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The dynamics of both filamentous and globular mammalian reovirus viral factories rely on the microtubule network

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ABSTRACT

Mammalian reovirus viral factories (VFs) form filamentous or globular structures depending on the viral strain. In this study, we attempt to characterize the dynamics of both filamentous and globular VFs. Here, we present evidence demonstrating that globular VFs are dynamic entities coalescing between them, thereby gaining in size and concomitantly decreasing in numbers during the course of the infection. Additionally, both kinds of VFs condense into a perinuclear position. Our results show that globular VFs rely on an intact MT-network for dynamic motion, structural assembly, and maintenance and for perinuclear condensation. Interestingly, dynein localizes in both kinds of VFs, having a role at least in large globular VFs formation. To study filamentous VF dynamics, we used different transfection ratios of μNS with filamentous μ2. We found a MT-network dependency for VF-like structures perinuclear condensation. Also, μNS promotes VFLSs perinuclear positioning as well as an increase in acetylated tubulin levels.

1. Introduction

Mammalian reovirus (MRV) is a non-fusogenic member of the *Reoviridae* family, genus *Orthoreovirus,* composed by ten dsRNA genome segments that encode for eight structural proteins (λ3, λ2, λ1, μ2, μ1,σ1, σ2, and σ3) and three non-structural proteins (μNS/μNSC, σ1s and σNS). This virus triggers in mice severe myocarditis and encephalitis (Sherry, 1998; Weiner et al., 1977). Despite being non-pathogenic for humans, lately, MRV has been correlated with intestinal autoimmune pathologies such as celiac disease (Bouziat et al., 2017). During its life cycle, MRV cores penetrate into the host cytosol, where the primary transcription of positive RNA strand of the ten-genome segments takes place, allowing for the expression of viral proteins required for the initiation of the viral replication. As a result, at early times post-infection, this replication process generates small, punctuated, membrane-less, cytosolic inclusions named viral factories (VFs). Biochemical evidence indicates that VFs are the sites in which several processes take place, including the secondary viral transcription, which increases viral protein levels, the dsRNA genome replication that involves the synthesis of (-) ssRNA, the packaging of genome segments as well as assembly of newly synthesized viral cores. The secondary synthesis of the viral transcripts is responsible for the enlargement of the VFs, which ultimately localize at the perinuclear region of the cells. The nonstructural proteins μNS and σNS and the minor core protein μ2 are the critical components required for the formation and maturation of the VFs. Morphologically, depending on the viral strains MRV VFs can be classified into filamentous (e.g., strain type 1 Lang (T1L)) and globular (e.g., certain strains of type 3 Dearing, including one from the Nibert lab ($T3D^N$)) (Broering et al., 2002; Parker et al., 2002). The genome segment M3 encodes for μNS, a 721 amino-acids protein (Mustoe et al., 1978), which directly associates at its N-terminal region (the amino acidic region 1-221) with μ2, σNS, λ1, λ2, σ2 and viral cores, and at the C-terminal region (the amino acidic region 221-471) with λ3, the RNA-dependent-RNA polymerase (Broering et al., 2004, 2002; Miller et al., 2010, 2003). It has been also described that μNS recruits the heavy and light chains from clathrin (the amino acidic motif, 711-LIDFS-715)(Ivanovic et al., 2011), the chaperone Hsc70 (Kaufer et al., 2012) as well as associated with stress granules (amino acids 78 and 79) (Carroll et al., 2014). Besides, μNS expressed in the absence of other viral proteins, generates cytosolic, phase-dense globular structures called viral factory-like structures (VFLS). These μNS-induced VFLSs are morphologically very similar to those formed in infected cells (Broering et al., 2002). The μNS C-terminal region (the amino acidic region 471–721) is critical for VFLS formation (Broering et al., 2005). Point mutations (H570A and C572A) in a conserved consensus zinc-hook motif across μNS homologs disrupt the VFLS phenotype (Arnold et al., 2008; Broering et al., 2005). Thus, a model appears in which μNS is a matrix protein for viral factory formation and

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nucleation of viral and host components, thought to be the primary location of virus transcription, replication and assembly (Kobayashi et al., 2009; Miller et al., 2010).

