapsids together with Tsg101 and IQGAP1 are then co-transported along actin filaments. We detected additional proteins (Alix, Nedd4 and the AAA-type ATPase VPS4) of the endosomal sorting complex required for transport (ESCRT) that are recruited into inclusions. Together, the results suggest that nucleocapsids recruit the machinery that enhances viral budding at the plasma membrane. Furthermore, we identified Lamp1 as a marker of the late endosomal compartment in inclusions, while ER, Golgi, TGN and early endosomal markers were absent. In addition, we observed that LC3, a marker of autophagosomal membranes, was present in inclusions. The 3D structures of inclusions show an intricate structure that seems to accommodate an intimate cooperation between cellular and viral components with the intention to support viral transport and budding.

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1. Introduction and state of the art

Marburg virus (MARV) and Ebola virus (EBOV) belong to the *Filoviridae* family of filamentous, highly pathogenic viruses, which are endemic in Central Africa. Filoviruses cause dramatic outbreaks of hemorrhagic fevers, and the most recent and most severe outbreak began in late 2013 in West Africa. More than 25,000 cases and over 10,000 deaths have been reported in the three most affected countries, Guinea, Sierra Leone and Liberia (Shears and O'Dempsey, 2015). Filoviruses contain a non-segmented negative-strand 19 kb RNA genome that encodes seven structural proteins. The genome is associated with five nucleocapsid proteins, NP, VP30, VP35, VP24 and L (Becker et al., 1998; Bharat et al., 2011). NP encapsidates the viral genome and, together with the polymerase L and the polymerase cofactor VP35, is necessary and sufficient for viral replication (Muhlberger et al., 1998). Viral transcription is dependent on the presence of VP30, an essential transcription factor in filovirus-infected cells (Biedenkopf et al., 2013; Modrof et al., 2002; Muhlberger et al., 1999). A layer of the matrix protein VP40 enwraps the nucleocapsid and connects the ribonucleoprotein complex with the lipid envelope (Bharat et al., 2011). The receptor-binding GP, as the only transmembrane glycoprotein, is inserted into the viral envelope. Homotrimeric GP is essential for recognizing target cells and induces fusion between cellular and viral membranes (Carette et al., 2011; Cote et al., 2011; Miller et al., 2012; Mittler et al., 2013).

1.1. Filoviral inclusions are sites of nucleocapsid formation

After filoviruses invade the target cell by macropinocytosis, GP recognizes its receptor, Niemann Pick C 1, in the endosome/lysosome and induces fusion of the viral envelope with the lysosomal membrane. This process releases the nucleocapsid into

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the cytoplasm, where all further steps of the replication cycle take place (Bhattacharyya et al., 2012). The incoming nucleocapsid contains all viral components to support primary transcription. The produced viral mRNAs are then translated by the host cell into the viral proteins (Muhlberger et al., 1998). It is presumed that the accumulation of the newly synthesized viral proteins in the infected cell triggers the synthesis of the viral genome (replication) (Muhlberger, 2007; Muhlberger et al., 1998, 1999). Nascent genomic RNA is encapsidated by a helical arrangement of NP, and VP35 and VP24 are intimately connected to the NP helix (Bharat et al., 2011). Newly formed ribonucleoprotein complexes accumulate in the perinuclear region to form inclusions (Becker et al., 1998; Kolesnikova et al., 2000).

Inclusion formation is a hallmark of filovirus-infected cells, which was documented by electron microscopy of infected cells during the first outbreak of Marburg virus in 1967 (Marini, 1971). The structural determinant of inclusion formation is NP. While all other nucleocapsid proteins are diffusely distributed in the cytosol upon single expression, recombinant expression of NP results in the formation of inclusions that contain helices of polymerized NP (Becker et al., 1998; Kolesnikova et al., 2000). The diameter of these helices is similar to the diameter of the inner ring of mature filoviral nucleocapsids (Kolesnikova et al., 2000; Bharat et al., 2012). Upon co-expression with NP, VP30, VP35, VP24 and VP40 are recruited into NP-induced inclusions that are closely associated with the rough endoplasmic reticulum (ER) (Becker et al., 1998; Huang et al., 2002; Kolesnikova et al., 2000; Watanabe et al., 2006). The ER association of inclusions is also detected in MARV-infected cells. In addition, individual electron-dense nucleocapsids associate with smooth membrane vesicles, indicating that NP is generally able to associate with cellular membranes (Dolnik et al., 2010; Kolesnikova et al., 2000). Inside inclusions, different maturation stages of nucleocapsids are detected. While thin-walled tubule-like structures prevail in the center of inclusions, the electron density of the nucleocapsids in the periphery is much higher, most likely because of additional nucleocapsid proteins that are recruited to the NP tubules. Co-expression studies of the different nucleocapsid proteins indicated that the association of NP-tubules with VP24 and VP35 results in the formation of electron dense nucleocapsid-like structures that are morphologically highly similar to the mature nucleocapsids in infected cells (Noda et al., 2006). Cryo-electron tomography of assembly intermediates showed that nucleocapsids have a defined orientation during transport and budding (Bharat et al., 2011).

