Establishment and application of an infectious virus-like particle system for Marburg virus

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The highly pathogenic Marburg virus (MARV) can only be investigated in high containment laboratories, which is time consuming and expensive. To investigate the MARV life cycle under normal laboratory conditions, an infectious virus-like particle (VLP) system was developed. The infectious VLP system is based on the T7-polymerase driven synthesis of a MARV-specific minigenome that encodes luciferase and is transcribed and replicated by the simultaneously expressed MARV nucleocapsid proteins NP, VP35, L and VP30. Transcription of the minigenome resulted in luciferase activity and replication resulted in encapsidated minigenomes. The encapsidated minigenomes, together with the viral matrix proteins VP40 and VP24 and the surface glycoprotein (GP), formed VLPs at the plasma membrane. Among the released pleomorphic VLPs, filamentous particles of 200–400 nm in length showed the highest capacity to induce reporter activity upon infection of target cells. To characterize the infectious VLP system, the intracellular concentration of one of the components was titrated, while all others were held constant. Intracellular concentrations of nucleocapsid proteins that resulted in highest replication and transcription activities also yielded VLPs with the highest ability to induce luciferase activity in target cells. High intracellular levels of VP40 maximized the release of VLPs, but reduced their ability to induce luciferase activity in target cells. The intracellular concentration of GP positively correlated with its incorporation into VLPs and their infectivity. Finally, we demonstrated that the infectious VLP system was suitable for rapid screening of neutralizing antibodies directed against MARV.

INTRODUCTION

Marburg virus (MARV), a member of the family Filoviridae, causes a haemorrhagic fever for which neither a specific therapy nor a licensed vaccine exists. MARV is a non-segmented negative-strand RNA virus whose genome consists of approximately 19 kb. The seven genes encode seven structural proteins that also function in transcription and replication of the viral RNA or interaction with host cell proteins, e.g. for intracellular transport or downregulation of host cell responses directed against the viral infection (Sanchez et al., 2007). Four proteins, the nucleoprotein (NP), the viral proteins VP35, VP30 and the polymerase L, together with the viral genome, make up the viral nucleocapsid complex, the template for transcription and replication of viral RNA (Becker et al., 1998; Mühlberger et al., 1998). The two matrix proteins of MARV, VP40 and VP24, bridge the space between the nucleocapsid and the viral envelope. VP40 functions as classical matrix protein and drives morphogenesis and budding of viral particles (Kolesnikova et al., 2004; Swenson et al., 2004), while the function of VP24 is unclear, although it seems to support the release of infectious virions (Bamberg et al., 2005). The surface glycoprotein (GP) is embedded in the viral envelope, and responsible for attachment and entry of virions (Feldmann et al., 1991; Will et al., 1993).

To understand the structure and function of the individual nucleocapsid proteins, a minigenome assay was established, which allows work to be performed under normal laboratory conditions. Using this assay it was established that NP, VP35 and L were necessary for transcription and replication of viral RNA (Mühlberger et al., 1998). The role of VP30, the fourth nucleocapsid protein, in viral transcription is still debated.

Recombinant expression of VP40, the major matrix protein, leads to the formation and release of virus-like particles (VLPs) that closely resemble viral particles

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(Kolesnikova et al., 2004; Swenson et al., 2004). This phenomenon has been used, in the form of a VLP system, to study VP40-induced budding and morphogenesis (Kolesnikova et al., 2004, 2007a, 2009; Mittler et al., 2007; Urata & Yasuda, 2010; Urata et al., 2007).

Thus, two systems are currently available to investigate specific steps in the MARV replication cycle: the minigenome system and the VLP system. In the present study, we have combined these two approaches to establish and characterize an infectious VLP system, which can be used to investigate virtually all aspects of the viral life cycle under normal laboratory conditions. The released infectious VLPs are of differing lengths and morphology, with both parameters seeming to influence infectivity. We have determined the optimal amounts of nucleocapsid proteins that result in maximal transcription/replication, and observed that the matrix proteins VP24 and VP40 influenced viral transcription and/or replication. Finally, we have demonstrated that infectious VLP systems are useful tools to partially replace screening experiments, e.g. testing of neutralization titres of antibodies, under BSL-4 conditions.

