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# Formation of high-order oligomers is required for functional bioactivity of an African bat henipavirus surface glycoprotein

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ABSTRACT

Hendra virus (HeV) and Nipah virus (NiV) are highly pathogenic henipaviruses originating from fruit bats in Australia and Asia that can cause severe infections in livestock and humans. In recent years, also African bat henipaviruses were identified at the nucleic acid level. To assess their potential to replicate in non-bat species, several studies were performed to characterize the two surface glycoproteins required for virus entry and spread by cell-cell fusion. It has been shown that surface expression and fusion-helper function of the receptor-binding G protein of Kumasi virus (KV), the prototypic Ghanaian bat henipavirus, is reduced compared to other non-African henipavirus G proteins. Immunostainings and pulse-chase analysis revealed a delayed export of KV G from the ER. As defects in oligomerization of viral glycoproteins can be responsible for limited surface transport thereby restricting the bioactivity, we analyzed the oligomerization pattern of KV G. In contrast to HeV and NiV whose G proteins are known to be expressed at a dimer-tetramer ratio of 1:1, KV G almost exclusively formed stable tetramers or higher oligomers. KV G also showed less stringent requirements for defined stalk cysteines to form dimers and tetramers. Interestingly, any changes in the oligomeric forms negatively affected the fusion-helper activity although surface expression and receptor binding was unchanged. This clearly indicates that the formation of mostly higher oligomeric KV G forms is not a deficiency responsible for ER retention, but is rather a basic structural feature essential for the bioactivity of this African bat henipavirus glycoprotein.

## 1. Introduction

Hendra virus (HeV) and Nipah virus (NiV) represent the highly pathogenic members of the genus *Henipavirus* within the *Paramyxoviridae* family. While the viruses do not cause clinical diseases in their natural reservoir, pteropid bats, they can cause severe illness after natural spillovers to livestock and humans. Due to their zoonotic potential and their high pathogenicity, HeV and NiV are classified as biosafety level 4 pathogens.

HeV infections have only been observed in Australia causing regular small outbreaks of severe respiratory diseases in horses. Rare spillovers to humans are reported, but neither bat-to-human nor human-to-human transmissions were observed yet (Marsh et al., 2010). In 2012, another henipavirus, Cedar virus (CedV), was isolated from Australian pteropid bats. But in contrast to HeV, CedV did not cause clinical diseases in experimental animal studies, most likely because of a compromised ability to counteract the IFN response (Lieu et al., 2015; Marsh et al., 2012).

The second highly pathogenic henipavirus, NiV, was first discovered in Malaysia in 1998 during an outbreak of acute respiratory diseases in pigs, the so-called barking pig syndrome (Chua et al., 1999; Mohd Nor et al., 2000). Pigs also transmitted the infection to humans who developed fatal encephalitis with high mortality rates (Chua et al., 2002). Since 2001, small regional outbreaks of human NiV encephalitis in India and Bangladesh are regularly reported and are caused by direct bat-to-human or human-to-human transmissions (Khan et al., 2010; Luby et al., 2009a,b; Rahman et al., 2012).

Although clinical henipavirus infections of livestock or humans are not reported in Africa so far, henipavirus-like viral RNA was isolated from African flying foxes (Baker et al., 2013; Drexler et al., 2009, 2012). Aside of viral RNA detection in bats, cross-reacting antibodies were identified in domestic pig populations in Ghana (Hayman et al., 2011). There is even some serological evidence for a potential spillover to humans in Cameroon (Pernet et al., 2014b). However, the potential of African bat henipaviruses to actually cause zoonotic diseases in livestock and humans is unclear yet. Though characterization is limited by the fact that none of the African bat henipaviruses could be isolated as live virus, the almost full-length genome of GH-M74a could be

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determined by sequencing viral RNA isolated from bat specimens in Kumasi, Ghana (Drexler et al., 2012). This prototypic Ghanaian bat henipavirus, now named Kumasi virus (KV) (Afonso et al., 2016), provides the basis for functional characterization of the viral proteins in comparison to their pathogenic Asian and Australian counterparts.

Henipaviruses carry a negative-sense single-stranded RNA genome of about 18 kB. Among the six structural henipavirus proteins, the two surface glycoproteins are the essential components for the pH-independent fusion processes during virus entry and cell-to-cell spread. Thus, characterization of their functional properties is of central importance to evaluate the ability of African bat henipaviruses to replicate in cells from livestock or humans.

For henipavirus induced virus-cell and cell-cell fusion processes, the coordinated activities of the receptor-binding glycoprotein (G) and the fusion protein (F) are required. Upon G attachment to henipavirus receptors on the cell surface, conformational alterations in G are triggered and lead to the activation of the F glycoprotein, which then mediates membrane fusion processes. The F protein, a typical class I viral fusion protein, needs to be proteolytically processed by host cell cathepsins into fusion-active F1/F2 subunits (Diederich et al., 2012; Pager et al., 2006). For finally causing fusion of two lipid membranes, trimeric F proteins need an allosteric triggering by the G protein, the so-called fusion-helper function (Chang and Dutch, 2012; Jardetzky and Lamb, 2014; Plattet and Plemper, 2013).

Compared to Asian and Australian henipavirus G proteins, the prototypic Ghanaian bat henipavirus attachment protein, KV G, revealed a restricted fusion-helper activity in henipavirus-permissive mammalian cell types (Krueger et al., 2013; Krüger et al., 2014; Pernet et al., 2014a; Weis et al., 2014). Functional and structural analyses demonstrated that the impaired fusion support of the KV G protein is not the result of a severely deficient binding to the main henipavirus receptor ephrin-B2, although the binding activity is somewhat reduced (Lee et al., 2015). Limited bioactivity is at least to a substantial extent the result of a restricted cell surface expression of the G protein (Krüger et al., 2014).

