Genus-specific recruitment of filovirus ribonucleoprotein complexes into budding particles

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The filoviral matrix protein VP40 orchestrates virus morphogenesis and budding. To do this it interacts with both the glycoprotein (GP₁₂) and the ribonucleoprotein (RNP) complex components; however, these interactions are still not well understood. Here we show that for efficient VP40-driven formation of transcription and replication-competent virus-like particles (trVLPs), which contain both an RNP complex and GP₁₂, the RNP components and VP40, but not GP₁₂ and VP40, must be from the same genus. trVLP preparations contained both spherical and filamentous particles, but only the latter were able to infect target cells and to lead to genome replication and transcription. Interestingly, the genus specificity of the VP40–RNP interactions was specific to the formation of filamentous trVLPs, but not to spherical particles. These results not only further our understanding of VP40 interactions, but also suggest that special care is required when using trVLP or VLP systems to model virus morphogenesis.

Ebola viruses (EBOV) and Marburg viruses (MARV) belong to two different genera in the family Filoviridae and both cause severe haemorrhagic fevers in humans and non-human primates. Their matrix protein, VP40, orchestrates morphogenesis and budding of virions, and virus-like particles (VLPs) are formed upon its expression in mammalian cells (Kolesnikova et al., 2004; Noda et al., 2002). During budding VP40 is known to interact with both the viral glycoprotein (GP₁₂) and the nucleoprotein (NP) (Noda et al., 2006), and this latter interaction is generally believed to be responsible for recruiting the ribonucleoprotein (RNP) complex into budding particles. In addition to NP, the RNP complex also contains the viral RNA genome, the polymerase (L), the polymerase cofactor (VP35) and the transcriptional activator (VP30). NP has been shown to directly interact with VP35 and VP30, both of which interact with L (Becker et al., 1998; Groseth et al., 2009).

EBOV and MARV VP40 are often thought to facilitate morphogenesis and budding by using the same molecular mechanisms. However, upon closer investigation there are clear differences between these two proteins, challenging this concept. For example, MARV VP40 contains only one late domain, which allows interaction with Tsg101 and facilitates budding (Uratæ et al., 2007), whereas EBOV VP40 contains two overlapping late domains (Licata et al., 2003), which interact with both Tsg101 and Nedd4 during budding. Furthermore, EBOV VP40 forms oligomers, which are critical for budding (Hoenen et al., 2010; Timmins et al., 2003b), whereas for MARV VP40 such structures have not yet been detected (Timmins et al., 2003a). EBOV VP40 is also known to have a specific RNA binding activity, which is essential for the viral life cycle (Hoenen et al., 2005) and might be of significance for RNP incorporation. In contrast, MARV VP40 does not appear to bind RNA (Timmins et al., 2003a).

In order to understand better the mechanisms by which EBOV and MARV VP40 orchestrate budding and interact with NP and GP₁₂, we have analysed their functions by using a transcription and replication-competent VLP (trVLP) assay (Hoenen et al., 2011), which was previously called an infectious VLP assay (Hoenen et al., 2006; Watanabe et al., 2004; Wenigenrath et al., 2010). This system represents a powerful tool to examine multiple steps of the viral life cycle, including genome replication and transcription, morphogenesis and budding of virus particles, and infection of target cells (Fig. 1a). Of particular interest was the ability of EBOV/MARV protein combinations to form chimeric trVLPs, as well as the ability of these particles to incorporate RNP complexes, which is a prerequisite for infectivity. If EBOV and MARV VP40 were interchangeable, this would support the idea that...
EBOV and MARV share a common mechanism for morphogenesis and budding. Initially, complete sets of entirely EBOV- or MARV-derived proteins (NP, VP35, VP30 and L, as well as GP1,2, VP40 and VP24) were expressed together with the homologous minigenome in 293T cells (producer cells). This leads to the formation of a vRNA-containing RNP complex, which is replicated via a cRNA intermediate (1) and transcribed into mRNAs (2), which in turn are translated into reporter protein (3). VP40 induces the formation of virus-like particles, which incorporate both the surface glycoprotein GP1,2 and RNP complexes (4), yielding replication- and transcription-competent virus-like particles (trVLPs). These trVLPs can infect target cells (5) pre-transfected with expression plasmids for NP, VP35, VP30 and L, but no minigenome. The minigenome brought into these target cells within the trVLPs serves as the template for replication and transcription, resulting in reporter activity which not only represents these processes, but also represents morphogenesis and entry of trVLPs.

