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Cell entry by a novel European filovirus requires host endosomal cysteine proteases and Niemann–Pick C1

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ABSTRACT

Lloviu virus (LLOV), a phylogenetically divergent filovirus, is the proposed etiologic agent of die-offs of Schreibers's long-fingered bats (*Miniopterus schreibersii*) in western Europe. Studies of LLOV remain limited because the infectious agent has not yet been isolated. Here, we generated a recombinant vesicular stomatitis virus expressing the LLOV spike glycoprotein (GP) and used it to show that LLOV GP resembles other filovirus GP proteins in structure and function. LLOV GP must be cleaved by endosomal cysteine proteases during entry, but is much more protease-sensitive than EBOV GP. The EBOV/MARV receptor, Niemann–Pick C1 (NPC1), is also required for LLOV entry, and its second luminal domain is recognized with high affinity by a cleaved form of LLOV GP, suggesting that receptor binding would not impose a barrier to LLOV infection of humans and non-human primates. The use of NPC1 as an intracellular entry receptor may be a universal property of filoviruses.

1. Introduction

Members of the family *Filoviridae* have non-segmented negative-strand RNA genomes and produce filamentous enveloped particles. Until recently, all known filoviruses belonged to one of two genera – *Ebolavirus* or *Marburgvirus* (Kuhn et al., 2011). Three ebolaviruses [Ebola virus (EBOV), Bundibugyo (BDBV), and Sudan virus (SUDV)], and two marburgviruses [Marburg virus (MARV) and Ravn virus (RAVV)] are associated with highly lethal outbreaks of filovirus disease in humans and non-human primates, and are endemic in equatorial regions of the African continent (recently reviewed in Feldmann and Geisbert, 2011; Hartman et al., 2010; MacNeil et al., 2011; Paessler and Walker, 2013; Pourrut et al., 2005). One ebolavirus, Reston virus (RESTV), is thought to be avirulent in humans (Morikawa et al., 2007), but has been associated with multiple incidents of filovirus disease in monkeys exported from the Philippines to the US or Europe for research (Miranda et al., 2002; Rollin et al., 1999). More recently, RESTV was shown to circulate in domesticated pigs in the Philippines (Barrette et al., 2009). There are currently no FDA-approved vaccines or therapeutics to prevent or treat filovirus infections.

Bats are long-suspected filovirus reservoirs (Leroy et al., 2009; 2005; Olival and Hayman, 2014; Pourrut et al., 2009; Swanepoel et al., 1996), but conclusive evidence for their role in the ecology of filoviruses was lacking until recently, when infectious MARV and RAVV were found to circulate in healthy Egyptian rousettes (*Rousettus aegyptiacus*) (Amman et al., 2012; Towner et al., 2009). Infectious EBOV has not yet been isolated from bats. However, EBOV-specific antibodies and viral nucleic acids have been detected in African fruit bats belonging to three species (*Hypsignathus monstrosus, Epomops franqueti,* and *Myonycteris torquata*) (Leroy et al., 2005).

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In the early 2000s, massive bat die-offs of Schreibers's long-fingered bats (*Miniopterus schreibersii*) occurred throughout the Iberian peninsula. Investigators working with bat carcasses from Cueva del Lloviu, Spain, were able to detect filovirus-like nucleic acids in the lung and spleen by PCR (Negredo et al., 2011). While attempts to isolate infectious virus from these carcasses were unsuccessful, a near-complete filovirus genome, equally divergent from those of ebolaviruses and marburgviruses (≈ 50% nucleotide sequence identity) was assembled (Negredo et al., 2011). Because this viral genome was detected only in carcasses of Schreibers's long-fingered bats and not in healthy long-fingered or mouse-eared myotis (*Myotis myotis*), the authors proposed that a new filovirus-like agent, named Lloviu virus (LLOV) after the site of detection, may have been responsible for the bat die-offs (Negredo et al., 2011). The Internal Committee of Taxonomy of Viruses (ICTV) placed LLOV in a new genus, *Cuevavirus*, within the family *Filoviridae* (Adams et al., 2014; Kuhn et al., 2010). LLOV represents the first filovirus discovered in Europe that was not transported there from an endemic area in Africa or Asia.

Since LLOV is phylogenetically divergent from ebolaviruses and marburgviruses, was discovered in a new geographic area, and may be virulent in bats, it is possible that it differs from other known filoviruses with regard to fundamental mechanisms of infection, multiplication, and *in vivo* pathogenesis. However, the lack of an isolate has severely impeded the study of LLOV. In this study, we exploited a vesicular stomatitis virus (VSV)-based surrogate system to investigate the structural and functional properties of the presumptive envelope glycoprotein (GP) of LLOV, and the mechanism by which it mediates viral entry into the cytoplasm of host cells.