The minor core protein μ2 is also a component of the VFs that can directly bind microtubules (MTs) (Kim et al., 2004), a feature that allows this protein to confer a filamentous or globular morphology to the VFs. The differences in morphology derive from amino acid polymorphisms in μ2 sequences at the amino-acidic position 208. Virus strains that produce filamentous VFs such as T1L, or globular, such as $T3D^N$, contain a proline or serine at position 208, respectively. Additionally, μ2 leads to stabilization of the MT network through acetylation in infected or transfected cells (Parker et al., 2002). When μ2 carrying a Pro^{208} is co-expressed with μNS in the absence of other viral proteins, the globular VFLS are redirected to MTs, developing a filamentous morphology typical of the filamentous strains (Broering et al., 2002). On the other hand, μ2-carrying Ser^{208} is more prone to form small aggregates through binding of μ2 with ubiquitin (Miller et al., 2004). It has been recently demonstrated that the association of μ2 to MTs relies on μ2 self-oligomerization (Eichwald et al., 2017). Additional work on this protein has identified a nuclear-localization signal motif (the amino acidic region 99–110), which allows μ2 accumulation in the nucleus when expressed in the absence of other viral proteins (Kobayashi et al., 2009). Interestingly, μ2 from T1L strain is essential in the repression of the IFN-α/β signaling pathway through a mechanism that involves an increase of IRF9 nuclear accumulation (Zurney et al., 2009). Moreover, nuclear localization of μ2 from T1L alters the subnuclear localization of the mRNA splicing factor SRSF2 by forming speckle complexes with it, resulting in a strain-specific modulation of the cell RNA splicing (Rivera-Serrano et al., 2017). Finally, it was also described that μ2 from T1L has a role influencing the cell tropism by promoting virion assembly in the VFs (Ooms et al., 2012, 2010).

An interesting aspect that remains to be explored is the role of the MTs and molecular motors in the dynamics of both filamentous and globular VFs. In the current study, we aimed to compare the dynamics of both filamentous and globular VFs in correlation with the MT network. Our data revealed that globular VFs are dynamic entities that rely on an intact MT network for their formation, coalescence, and structural maintenance. The molecular motor dynein also localizes in filamentous and globular VFs, playing a role in the assembly and the structural maintenance of at least globular VFs. Based on co-expression at different ratios of μNS and μ2, we demonstrate that the MT-association to VFLSs also relies on μNS expression.

