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Elucidating virus entry using a tetracysteine-tagged virus

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ABSTRACT

Fluorescent tags constitute an invaluable tool in facilitating a deeper understanding of the mechanistic processes governing virus-host interactions. However, when selecting a fluorescent tag for *in vivo* imaging of cells, a number of parameters and aspects must be considered. These include whether the tag may affect and interfere with protein conformation or localization, cell toxicity, spectral overlap, photostability and background. Cumulatively, these constitute challenges to be overcome. Bluetongue virus (BTV), a member of the *Orbivirus* genus in the *Reoviridae* family, is a non-enveloped virus that is comprised of two architecturally complex capsids. The outer capsid, composed of two proteins, VP2 and VP5, together facilitate BTV attachment, entry and the delivery of the transcriptionally active core in the cell cytoplasm. Previously, the significance of the endocytic pathway for BTV entry was reported, although a detailed analysis of the role of each protein during virus trafficking remained elusive due to the unavailability of a tagged virus. Described here is the successful modification, and validation, of a segmented genome belonging to a complex and large capsid virus to introduce tags for fluorescence visualization. The data generated from this approach highlighted the sequential dissociation of VP2 and VP5, driven by decreasing pH during the transition from early to late endosomes, and their retention therein as the virus particles progress along the endocytic pathway. Furthermore, the described tagging technology and methodology may prove transferable and allow for the labeling of other non-enveloped complex viruses.

Contents

Abbreviations: FP, Fluorescent protein; TC, biarsenical tetracysteine; BTV, Bluetongue virus; dsRNA, double-stranded RNA; RG, reverse genetics; cDNA, Complementary DNA; NS2, nonstructural protein 2; EEA1, early endosome marker; CD63, late endosome marker; FRET, fluorescence resonance energy transfer; EDT_2, ethane dithiol; BAL, 2,3-dimercaptopropanol or British anti-Lewisite.

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1. Introduction

Since its inception, visualization of live-virus trafficking in host cells has been highly informative, furthering our understanding of the interactions between virus and host cells. To date, enveloped viruses have been at the forefront of such studies through the insertion of fluorescent markers into the viral genomes, enabling the study of their trafficking [1–3], or the labeling of lipid envelopes using lipophilic tracers, such as 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), 3,3′-Dioctadecyloxacarbocyanine perchlorate (DiO), 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate (DiD), 4-(4-(Dihexadecylamino)styryl)-N-Methylpyridinium Iodide (DiA), and 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindotricarbocyanine Iodide (DiR) [4]. In all cases, it is paramount that any modification does not inhibit cellular functions, nor affect viral infectivity [5]. To date, two general strategies exist for the labeling of viruses. It is possible to label a target viral protein by fusing it with a fluorescent protein (FP) or by chemical labeling using a fluorescent dye [6].

Fusing a FP to the target viral protein is achieved by inserting the FP gene into the open reading frame of the viral protein [7,8]. When the virus replicates within host cells it is thus labeled. However, while this allows for the study of virus replication [9], or the dynamics of viral entry [10], a caveat that can arise is a significant amount of background signal due to overexpression of the protein in infected cells.

An alternatively method to introducing a FP into the open reading frame is chemical labeling, such as the introduction of a biarsenical tetracysteine (TC) tag with a CCPGCC motif into the open reading frame of the target viral protein [11–13]. Proteins with the TC tag are recognized and specifically bound by membrane-permeable biarsenical dyes, such as FlAsH (green) and ReAsH (red), which fluoresce when bound to the TC motif [11,12].

When labeling architecturally complex non-enveloped capsid viruses, there are physical constraints and limitations. Alterations, such as the introduction of a FP, could disrupt the structure-function relationship of the virus particles, interfering with their assembly or infectivity. This was evidenced by the lack of similar developments for the capsid virus members of the *Reoviridae* family. Recently, it was shown that such limitations could be overcome by the successful genetic engineering of the segmented RNA genome of Bluetongue virus (BTV), a complex non-enveloped Orbivirus belonging to the *Reoviridae* family. This facilitated the generation of fluorescent virus particles that could be visualized in virus entry studies for both live and fixed cells [14].