(b) EBOV (upper panels) or MARV (lower panels) trVLPs were produced by co-expressing either EBOV (E) VP40 or MARV (M) VP40 with the other viral proteins and a minigenome in HEK293T cells. Reporter activity was determined 72 h post-transfection (left panels). As negative control the viral polymerase L was omitted (−L). Target cells were infected with trVLP-containing supernatant from the producer cells, and reporter activity was determined 72 h post-infection (right panels). (c) trVLPs were produced by co-expressing either EBOV (E) or MARV (M) RNP components (NP, VP35, VP30 and L), VP40 and GP 1,2. After 72 h, supernatant was passaged onto target cells, and after a further 72 h reporter activity in these cells was determined. Numbers above the bars indicate mean reporter activity in per cent. Shown are the mean and SD from three independent experiments.

Fig. 1. Role of the phylogenetic relationship of viral proteins in trVLP assays. (a) Schematic overview of a trVLP assay. All filoviral proteins and a minigenome are expressed in human embryonic kidney cells (HEK) 293T cells (producer cells). This leads to the formation of a vRNA-containing RNP complex, which is replicated via a cRNA intermediate (1) and transcribed into mRNAs (2), which in turn are translated into reporter protein (3). VP40 induces the formation of virus-like particles, which incorporate both the surface glycoprotein GP1,2 and RNP complexes (4), yielding replication- and transcription-competent virus-like particles (trVLPs). These trVLPs can infect target cells (5) pre-transfected with expression plasmids for NP, VP35, VP30 and L, but no minigenome. The minigenome brought into these target cells within the trVLPs serves as the template for replication and transcription, resulting in reporter activity which not only represents these processes, but also represents morphogenesis and entry of trVLPs. (b) EBOV (upper panels) or MARV (lower panels) trVLPs were produced by co-expressing either EBOV (E) VP40 or MARV (M) VP40 with the other viral proteins and a minigenome in HEK293T cells. Reporter activity was determined 72 h post-transfection (left panels). As negative control the viral polymerase L was omitted (−L). Target cells were infected with trVLP-containing supernatant from the producer cells, and reporter activity was determined 72 h post-infection (right panels). (c) trVLPs were produced by co-expressing either EBOV (E) or MARV (M) RNP components (NP, VP35, VP30 and L), VP40 and GP 1,2. After 72 h, supernatant was passaged onto target cells, and after a further 72 h reporter activity in these cells was determined. Numbers above the bars indicate mean reporter activity in per cent. Shown are the mean and SD from three independent experiments.
Owing to the presence of VP40, producer cells also generated trVLPs containing RNP complexes. These trVLPs can enter target cells and deliver their minigenome-containing RNP complexes, which serve as templates for genome replication and transcription. The resulting reporter activity therefore reflects, in addition to genome replication and transcription, production of trVLPs in producer cells and their entry and delivery of the minigenome into target cells. Reporter activity in target cells was readily detectable after infection with trVLPs containing either only EBOV or only MARV components, thus showing that both sets of proteins are capable of supporting the formation of homologous trVLPs (Fig. 1b, right panels). In contrast, when trVLPs were produced by co-expression of EBOV proteins, infection with MARV VP40 resulted in no reporter activity in target cells (Fig. 1b, upper right panel, right bar). Similar results were obtained when trVLPs were produced by co-expressing MARV RNP proteins with MARV GP1,2 and VP24, and with EBOV VP40 infection (Fig. 1b, lower right panel, middle bar). This clearly suggests a functional incompatibility of VP40 with either the RNP components or with GP1,2.