2. Results

2.1. Quantifications of MRV factory dynamics

We performed a detailed characterization of MRV factory dynamics using two different MRV strains, $T3D^N$ and T1L, which respectively form factories of primarily globular or filamentous morphology late in infection (Broering et al., 2002; Miller et al., 2004; Parker et al., 2002; Shah et al., 2017; Spendlove et al., 1963). After infecting CV-1 cells with both strains, we acquired images at pre-determined time points between 4 and 24 hpi (hours post-infection) (Fig. S1a) and then quantified various aspects of the VFs, including their perinuclear localization, numbers, size and frequency distributions. In the case of the $T3D^N$ VFs, they were indeed globular and remained so in all cells at all time points. The $T3D^N$ VFs showed to be dynamic entities, quickly increasing in number and gradually increasing in size between 4 and 12 hpi. At late times post-infection the VFs keep increasing in size but their number decrease, suggesting a coalescence mechanism of the VFs (Fig. 1a). The increase in $T3D^N$ VFs size was substantial, with the projected, two-dimensional surface area of the largest factories at 24 hpi approaching 300 times that of the smallest detectable factories at the same time post-infection (Fig. 1b), consistent with a volume increase of ~5000 times. Noteworthy, smaller VFs (0–40 μm2) undergo number fluctuations ranging from reduced amounts at early time post-infection (6 hpi), to larger amounts between 10 and 14 hpi and back to small amounts at late times post-infection (18–24 hpi). In the case of the T1L factories, another noteworthy observation was the high percentage of globular morphology at earlier times post-infection, 6–14 hpi, whereas those with filamentous morphology gradually increased in number to become predominant only at later times, 18–24 hpi (Fig. 1c). Upon quantifying the relative ability of the $T3D^N$ and T1L VFs to condense in the perinuclear region of the infected cell, we found that T1L VFs show a notable correlation between three parameters: i) the timing of perinuclear condensation (Fig. 1d), ii) the conversion to VFs of filamentous morphology (Fig. 1c), and iii) a substantial accumulation of the μ2 protein detected by immunoblotting (Fig. S1b). Of note, the perinuclear ratio has a low value when the measured entities are in near vicinity of the nuclear edge and a high value when they are further away from the nuclear edge. The perinuclear condensation of globular $T3D^N$-VFs is similar to the observed for T1L up to 14 hpi. At increasing times post-infection (18 and 24 hpi), globular VFs are apparently less condensed than the filamentous VFs. However, this apparent discrepancy is explained by the distance from the nuclear edge to outside edge of the massive globular VFs at these times post-infection.

2.2. MT dependence of factory dynamics

The direct association of μ2 with MTs is necessary for the filamentous VFs morphology and this association is disrupted by treatment with depolymerizing drugs as nocodazole, in which case the VFs switch to globular morphology (Parker et al., 2002). In this study, we investigated whether the MT network was directly involved in assembly of globular VFs. For this purpose, $T3D^N$–infected CV-1 cells were treated at 1 hpi with MT-depolymerizing agents, such as nocodazole and vinblastine, or with MT-stabilizing compounds, as taxol (Fig. 2a). It has been previously described that reovirus requires 20 min for its internalization (Mainou and Dermody, 2011; Mainou et al., 2013). Therefore, we added the drug at 1 hpi to ensure that the viral entry process was not affected by our analysis. At 16 hpi, cells were fixed, and then VFs were evaluated concerning several aspects such as number, size, and perinuclear condensation. The VFs treated with MT-depolymerizing agents showed to be significantly higher in number but smaller than the untreated VFs (Fig. 2b, c, and d) and also VFs cannot condensate in the perinuclear region (Fig. 2e). Interestingly, upon taxol treatment, VFs show no differences in these measured aspects compared to the untreated samples. These data strongly suggest that the MT network is required for at least two events: i) assembly of VFs by coalescence and ii) VFs condensation to the perinuclear area. We next investigated the role of the MT network in the maintenance of the $T3D^N$-VFs structure as well as their cytosolic positioning. Thus, $T3D^N$ infected CV-1 cells at 18 hpi were treated for 3 h with nocodazole, vinblastine, or taxol (Fig. 3). Under these conditions, VFs treated with nocodazole or vinblastine disaggregated by increasing in number and reducing in size (Fig. 3b, c, and d). Interestingly, only VFs treated with vinblastine delocalized from the perinuclear region (Fig. 3e). As expected, taxol treated VFs behaved similarly to untreated VFs. We noticed, however, that T1L and $T3D^N$- infected cells treated for a more extended period (1 hpi to 24 or 48 hpi) with either MT-depolymerizing or MT-stabilizing drugs had a mild reduction (approx 1–1.5 log) in the infectivity of the viral progeny (Fig. S2). Our data suggest that while the MT network is required for the formation, structure maintenance, and cytosolic positioning of the globular VFs, it could also have a role in viral replication, transcription or translation.

2.3. The role of the molecular motors in the dynamics of globular VFs

Since the MT network requires of molecular motors to transport cargoes along the rails, we investigated if the molecular motor dynein has a role in the dynamics of both filamentous and globular VFs. In a