Virology 454-455 (2014) 280–290

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Differential segregation of nodaviral coat protein and RNA into progeny virions during mixed infection with FHV and NoV

Radhika Gopal, P. Arno Venter [1], Anette Schneemann *

Department of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, CA, USA

ARTICLE INFO

Article history:
Received 30 December 2013
Returned to author for revisions
27 January 2014
Accepted 3 March 2014
Available online 21 March 2014

Keywords:
Flock House virus
Nodamura virus
Mixed infection
Viral assembly
RNA encapsidation
Viral reassortant

ABSTRACT

Nodaviruses are icosahedral viruses with a bipartite, positive-sense RNA genome. The two RNAs are packaged into a single virion by a poorly understood mechanism. We chose two distantly related nodaviruses, Flock House virus and Nodamura virus, to explore formation of viral reassortants as a means to further understand genome recognition and encapsidation. In mixed infections, the viruses were incompatible at the level of RNA replication and their coat proteins segregated into separate populations of progeny particles. RNA packaging, on the other hand, was indiscriminate as all four viral RNAs were detectable in each progeny population. Consistent with the *trans*-encapsidation phenotype, fluorescence *in situ* hybridization of viral RNA revealed that the genomes of the two viruses co-localized throughout the cytoplasm. Our results imply that nodaviral RNAs lack rigorously defined packaging signals and that co-encapsidation of the viral RNAs does not require a pair of cognate RNA1 and RNA2.

Introduction

The nodaviruses are a family of non-enveloped, icosahedral viruses that have a bipartite, positive-sense RNA genome. They are divided into two genera, Alphanodavirus and Betanodavirus, whose members were originally isolated from insects and fish, respectively (Thiery et al., 2012). In the Alphanodavirus genus, Flock House virus (FHV) is the most thoroughly characterized virus, whereas Nodamura virus (NoV) represents the type species. NoV is distinct from other alphanodaviruses in its ability to infect some mammals, including suckling mice and hamsters (Garzon and Charpentier, 1991; Scherer and Hurlbut, 1967; Scherer et al., 1968). Studies of NoV have lagged behind those of FHV primarily due to lack of a cell culture system in which the virus can be efficiently propagated. Its life cycle can be studied, however, upon transfection of the viral RNAs into a variety of cell types, including vertebrate and invertebrate cells.

The nodaviral genome divides replication and packaging functions between two RNA segments. The larger segment, RNA1, encodes the RNA-dependent RNA polymerase (RdRp), which establishes replication complexes on the surface of mitochondria (Miller and Ahlquist, 2002; Miller et al., 2001). More specifically, RNA synthesis occurs in so-called spherules, which represent invaginations of the outer membrane of the organelle (Kopek et al., 2007). The smaller genome segment, RNA2, encodes capsid protein alpha, which co-packages one molecule of RNA1 and RNA2 into progeny particles that have $T=3$ icosahedral symmetry (Fisher and Johnson, 1993; Friesen and Rueckert, 1981; Krishna and Schneemann, 1999). A third, subgenomic RNA3 is synthesized from RNA1 and encodes protein B2, a suppressor of RNA silencing. RNA3 is not packaged into particles (Chao et al., 2005; Galiana-Arnoux et al., 2006; Li et al., 2002).

X-ray crystallography and cryo-electron microscopy of several alphanodaviruses have provided detailed insights into the structure of the protein capsid including important information on the arrangement of the packaged RNA (Fisher and Johnson, 1993; Tang et al., 2001; Tihova et al., 2004). A significant portion of the genome (13–35%) forms double-stranded (ds) regions that sit directly underneath the 30 edges of the icosahedral capsid, where they interact with positively charged amino acid side chains located primarily in the N and C termini of the coat protein. The remaining RNA takes an unknown pathway but is thought to drop down into the interior of the capsid and return back up to connect the dsRNA regions at the twofold contacts (Devkota et al., 2009; Tihova et al., 2004). Because the density representing RNA in these particles is icosahedrally averaged, information about the location of specific bases or sections of the genomic RNAs is not available. It is also not known whether the RNA that is visible in the structure represents RNA1, RNA2 or both.

In contrast to many other non-enveloped, icosahedral, positive strand RNA viruses, nodaviral coat proteins do not form empty

* Corresponding author. Tel.: +1 858 784 8643; fax: +1 858 784 7979.
E-mail address: aschneem@scripps.edu (A. Schneemann).
[1] Current address: Vertex Pharmaceuticals, Boston, MA, USA.

http://dx.doi.org/10.1016/j.virol.2014.03.003
0042-6822

particles but need nucleic acid to establish stable quaternary interactions. Regardless of whether particles contain the viral genome or random cellular RNAs, as for example in virus-like particles, their sedimentation rate and density remain unchanged. This indicates that the amount of RNA packaged in each capsid is relatively constant. It is often referred to as a "headful" and probably represents a compromise between the limited capacity of the particle's internal volume and the requirement for a minimum nucleic acid scaffold during and after assembly.

Although FHV is a generally well-characterized nodavirus, several aspects of its life cycle remain poorly understood. One of them is the mechanism by which RNA1 and RNA2 are recognized and packaged into a single particle. The two RNAs lack notable regions of sequence identity or obvious secondary structures that could serve as common signals for packaging as has been observed for some other segmented positive strand RNA viruses (Choi et al., 2002). Instead, nodaviral RNA1 and RNA2 appear to have unique features that ensure their recognition and encapsidation.

