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Visual detection of Flavivirus RNA in living cells

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ABSTRACT

Flaviviruses include a wide range of important human pathogens delivered by insects or ticks. These viruses have a positive-stranded RNA genome that is replicated in the cytoplasm of the infected cell. The viral RNA genome is the template for transcription by the virally encoded RNA polymerase and for translation of the viral proteins. Furthermore, the double-stranded RNA intermediates of viral replication are believed to trigger the innate immune response through interaction with cytoplasmic cellular sensors. Therefore, understanding the subcellular distribution and dynamics of Flavivirus RNAs is of paramount importance to understand the interaction of the virus with its cellular host, which could be of insect, tick or mammalian, including human, origin. Recent advances on the visualization of Flavivirus RNA in living cells together with the development of methods to measure the dynamic properties of viral RNA are reviewed and discussed in this essay. In particular the application of bleaching techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are analysed in the context of tick-borne encephalitis virus replication. Conclusions driven by this approached are discussed in the wider context Flavivirus infection.

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1. Introduction

Flaviviridae is a family of viruses causing severe diseases and mortality in humans and animals. The Flavivirus genus is the largest in the family and includes among the most important emerging viruses known to man, which are mostly transmitted by mosquitoes or ticks (arthropod-borne viruses), such as yellow fever virus (YFV), the dengue viruses (DV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV). Hepatitis C virus (HCV, genus Hepacivirus) is the unique blood-borne virus in the family [1]. HCV is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. Although these viruses belong to different genera with different biological properties, the members of this family share similarity in terms of virion morphology, genome organization and replication strategies [2–5].

Flaviviruses are icosahedral enveloped 50 nm viruses with a RNA genome packaged by the viral capsid protein (C). The spherical nucleocapsid core of about 30 nm is covered by a host-derived lipid bilayer with two surface glycoproteins, membrane (M, which is expressed as prM, the precursor to M) and envelope (E), that have double-membrane anchors at the C-terminus. The Flavivirus genome is an 11-kilobase single-stranded RNA molecule of positive polarity that encodes a single long open reading frame (ORF). The ORF of all Flaviviruses is flanked by 5′ (about 100 nucleotides) and 3′ (400–700 nucleotides) untranslated regions (UTR) carrying RNA sequence motifs and secondary structures that function as *cis*-acting regulatory elements for genome amplification, translation or packaging [3]. Translation of the genome by the host cell machinery produces a long polyprotein precursor that is co- and post-translationally cleaved into at least 10 proteins. The N-terminal end of this polyprotein encodes the structural proteins (C-prM-E) followed by seven non-structural (NS) proteins (i.e. for TBEV: NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) each of which is an essential component of the viral replication complex (RC). Processing of the polyprotein is carried out by both host proteases and the viral protease NS3/NS4B in the lumen of the ER.

Flavivirus infection is initiated when mature viral particles attach to the target cell surface through interaction of the large glycoprotein E with cellular receptors. After attachment, the virus is internalized by receptor-mediated endocytosis and delivered to the endosome. The low pH in the endosomal compartment induces a conformational change in the surface protein E that triggers the fusion of the viral and host cell membrane [6,7]. This process

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results in the release of the nucleocapsid and viral RNA into the cell cytoplasm. At this stage, the uncoated genome can be translated by the cell machinery to generate the polyprotein precursor. Processing of the polyprotein by host and viral proteases then leads to the generation of the individual viral proteins and to the initiation of genome replication. The viral RNA-dependent RNA polymerase (RdRp) NS5 copies complementary minus-strand RNA from genomic RNA, which then serves as template for the synthesis of new positive strand viral RNAs. Viral RNA synthesis is asymmetric, with the plus-sense RNA synthesized in excess over minus-sense RNA [8–10]. The newly synthesized plus-sense RNA is subsequently: (i) used for translation of further viral proteins; (ii) used for the synthesis of additional minus-sense RNA; (iii) incorporated as genomic viral RNA into new viral particles. Hence, the viral RNA has at least three different functions (translation, replication, and association with nascent viral particles), which need to be tightly regulated and coordinated during the viral replication cycle. More recently, an abundant non-coding RNA derived from incomplete degradation of the viral 3′UTR by the cellular 5′-3′ exonuclease Xrn1 and produced by all Flaviviruses (termed sfRNA for subgenomic flaviviral RNA) was reported to be required for viral pathogenicity, possibly by regulating the interferon response (see below) [11–13].

Viruses are obligate intracellular parasites, therefore they must exploit the host cell machinery to efficiently replicate and produce infectious progeny. Flaviviruses usurp and modify cytoplasmic membranes in order to build functional sites of protein translation, processing and RNA replication [5,14–19]. These sites, enriched in cellular membranes, viral RNA and virus- and host-encoded proteins, are generally defined as replication complexes. Membrane wrapping of the RC is thought to provide a physical framework in which RNA synthesis can occur and to ensure protection from host-response proteins recognizing the viral RNA. Recently, elegant three-dimensional EM tomography studies have shown that Flaviviruses such as DENV, TBEV and WNV share the property of forming vesicles of approximately 80 nm of diameter as invaginations towards the lumen of the ER bearing necked connections to the cytoplasm [19–21]. At variance, HCV induces the formation of double-membrane extrusions from the ER membrane, probably with the same purpose of protecting the viral RC [22,23].