While this manuscript was in preparation, a study describing some entry-related properties of LLOV GP was published (Maruyama et al., 2013). That study employed vesicular stomatitis virus (VSV) single-cycle pseudotypes bearing LLOV GP. Here, we used reverse genetics to generate a recombinant VSV containing LLOV GP that is capable of multiple rounds of multiplication in tissue culture, thus providing a robust model for early steps in infection by the authentic virus. Our findings are in agreement with those of Maruyama and co-workers, and extend them in several important respects. Most significantly, we demonstrate that the late endosomal membrane protein Niemann–Pick C1 (NPC1) is a critical entry receptor for LLOV that binds directly and with high affinity to a cleaved form of LLOV GP.

2. Results

2.1. Generation of an infectious recombinant vesicular stomatitis virus expressing the LLOV transmembrane envelope glycoprotein

To study the mechanism by which LLOV enters host cells, we focused on the LLOV *GP* gene, which is predicted to encode a single transmembrane glycoprotein and multiple secreted glycoproteins homologous to their counterparts from African and Asian filoviruses (Figs. 1 and S1) (Negredo et al., 2011). Using reverse genetics, we generated the first recombinant vesicular stomatitis virus (rVSV) expressing the LLOV transmembrane glycoprotein GP in place of the orthologous VSV entry glycoprotein, G. The rescued virus was amplified in Vero cells, and the integrity of the LLOV *GP* gene in multiple viral clones was verified by RT-PCR and sequencing. We found that rVSV-GP-LLOV was competent to form plaques on Vero cell monolayers, and to replicate in these cells at levels comparable to rVSVs bearing other filovirus glycoproteins (Fig. 1A) (Carette et al., 2011; Wong et al., 2010).

Because the LLOV glycoprotein in these virus particles was not detected by several EBOV- and MARV GP-specific antibodies, we raised a rabbit polyclonal antiserum against a sequence in the N-terminal region of the LLOV GP1 subunit (residues 83–97; TKRWGFRSDVIPKIV). Western blotting of concentrated

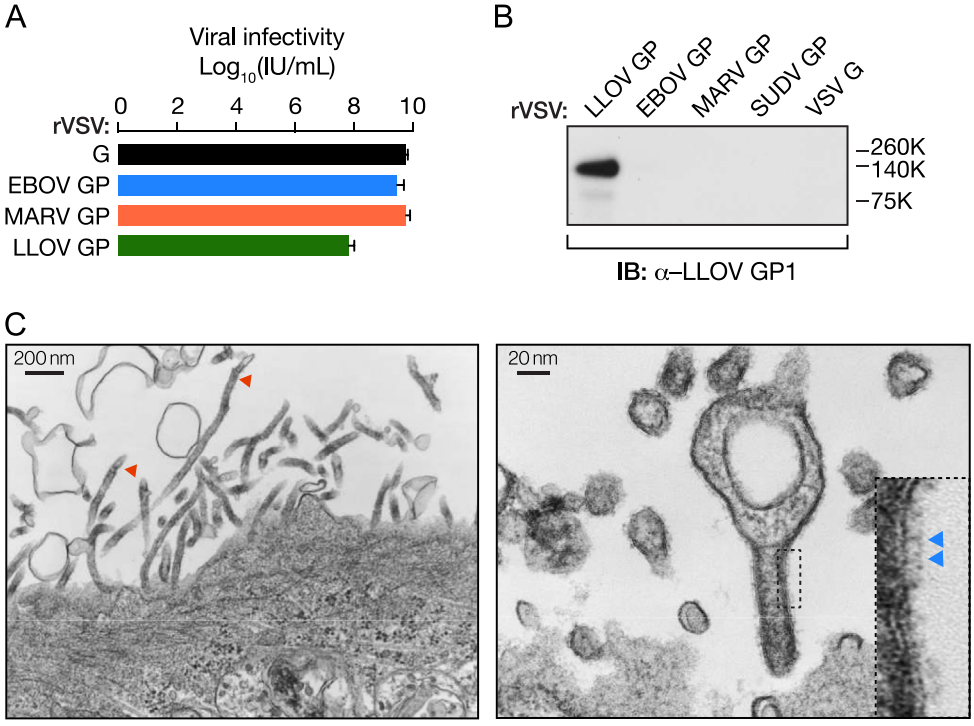

Fig. 1. LLOV GP is incorporated into VSV particles and filovirus-like particles, and is competent to mediate cell entry. (A) Vero cells were exposed to recombinant VSVs (rVSVs) expressing eGFP, and VSV G or different filovirus glycoproteins. Infected cells were visualized by fluorescence microscopy at 12–16 h post-infection. Averages ± SD (n=4) are shown. (B) Incorporation of LLOV GP into rVSVs was determined by SDS-PAGE and western blotting with an LLOV GP1-specific antiserum. (C) Vero E6 cells generating filovirus-like particles (VLPs) consisting of EBOV VP40 and LLOV GP were visualized by transmission electron microscopy. Red arrows, filamentous VLPs released from cells. Blue arrows, spike-like projections from the surface of a VLP, likely corresponding to LLOV glycoprotein spikes.