1.2. Cellular factors are recruited into inclusions

Recently, inclusions have been shown to represent the sites of viral RNA synthesis in EBOV-infected cells. Interestingly, during mitosis, the inclusions undergo dramatic reorganization resulting in the formation of inclusions in both daughter cells (Hoenen et al., 2012). These observations suggest specific interactions with cellular factors, which allow for the structural rearrangement and dynamics of the inclusions.

For example, Tsg101, a component of the endosomal complex required for transport (ESCRT) I, is recruited to the inclusions by a PSAP late domain motif in NP. Functionally, the interaction between Tsg101 and NP enhanced the release of virus-like particles (VLPs) (Dolnik et al., 2010). A recombinant MARV with a mutated PSAP late domain in the NP showed defects in the transport of nucleocapsids and budding (Dolnik et al., 2014). Interestingly, as a result of its inability to interact with Tsg101, mutant NP was also unable to recruit the Tsg101 interacting protein IQGAP1, an actin cytoskeleton regulator.

1.3. Nucleocapsid transport from inclusions to the budding sites

Recent live cell imaging experiments of MARV- and EBOV-infected cells revealed that the inclusions are indeed the source of nucleocapsids (Schudt et al., 2015, 2013). Single nucleocapsids are released from the inclusions and transported through the cytoplasm. Most interestingly, although VP40 is present in the inclusions, the released nucleocapsids were devoid of VP40 after being released from inclusions. The interaction between nucleocapsids and VP40 was detected in the cell periphery and the interaction with VP40 was the prerequisite for the entry of nucleocapsids into filopodia. The transport of nucleocapsids was powered by the condensation of actin over surprisingly long distances (up to 25 μm). In addition, it was observed that nucleocapsids were transported along actin filaments (Schudt et al., 2015, 2013).

Taken together, inclusions play a central role in the replication cycle of filoviruses and represent an interface to recruit cellular proteins. We were therefore interested to screen for further cellular factors that are recruited to inclusions.

2. Results and discussion

2.1. MARV RNA synthesis occurs in inclusions

For many viruses, it has been shown that viral inclusions are sites of viral transcription and replication (Netherton et al., 2007). For EBOV, it was reported that inclusions are sites of viral RNA synthesis (Hoenen et al., 2012). Because this experimental evidence was missing for MARV, we performed 5′bromouridine (Br-Uridine) labeling of nascent viral RNA in MARV-infected cells (Wansink et al., 1993). Huh-7 cells were infected with MARV and treated with actinomycin D (ActD) at 17 h p.i. to shut down cellular transcription. At 18 h p.i., Br-Uridine was added for 1 h, the cells were subsequently fixed, and the intracellular localization of MARV NP and Br-Uridine was determined. In non-infected control cells (Mock), newly synthesized RNA was detected mainly in the nucleus. In the presence of ActD, nuclear staining disappeared due to the inhibition of cellular transcription (Fig. 1). Upon ActD treatment of MARV-infected cells, clear signals for nascent viral RNA (Br-Uridine) were detected in inclusions, indicating that these are sites of MARV RNA synthesis.

Inclusion formation is a phenomenon that is observed in many cells infected by a wide variety of RNA and DNA viruses (Moshe and Gorovits, 2012). Well-investigated examples are the vaccinia virus, African swine fever virus and iridoviruses. While the phenomenon was often observed, its functional contribution to the viral replication cycle is still not understood in detail. Some viruses use the inclusions as sites for replication hidden from the innate immune recognition of the cell. Viruses also make use of the cell's ability to sequester malformed proteins into special structures, aggresomes, to avoid cellular disturbances (Heath et al., 2001). Viruses possibly usurp those cellular salvage pathways that are intended to recruit chaperones to these sites in an attempt to overcome a transient stress situation. As we have shown here, MARV also sequesters its replication machinery to special intracellular sites close to the nucleus where newly formed nucleocapsids accumulate.

2.2. ESCRT-associated proteins are recruited into NP inclusions

The morphogenesis of nucleocapsids occurs inside virally induced inclusions where nucleocapsids of different electron densities and representing different maturation stages can be detected (Kolesnikova et al., 2000; Schudt et al., 2013). Because the size of the MARV nucleocapsids (60 nm in diameter and 900 nm in length) prevents their transport over long distances by diffusion, they need support from cellular factors for their transport through the

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