RESULTS AND DISCUSSION

To advance the currently available MARV minigenome system, with the aim of making the investigation of morphogenesis and release of MARV particles and infection of new target cells accessible under controlled conditions, we started to establish an infectious VLP assay for MARV. As a first step, the CAT reporter gene in the existing minigenome-encoding plasmid 3M-5M (Mühlberger et al., 1998) was replaced by the Renilla luciferase gene by using recombinant PCR, and the functionality of the constructed 3M-5M-Luc was tested. Human embryonic kidney (HEK) 293 cells were transfected with plasmids encoding the MARV nucleocapsid proteins NP, VP35, VP30 and L, the T7 DNA-dependent RNA polymerase and 3M-5M-Luc. We detected luciferase activity in the cell lysates at 48 h post-transfection (not shown). Next, we included plasmids encoding the MARV proteins VP40, VP24 and GP in the system to allow the synthesized minigenome-containing nucleocapsids to be transported to the plasma membrane and released into the supernatant (Dolnik et al., 2008; Hoenen et al., 2006b; Watanabe et al., 2004). Ultrastructural analyses of transfected cells (producer cells) revealed budding of filamentous particles from the plasma membrane (Fig. 1a, upper row). Budding profiles appeared to be similar to budding sites in MARV-infected cells (Fig. 1a, bottom row). Negative staining of released particles and electron microscopic analysis showed that infectious VLPs in the supernatant represented a mixture of spherical and filamentous particles; the latter were of high variability in length (Supplementary Fig. S1, available in JGV Online).

The released filamentous VLPs have a similar morphology to particles released from cells expressing MARV VP40 (Kolesnikova et al., 2009; Mittler et al., 2007). A fraction of the filamentous VLPs contained short electron-dense cores whose structure was similar to MARV nucleocapsids (Supplementary Fig. S1). In comparison to the MARV nucleocapsids, which span the whole length of the virions (Fig. 1a, bottom panel), the average length of the cores was considerably shorter. We found that the length of the minigenome-containing nucleocapsids varied from 104 to 750 nm, with the mean value being 263 ± 162 nm (n=17). These nucleocapsids were found in VLPs of different lengths. Some VLPs seemed to be completely filled with the nucleocapsids, while others contained nucleocapsids at one end while the other part seemed to be empty (Supplementary Fig. S1). Since the minigenome RNA used 3M-5M-Luc that comprises 2216 nt, which is 8.6 times less than the original MARV genome (19 kb), it is conceivable that encapsidation of the minigenome resulted in shorter nucleocapsids. Nevertheless, the average size of the minigenome containing nucleocapsids is approximately double the expected size (865–890 nm divided by 8.6 ± 100 nm) (Geisbert et al., 1995). The average length of the nucleocapsids might, therefore, correspond to two encapsidated minigenomes in a row. However, it is currently unknown how many genomes are encapsidated in MARV particles, and further experiments are needed to ascertain whether the genome length determines the length of the nucleocapsid.

To separate the different forms of VLPs, the preparations were fractionated in a Nycodenz gradient (Kolesnikova et al., 2009). Western blot analysis showed that harvested gradient fractions 2–6 contained viral proteins (Fig. 1b; exemplified by NP and VP40) and particles of different morphology (Fig. 1c). Fractions 2 and 3 mainly contained short filamentous and spherical particles. Fractions 4–6 exclusively contained filaments of up to 6 μm in length (Fig. 1c). We were then interested as to whether the different forms of VLPs were infectious. Therefore, hepatoma (Huh-7) cells pre-transfected with plasmids encoding the MARV nucleocapsid proteins NP, VP35, VP30 and L (indicator cells) were incubated with VLPs derived from the different fractions. We observed that fractions 2–5 all contained infectious VLPs (not shown). The viral proteins incorporated in VLPs of the different fractions were quantified and aliquots containing the same amounts of VP40 were used to infect indicator cells. We found that particles of fraction 3 showed the highest relative ability to induce reporter gene activity in indicator cells (Fig. 1d). The longest VLPs, found in fraction 6, were not able to induce reporter gene activity (Fig. 1c, d). These results demonstrated that VLPs were released from producer cells and were able to infect new target cells. The infectivity of the released VLPs was influenced by length and morphology.

Next, we analysed how changes in the ratio between individual components influenced the established infectious VLP system. Therefore, we titrated the amount of one of the plasmids, while the amount of all other transfected
expression plasmids was left constant. Then the following parameters were monitored: in producer cells (i) amount of protein translated from the titrated plasmid, (ii) the level of tubulin as a cellular control protein, (iii) reporter gene activity, (iv) the release of VLPs, and (v) in indicator cells the ability of VLPs to induce reporter gene activity upon infection.