Mammalian cells have evolved a variety of defence mechanisms to detect, contain and clear viral infections. There are two fundamentally different types of responses to invading pathogens: the innate and the acquired immune response. The innate immune response offers the first early protection against foreign invaders and is mediated by a limited number of pattern-recognition receptors (PRRs). In contrast, acquired immunity is implicated in pathogens clearance during the late phase of the infection and long-term protection, which involves lymphocytes (T and B cells) clonally expressing a large repertoire of rearranged antigen-specific receptors. An effective innate immune response to viral infection involves two phases: an early phase of interferon production, triggered by the recognition of conserved "non-self" signatures, also known as pathogen-associated molecular patterns (PAMPs), by host PRRs, and a later phase of IFN signalling and interferon stimulated genes (ISGs) expression [24]. Indeed, upon recognition, PRRs initiate signalling cascades that result in the activation of transcription factors critical for type I interferons (INFα and IFNβ) expression. Thereafter, as secreted factors, type I IFNs can regulate a variety of immune responses through interaction with the type I IFN receptor. These responses include induction of a protective antiviral state in the infected and neighbouring cells as well as initiation of the acquired immunity. To date, two distinct families of sensors have been characterized as key players in the detection of RNA viruses: the Toll-like receptor (TLR) and the RIG-I (retinoic acid inducible gene-I)-like receptor (RLR) families [25–30]. RNA viruses are specifically sensed by the intracellular TLRs such as TLR3, TLR7 and TLR8, which recognise dsRNA and ssRNA [31,32]. Whereas TLRs detect viruses-derived nucleic acids within intracellular compartments of specific cell types, such as dendritic cells and macrophages, RLRs sense viral components that are present in the cytoplasm of most infected cells. The RLR family is composed of three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [33,34]. RIG-I and MDA5 are DExD/H-containing RNA helicases with two caspase activation and recruitment domains (CARD), which are essential for the interaction with the IFNβ promoter stimulator (IPS)-1 adaptor protein (also known as mitochondrial anti-viral signalling protein (MAVS)). Interestingly, IPS-1 is localized on specialized ER membranes associated to mitochondria or peroxisomes, suggesting a critical function of these organelles as a signalling platform for antiviral innate immunity [35–38]. IPS1 activation then associates with tumour necrosis factor (TNF) receptor-associated factor (TRAF) 3 leading to TBK1 and inhibitor of kB kinase (IkB) ε (IKKε) activation and subsequent phosphorylation of the IRF3 transcription factor. Alternatively, IPS-1 recruits the adaptor Fas-associated death domain (FADD) and the kinases receptor- interacting protein 1 (RIP1) in order to trigger the NF-kB pathway. Upon activation, IRF3 and NF-kB translocate to the nucleus to drive type I IFN transcription and subsequent induction of the antiviral state [39].

Originally, both RIG-I and MDA5 were thought to sense cytoplasmic dsRNA during viral infection [40]. However, through numerous studies, it has been clearly demonstrated that, despite their structural and functional similarities, the two sensors are not redundant in their ability to recognize non-self RNA [41]. Poly (I:C) as well as chemically synthesized RNA oligonucleotides annealed to a complementary strand trigger RIG-I [42,43]. RNAs carrying a 5′-triphosphate (5′-PPP) moiety, generally produced during infection by influenza and other negative-strand RNA viruses, are RIG-I agonists as well [44,45]. Long, possibly branched dsRNAs found for example in picornaviruses [45] and mRNAs lacking 2′-O-methylation at their 5′ cap structure [46,47] are MDA5 agonists. Total RNA extracted from virally infected cells can also stimulate specific RLRs. However, the form of viral RNA that is recognized depends on the specific virus. Concerning *Flaviviridae*, while RIG-I knockout mice demonstrated increased susceptibility to JEV infection compared to control mice, MDA5 deficient mice responded normally to infection [41]. Furthermore, siRNA-mediated knock out RIG-I, but not MDA5, affected TBEV-mediated induction of interferon [48]. In contrast, WNV and DV were shown to induce both RLRs-dependent pathways of PAMP recognition. Consistently, cells lacking RIG-I or MDA5 were not able to properly counteract viral infection [49–51]. Interestingly, temporal regulation of PRR appears to take place in WNV, with early activation of RIG-I and a later role for MDA5 [52]. Altogether, these studies suggest that RIG-I plays a critical early role in establishing effective immune responses to all Flaviviruses, whereas MDA5 role appear to be virus-dependent and acting at later stages. However, the real Flavivirus RNA structure that is recognized by cellular PRRs has not been identified so far and only few reports investigated this topic in the context of the whole cell [53].

As mammalian hosts have evolved several sensors for viral infection, viruses, on the other hand, adapted multiple tricks to escape or at least counteract innate immune response. These can be distinguished in those that target PRR signalling, thus delaying the first induction of IFNs, and those that target IFNs signalling, thus limiting their antiviral potential [54]. Typically, evasion of IFN induction is accomplished by viral proteins that directly inhibit the function of PRRs. For example, the NS1 viral protein is the main IFN antagonist of influenza A viruses [55]. NS1 acts both by preventing the nucleation of the IFN enhanceosome [56–58] and

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