Some information regarding the packaging mechanism has come from mutational analyses of the coat protein. These analyses showed that the N- and C-termini of alpha protein play a critical role in the recognition of the viral RNAs. Deletion of N-terminal residues 2–31 results in inefficient packaging of RNA2, without affecting packaging of RNA1 (Marshall and Schneemann, 2001). In contrast, deletion of C-terminal residues 382–407 results in packaging of random cellular RNA (Schneemann and Marshall, 1998). An arginine-rich motif proximal to the N terminus was found to be important for specific packaging of RNA1 (Venter et al., 2009). Together, these results imply that, at least in case of these coat protein mutants, recognition of the two genomic segments occurs independently of each other and assembly is not initiated on a non-covalent complex of the two RNAs.

Additional hints about RNA packaging came from experiments in which FHV-infected cells were engineered to produce two types of coat protein: one from an mRNA that was generated in the nucleus, the other from a viral RNA2 replicating in the cytoplasm. Only coat protein translated from the RNA2 replicon packaged the FHV genome, while coat protein translated from non-replicating RNA packaged cellular RNA (Venter and Schneemann, 2007). These results were interpreted to indicate that viral RNA replication, translation and packaging are coupled events and that they may occur in separate cellular microdomains or viral factories to prevent interference by other cellular components.

The need to evolve and maintain a mechanism that ensures co-packaging of multiple genome segments puts nodaviruses, as well as other viruses with segmented genomes, such as reoviruses, bunyaviruses and influenza viruses, at a disadvantage when compared to non-segmented viruses. On the other hand, these viruses have the ability to form reassortants, which increases their genetic diversity and promotes rapid evolution in the face of environmental pressures. The formation of reassortants implies that the genome packaging mechanisms used by these viruses have a certain level of flexibility built into them to allow incorporation of segments whose sequence may differ significantly from the parental segment. Specificity must be maintained, however, with regard to the number of segments packaged and the proteins they encode.

We chose FHV and NoV to explore the formation of viral reassortants as a means to further understand nodaviral genome recognition and packaging. The two viruses, which were isolated from different insects in separate geographic locations at different times (Scherer and Hurlbut, 1967; Scotti et al., 1983), are genetically only distantly related to each other. FHV RNA1 (3.1 kb) shares 50% identity with NoV RNA1 (3.2 kb), while FHV RNA2 (1.4 kb) shares 56% identity with NoV RNA2 (1.3 kb). RdRp and coat protein are 40% and 47% identical, respectively. We found that the two viruses are incompatible at the level of RNA replication and that their capsid proteins segregate into separate particles. These particles, however, packaged not only their cognate RNAs but also those of the other virus. Consistent with the observed *trans*-encapsidation phenotype, fluorescence *in situ* hybridization of co-infected cells revealed that the RNAs of the two viruses largely co-localized in the cytoplasm. Overall, the results presented here combined with previous data suggest that nodaviral RNAs lack rigorously defined packaging signals and that co-encapsidation is likely based on molecular features that emerge subsequent to the initial interaction of coat protein subunits with the individual RNAs.

Results

Demonstration of mixed nodaviral infection in BHK21 cells

We initially planned to study the outcome of mixed nodaviral infections in cultured *Drosophila* S2 cells, which have been used extensively to investigate the FHV life cycle. Because S2 cells cannot be infected with NoV, we employed liposome-mediated transfection of viral RNAs. Specifically, S2 cells were transfected with a mixture containing equal amounts of FHV and NoV genomic RNAs extracted from purified virus particles and infection was monitored by confocal immunofluorescence microscopy with antibodies against the coat proteins of the two viruses. Surprisingly, the vast majority of transfected cells contained only one type of coat protein, that of FHV or NoV, whereas few contained both (data not shown). The inefficiency with which the transfection procedure gave rise to co-infected cells precluded use of the S2 cell line as a suitable system of investigation. We therefore turned our attention to mammalian BHK21 cells, which also support FHV and NoV replication upon transfection of the viral RNAs as long as the cells are cultured at $\leq 33\,^{\circ}C$ (Ball et al., 1992). When BHK21 cells were transfected with equal amounts of FHV and NoV RNAs, the average transfection efficiency in five independent experiments was $30 \pm 8\%$. This was based on examining a total of 444 cells processed for immunofluorescence microscopy and scoring as positive those that contained at least one type of nodaviral coat protein. The majority, $79 \pm 6\%$, of these positive cells contained both FHV and NoV coat proteins, whereas $6 \pm 1\%$ contained only FHV protein and $15 \pm 2\%$ only NoV protein (Fig. 1).

Monitoring the transfection efficiency of BHK21 cells by confocal immunofluorescence microscopy revealed the subcellular localization of the viral coat proteins. As previously observed (Petrillo et al., 2013), cells transfected with FHV RNA contained coat protein throughout the cytoplasm and distributed in a somewhat reticular pattern (Fig. 1A). This pattern was mirrored by coat protein in cells transfected with NoV RNAs (Fig. 1B). In cells co-transfected with the genomes of both viruses, the signal for the two coat proteins largely overlapped (Fig. 1C and D), indicating that NoV and FHV did not appear to segregate into separate cellular microenvironments where each type of coat protein accumulated for subsequent assembly and RNA packaging.

The involvement of mitochondria in nodaviral infections is well established (Garzon et al., 1990; Miller et al., 2001). In contrast to NoV, however, it had not yet been confirmed that these organelles also serve as a site of RNA replication when FHV infects mammalian cells instead of insect cells. We therefore performed additional confocal immunofluorescence analyses as well as electron microscopic analyses on BHK21 cells transfected with FHV RNA. Using antibodies against the polymerase combined with MitoTracker red staining, we reproduced earlier results that RdRp is located on mitochondria in these cells (Fig. 2A) and that the organelles