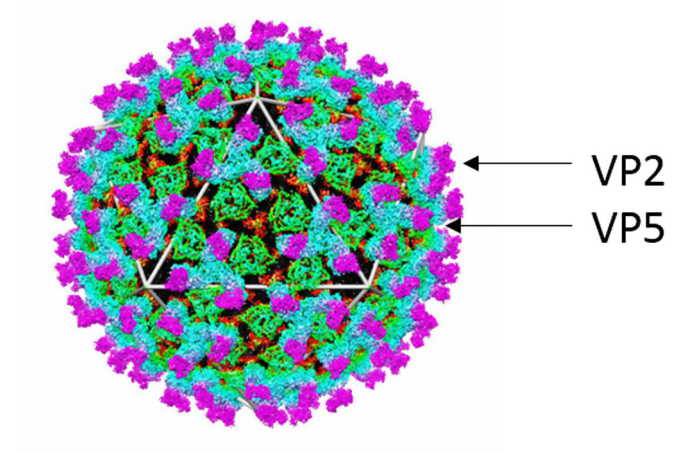

Fig. 1. BTV capsid structure. Electron-Cryomicroscopy high-resolution (7-Å) image of BTV. Outer-capsid coat comprised of VP2 (cyan and magenta) and VP5 (green). Inner capsid comprised of VP7 (black and red), whereby VP3 (not visible) is occluded by the VP7 layer (Adapted from Zhou, Roy & associates, PNAS, 2010). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BTV, with multiple serotypes (27 recognized to date) is endemic in most parts of the world, often resulting in high morbidity and mortality in ruminants. The BTV viral genome, comprising of 10 double-stranded RNA (dsRNA) segments (S1 to S10) encodes 7 structural (VP1 to VP7) and 4 non-structural proteins (NS1 to NS4) [15,16]. In the virus particles, the structural proteins are organized in two capsids that form concentric protein shells. The outer capsid is comprised of VP2 and VP5, while an inner capsid, or "core", comprised of VP7 and VP3. The core encloses the viral transcription complex (VP1, VP4 and VP6), in addition to the viral genome [15,17].

The constitutive components of the outer capsid (VP2 and VP5) have demonstrated a functional division to facilitate membrane attachment and membrane penetration. Three-dimensional structural studies of virions by cryo-electron microscopy (Fig. 1) revealed that VP2 arranges as trimers on the virion surface protruding as spike-like structures from the surface of the virus particles, facilitating viral attachment to the host cell [18,19]. The second outer capsid protein, VP5, is less exposed than VP2, but is also arranged into globular trimers that are mostly covered by VP2 [19]. Structurally, VP5 resembles the fusion proteins of enveloped viruses and consists of amphipathic α-helical regions on its external surface that facilitate penetration of endosomal membranes to release BTV cores into the cytoplasm [20].

A recent cryo-electron microscopy study at atomic resolution of BTV virus particles, under both physiological and low-pH conditions, has further elucidated how the VP2 and VP5 proteins sense endosomal pH. Furthermore this study revealed how VP2 and VP5 coordinate during cell entry and how VP5 acquires membrane-penetration activity by a dramatic conformational change induced by low pH [21], supporting and validating earlier observations.

The 10 segmented dsRNA molecules comprising the BTV genome, ranging in size from 0.8 to 3.9 kb, have a limited capacity to accommodate foreign genes [22]. Thus, to avoid disruption of the overall structure of the protein by introducing a FP, an alternate strategy of introducing a small TC tag with a CCPGCC motif was selected [11–13]. Previously, this technique had been successfully applied to enveloped viruses [23–26], but not for any complex capsid virus such as BTV. In this report we described a methodical approach that required combinations of sequence comparison data, biochemical methods and the BTV reverse genetics (RG) system [27] to generate a virus with the TC tags introduced into VP2 in such a way that they do not disrupt the structure-function relationship of the virus particles. The power of this technology was demonstrated through the enabling of the delineation of the dynamics of the two outer capsid proteins, which were shown to dissociate from one another during the early stages of virus entry into mammalian cells [14]. Here we describe the prerequisite methodology that led to this observation.

2. Methods and results

2.1. Identification of putative exposed regions in VP2 for subsequent TC tag insertion

As the host attachment protein responsible for virus entry, the exposed outer capsid protein VP2 was the prime candidate for labeling the virus particle. The insertion of any tag had to minimize any potential disruptions of the protein conformation, as this was likely to have proven an impediment to virus infectivity. Optimally,