Henipavirus G proteins are type II membrane glycoproteins. Full-length NiV G, as an example for an attachment protein of pathogenic henipaviruses is depicted in Fig. 1A. It consists of 602 amino acids and is subdivided into a 46-residue cytoplasmic tail, one membrane spanning segment, and a C-terminal globular head domain which is responsible for binding to the henipavirus receptors ephrin-B2/-B3 (Bonaparte et al., 2005; Bowden et al., 2008; Negrete et al., 2005, 2006). The receptor-binding head domain is linked to the transmembrane domain by a 106-residue stalk domain which is essentially required for receptor-induced conformational changes in G, allosteric F triggering and G oligomerization (Liu et al., 2015). Three cysteine residues are responsible for the formation of disulfide-linked dimers (C158, C162) and the formation of stable, disulfide-linked tetramers (C146; Fig. 1B) (Maar et al., 2012). NiV and HeV G proteins are expressed as disulfide-linked dimers that form stable tetramers at a ratio of 1:1, and changes in oligomerization were found to critically affect the fusion-helper activity of pathogenic henipaviral G proteins (Maar et al., 2012).

Similar to NiV and HeV G, KV G contains three stalk cysteines (Fig. 1A). However, neither the oligomeric pattern, nor the role of the cysteines for dimer and tetramer formation, or the role of oligomerization for fusion-helper function is known yet. To address in how far KV G oligomerization affects surface expression, receptor binding and allosteric changes needed for fusion-helper function (Bradel-Tretheway et al., 2015; Kreis and Lodish, 1986; Maar et al., 2012), KV G proteins with systematic mutations in the stalk cysteines were generated and characterized.

## 2. Material and methods

### 2.1. Cells and plasmids

Vero76 and HeLa cells were cultivated in Dulbecco's modified Eagle's medium (Gibco) with 10% FCS (Life Technologies), 100 U/ml penicillin, and 0.1 mg/ml streptomycin and 4 mM l-glutamine (Gibco). Cloning and characterization of the C-terminally tagged KV (formerly designated GH-M74a) and NiV G proteins have been described previously (Diederich et al., 2012; Weis et al., 2014). All substitution mutants were generated with the Q5 Site-Directed Mutagenesis Kit (NEB).

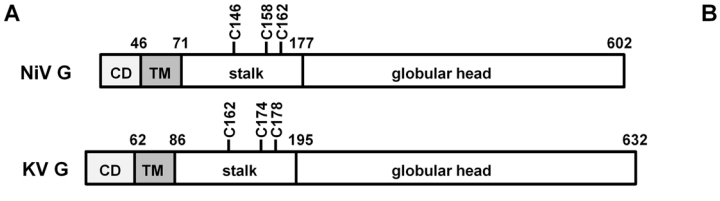
Cells were transfected with FuGENE HD transfection reagent (Promega) and Lipofectamine 2000 (Life Technologies) according to the protocols of the manufacturers.

### 2.2. Fusion assays

To analyze fusion-helper activities of wildtype and mutant henipavirus G proteins, Vero cells were co-transfected with plasmids encoding HA-tagged G proteins in combination with a plasmid encoding a HA-tagged NiV F protein (Weis et al., 2014). To visualize syncytia formation by Giemsa staining, Vero cells co-expressing henipavirus F and G proteins were fixed with ethanol at 48 h post transfection and were stained with 1:10 diluted Giemsa staining solution for 30 min. To quantify fusion, the number of nuclei of fifty randomly chosen microscopic fields of three different experiments were counted and averaged.

### 2.3. Surface biotinylation analysis

For surface biotinylation, $10^6$ G-expressing Vero cells were washed and incubated for 30 min at 4 °C with 1 mg/ml EZ-Link Sulfo-*N*-hydroxysuccinimido-LC-Biotin (Thermo Scientific). After several washings with PBS containing 0.1 M glycine, cells were lysed for 30 min in Triton-X buffer (20 mM Tris, pH 8.0; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100). After centrifugation for 15 min at 14,000 × *g*, biotinylated surface proteins were precipitated from supernatants with NeutrAvidin agarose (Thermo Scientific). Supernatants of the precipitates containing the unbound, non-biotinylated intracellular G proteins and the NeutrAvidin precipitates containing the surface-expressed G proteins were mixed with 2× SDS sample buffer with or without 0.1 M Dithiothreitol (DTT) and separated on a 10% SDS gel. Surface-expressed biotinylated G proteins and intracellular G proteins



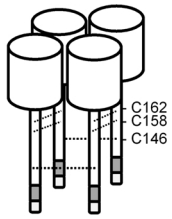




**Fig. 1.** Schematic model of NiV G and KV G proteins. (A) Schematic representation of the G glycoproteins. CD, cytoplasmic domain; TM, transmembrane domain. Numbers represent the first amino acid of the TM, the stalk domain and the head domain. The definition of the TM region of KV G is based on the prediction of transmembrane regions by the online tool TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Cysteines in the stalk domain are indicated as vertical black lines.
(B) Model for NiV G tetramer formation according to Maar et al. (2012). Cysteine residues at positions 158 and 162 are implicated in disulfide bond formation responsible for dimer formation. Tetramer stabilization is proposed to occur via intersubunit disulfide bond formation through C146.