In order to determine the source of this incompatibility, trVLP assays were performed in which the RNP proteins VP40 and GP1,2 from either EBOV or MARV were combined in various permutations. When EBOV GP1,2 was exchanged for MARV GP1,2 in trVLPs otherwise consisting of only EBOV proteins, we did not observe any reduction in reporter activity in target cells, but rather a dramatic increase in activity of approximately 15-fold (Fig. 1c, left panel). Similarly, when trVLPs consisting of MARV VP40 and MARV RNP components contained MARV GP1,2, a 20-fold higher reporter activity in target cells was observed than when they contained EBOV GP1,2 (Fig. 1c, right panel). The molecular mechanism underlying this increased infectivity of trVLPs containing MARV GP1,2 remains to be determined. It will be particularly interesting to investigate whether this increase is target cell type specific and whether it is mediated by the ectodomain of GP1,2, which would suggest a more efficient entry process. Alternatively, involvement of the GP1,2 transmembrane domain would suggest more efficient incorporation of GP1,2 into trVLPs, since this domain has been shown to be responsible for the interaction of VP40 and GP1,2 in the case of MARV (Mittler et al., 2007). Nevertheless, while the use of MARV GP1,2 positively influenced either the production of infectious trVLPs or their specific infectivity regardless of the trVLP system used, GP1,2 and VP40 did not have to be from the same genus for the efficient formation of infectious trVLPs.

In contrast, whenever the RNP components and VP40 were of different origin, reporter activity in target cells dropped dramatically (Fig. 1c, lanes EME, EMM, MEE and MEM). These data suggest that a functional interaction between the RNP components and VP40 is critical for the production of infectious trVLPs, and that this interaction is not conserved between proteins from the different filovirus genera.

One obvious application of this phenomenon is the mapping of regions of VP40 that are important for this interaction. Since EBOV and MARV VP40 show a high degree of sequence homology, it should be possible to swap regions of these proteins and analyse the resulting VP40 chimeras for their ability to form infectious trVLPs in the presence of homologous or heterologous RNP complex components. However, none of the chimeras created in our laboratory by using this approach were able to drive the formation of any VLPs, although they were all readily expressed. In addition, their loss of budding ability was accompanied by aberrant oligomerization behaviour (data not shown). Particularly surprising was the inability to exchange the complete N- and C-terminal sections of EBOV VP40 (i.e. aa 1–194 and 195–324) with their MARV VP40 counterparts, since X-ray crystallography clearly shows that these regions form structurally independent domains (Dessen et al., 2000). This suggests that the interaction of the N- and C-terminal domains of VP40 with one another may be important for its function.

The most likely explanation for the need to maintain the phylogenetic relationship between VP40 and the RNP components to generate functional trVLPs is that the incorporation of RNP complexes into trVLPs is directly controlled via their interaction with VP40, which is impaired when these components are derived from different filovirus genera. To test this hypothesis, we analysed the incorporation of the RNP component NP into trVLPs by using Western blotting. trVLPs were readily produced by EBOV
VP40 and they contained EBOV NP, thus indicating efficient RNP incorporation (Fig. 2). Some release of EBOV NP was also detected in the absence of VP40, which was possibly caused by either the release of membrane-enclosed pleomorphic particles driven by GP1,2, or by released cell-derived particles in which NP was incorporated non-specifically, as previously reported (Kolesnikova et al., 2009; Noda et al., 2002). Surprisingly, a significant amount of NP was also found in trVLPs if their production was driven by MARV VP40 instead of EBOV VP40. This speaks against a genus-specific interaction between NP and VP40 and conflicts with the functional findings of the trVLP assays, which clearly suggested that this interaction is genus specific.

We have recently shown that MARV VLP and trVLP preparations contain a multitude of morphologically distinct particles, which can be fractionated by centrifugation through a Nycodenz gradient (Sigma-Aldrich) (Kolesnikova et al., 2009; Wenigenrath et al., 2010). Furthermore, only a fraction of the crude trVLP preparations (i.e. short filamentous trVLPs) show high levels of infectivity. Therefore, we considered that RNP incorporation may vary between infectious and non-infectious trVLP fractions, which could explain the apparent conflict between the functional trVLP data and the Western blot data. To examine this, EBOV trVLPs were purified over a sucrose cushion and then separated into three fractions by using a Nycodenz gradient. Analysis of negatively stained trVLPs showed that fraction 1 contained mostly spherical particles with a diameter ranging from 99 to 424 nm (mean 271 ± 94 nm) (Fig. 3a, left panel). In contrast, fractions 2 and 3 contained filamentous particles, with fraction 2 containing shorter particles ranging from 360 to 1422 nm.