**NP**

First, we titrated the plasmid encoding the major nucleocapsid protein NP in the background of the established infectious VLP system. As expected, increasing amounts of transfected pCAGGS-NP led to enhanced expression of NP, which did not influence the level of the cellular control protein tubulin (Fig. 2a). NP was essential for the development of reporter gene activity which rose sharply with increasing intracellular amounts of NP and reached a plateau at 0.5 μg of transfected pCAGGS-NP (Fig. 2b; Mühlberger et al., 1998). Western blot analysis of the supernatant of the producer cells showed that the release of VLPs was supported by the presence of NP, as has been previously reported (Fig. 2c; compare amount of VP40 in lanes 1 and 2; Kolesnikova et al., 2004; Swenson et al., 2004).

![Expression of NP in producer cells](image)

**Fig. 1.** Release, morphology and infectivity of VLPs. (a) HEK 293 cells were transfected with plasmids encoding NP, VP35, VP40, GP, VP30, VP24 and L, together with plasmids encoding the T7 polymerase and a MARV-specific minigenome containing the Renilla luciferase reporter gene. At 48 h post-transfection, cells were washed and fixed with paraformaldehyde and subsequently embedded in epon. Ultrathin sectioning was performed and sections were analysed by transmission electron microscopy. (b–d) VLPs were produced as described under (a) and separated over a Nycodenz gradient by ultracentrifugation. The gradient was fractionated and the six fractions were pelleted by ultracentrifugation. The pellets were resuspended and divided into three aliquots. (b) One aliquot was separated by SDS-PAGE and analysed by Western blot by using antibodies against MARV NP and VP40. (c) Another aliquot of the pelleted fractions was fixed with paraformaldehyde, negatively stained and analysed by electron microscopy. The numbers in the panels correspond to the numbers of the fractions. Fraction 1, top; fraction 6, bottom. (d) VLPs from the different fractions containing the same amounts of VP40 were used to infect Huh-7 cells that were pre-transfected with plasmids encoding the MARV nucleocapsid proteins. The cells were lysed at 48 h post-infection, and Renilla luciferase activity was determined.
et al., 2004). The ability to induce reporter gene activity in indicator cells was highest with VLPs released from producer cells that were transfected with 0.2 μg pCAGGS-NP and dropped when VLPs contained more NP (Fig. 2c, d).

**VP35**

VP35 is the polymerase cofactor of MARV, which recruits the polymerase to the nucleocapsid (Becker et al., 1998; Möller et al., 2005; Mühlberger et al., 1998). The intracellular level of VP35 increased with increasing amounts of pCAGGS-VP35 and, for so far unknown reasons, the expression of tubulin was decreased (Fig. 3a).

It has been published previously that VP35 is essential for viral transcription and replication in a MARV-specific minigenome system, and this was supported by our experiments (Fig. 3b; Mühlberger et al., 1998). Transfection of 0.01 μg pCAGGS-VP35, resulted in a 10-fold increase in the reporter signal over background. Reporter signal peaked at 0.1 μg pCAGGS-VP35, dropped more than 10 times at 0.5 μg and reached background levels at 1 μg pCAGGS-VP35 (Fig. 3b).

Increasing intracellular levels of VP35 resulted in an enhanced incorporation of the protein into VLPs (Fig. 3c), but even tiny amounts of VP35 were sufficient to yield infectious VLPs (Fig. 3d). Higher expression rates of VP35, induced by the transfection of more than 0.2 μg pCAGGS-VP35, strongly inhibited the activity of VLPs (Fig. 3d). These results are in line with published data showing that an out-of-balance ratio between NP and VP35 strongly inhibited transcription and replication (Mühlberger et al., 1998), resulting in fewer minigenomes and consequently in fewer infectious VLPs.

**Polymerase L**

Because of a lack of specific antibodies, titration of pCAGGS-L in the background of the infectious VLP system could not be monitored by Western blot (Supplementary Fig. S2a, available in JGV Online). However, even transfection of tiny amounts of L were sufficient to increase the reporter signal by 100-fold over background which reached a plateau at 1 μg pCAGGS-L (Supplementary Fig. S2b, available in JGV Online). L had no influence on the release of VLPs, but the ability of the released particles to induce reporter gene activity in indicator cells was increased with increasing amounts of transfected pCAGGS-L, suggesting that more
minigenome-containing nucleocapsids had been incorporated (Supplementary Fig. S2c, d, available in JGV Online).