**Fig. 3.** Separation of trVLPs via Nycodenz gradient centrifugation. EBOV trVLPs were purified via centrifugation on a sucrose cushion and then separated into three fractions by centrifugation on a Nycodenz gradient consisting of seven steps of 30 % (730 μl), 20 % (490 μl), 15 % (490 μl), 10 % (490 μl), 7.5 % (490 μl), 5 % (490 μl) and 2.5 % (490 μl) Nycodenz in TNE buffer [10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA] followed by centrifugation in an SW60 rotor (Beckman) for 15 min at 16 000 r.p.m. and at 4 °C. Three 980 μl fractions were taken off the top of the gradient and the trVLPs in each fraction were pelleted and analysed. (a) Fractions were analysed by electron microscopy after negative staining. Bars, 500 nm. (b) The relative amounts of short (length < 1200 nm) and long (length ≥ 1200 nm) filamentous particles in fractions 2 and 3 were determined by measuring 94 particles using the AMT Image Capture Engine software. (c) Fractionated trVLPs were used to infect target cells, in which reporter activity was measured 72 h post-infection. Reporter activity was normalized to the amount of VLPs present in each fraction, as determined by quantification of GP1,2 content. (d) Fractionated trVLPs were analysed by Western blotting. A representative blot of three independent experiments is shown.
in length (mean ± SD, 800 ± 323 nm), and fraction 3 containing longer particles ranging from 561 to 8224 nm in length (mean ± SD, 2376 ± 1394 nm) (Fig. 3a, middle and right panel). Of the trVLPs in fraction 2, 93% were shorter than 1200 nm, which is the reported mean length of infectious Zaire ebolavirus virions (Geisbert & Jahrling, 1995), whereas in fraction 3 the majority (65%) of trVLPs were longer than 1200 nm (Fig. 3b). When we infected target cells with the individual fractions, infectious trVLPs were found mainly in fraction 2 (Fig. 3c). This suggests that, as with MARV (Wenigenrath et al., 2010), for EBOV the infectious trVLPs are short filamentous particles, whereas spherical or long filamentous trVLPs are non-infectious. Furthermore, when we analysed these Nycodenz-fractionated trVLP populations by Western blotting, we could show that in fraction 1 (containing spherical particles) NP was incorporated regardless of the origin of VP40 (Fig. 3d). However, in fractions 2 and 3, in which predominantly filamentous particles are located, NP was only incorporated into particles produced by expression of homologous VP40. This observation clearly shows that incorporation of NP into filamentous and infectious trVLPs is indeed genus specific, whereas its incorporation into non-infectious spherical particles seems to be genus independent. It also suggests that spherical particles, which are also found in VLP preparations produced solely by VP40 and GP1,2 (Noda et al., 2002), might be produced via a mechanism distinct from that involved in the budding of actual filovirus particles, where the vast majority of particles have a characteristic filamentous morphology.

In summary, our data show that, for formation of infectious filovirus trVLPs, the RNP components and VP40 must be derived from the same genus, and that this phenomenon correlates with the incorporation of the RNP components into these particles, thus suggesting that VP40 plays a direct role in the incorporation of the filovirus RNP complex into budding particles. Furthermore, we show that incorporation of RNP components into non-infected spherical particles is genus-independent. Since these spherical particles have no biological equivalent and are produced non-specifically as a by-product of VLP and trVLP systems, this finding underlines the importance of careful separation of these two types of VLPs prior to biochemical analysis and emphasizes their potential to confound analyses of the mechanisms that contribute to filovirus morphogenesis and budding. Nevertheless, when used with care trVLP systems remain very powerful tools for studying the virus life cycle.

Acknowledgements

The authors are grateful to John Bernebaum, Astrid Herwig, Julie Callison and Les Shupert for technical assistance, to Verena Krähling for providing the anti-NP antibody and to Allison Groseth for critical reading of the manuscript. This work was supported by the Schering Foundation (T. H.) and the German Research Association (SPP1175 and SFB 593, TPB12) (S. B.), and in part by the Intramural Research Program of the NIH, NIAID. Disclaimer: the content of this publication does not necessarily reflect the views or policies of the US Department of Health and Human Services or of the institutions and companies affiliated with the authors. V. W.-J. performed this work as an employee of Tunnell Consulting, Inc., a subcontractor to Battelle Memorial Institute under its prime contract with NIAID, under contract no. HHSN27200200016I.

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