**VP30**

The influence of VP30 on MARV transcription and/or replication is currently debated. While it has been published that MARV transcription in a minigenome system is independent of the presence of VP30 (Mühlberger et al., 1998), more recent publications showed that inhibition of VP30 in MARV-infected cells by siRNAs not only shuts down VP30 expression, but also shuts down the expression of other tested viral proteins, suggesting that VP30 positively influences transcription (Fowler et al., 2005). Moreover, attempts to rescue MARV entirely from cDNA were only successful in the presence of VP30 (Enterlein et al., 2006).

In the present study, we found a two- to threefold increase in reporter gene activity in the presence of low amounts of VP30 (Supplementary Fig. S3a, b, available in JGV Online). At higher levels of intracellular VP30, i.e. after transfection of more than 0.1 μg pCAGGS-VP30, the reporter gene activity was slightly decreased. The incorporation of VP30 into released VLPs was enhanced with increased intracellular levels of VP30 (Supplementary Fig. S3c, available in JGV Online). VLPs from producer cells expressing small amounts of VP30 induced more reporter gene activity in indicator cells compared with VLPs from cells without VP30 (Supplementary Fig. S3d, available in JGV Online). Taken together, these data suggest that VP30 plays a role in the process of MARV mRNA synthesis. In contrast to Ebola virus (EBOV) VP30, which dramatically increases EBOV transcription (Mühlberger et al., 1999), MARV VP30 is not absolutely required for this process.

**VP40**

VP40 is the major MARV matrix protein and is able to induce the budding of filamentous particles (Jasenosky et al., 2001; Kolesnikova et al., 2004; Swenson et al., 2004). Matrix proteins of other Mononegavirales were shown to inhibit cellular gene expression, as well as viral RNA synthesis (Ferran & Lucas-Lenard, 1997; Finke et al., 2003; Liu & Ye, 2002; Pulmanasahakul et al., 2008). It was, therefore, interesting to analyze the effect of MARV VP40 on steps in the viral life cycle that can be tested with the newly established infectious VLP system. While upon transfection of increasing amounts of pCAGGS-VP40, the intracellular levels of VP40 were enhanced, the concentration of intracellular tubulin was decreased in samples that have been transfected with more than 0.2 μg pCAGGS-VP40 (Fig. 4a). Increasing VP40 levels also slightly inhibited the reporter gene activity by two- to threefold (Fig. 4b).

The release of VLPs was absolutely dependent on the presence of VP40 and the amount of released VLPs positively correlated with the intracellular level of VP40. The ratio between VP40 and NP in released VLPs was stable, indicating that the number of VLPs in response to higher intracellular levels of VP40 was increased and not the amount of VP40 per particle (Fig. 4c). Interestingly, though the amount of VLPs steadily increased in response to the amount of intracellular VP40, their ability to induce luciferase activity in indicator cells reached a plateau at 0.1 μg transfected pCAGGS-VP40 (Fig. 4d). To further analyze this phenomenon, indicator cells were infected with normalized aliquots of VLPs derived from producer cells expressing different levels of VP40 (Fig. 4e). We observed that particles produced in cells with low level expression of VP40 (0.1 μg transfected plasmid) had the highest capacity to induce reporter gene activity, which dropped sharply with particles derived from cells expressing higher amounts of VP40.

These results indicated that intracellular levels of VP40 control the release of particles, which is consistent with the idea that VP40 orchestrates viral budding (Kolesnikova et al., 2007b). Moreover, the presented results suggested that high concentrations of VP40 impair viral replication and/or transcription as well as expression of a cellular marker protein. It is currently under investigation, whether these two observations are linked and have a common origin, e.g. downregulation of cellular transcription. Finally, VP40 determines infectivity of released VLPs by a so far unknown mechanism.

**GP**

The surface GP, the only transmembrane protein of MARV, is responsible for receptor recognition and fusion
of the virus with the cellular membrane in the endosomal compartment (Becker et al., 1995, 1996; Chandran et al., 2005; Schornberg et al., 2006; Will et al., 1993). In the present study it was of interest to analyse whether intracellular expression of GP influenced viral replication and/or transcription or cellular gene expression, since cytotoxic effects of filovirus glycoproteins have been described before (Alazard-Dany et al., 2006; Volchkov et al., 2001; Yang et al., 2000). Additionally, we wanted to investigate whether the concentration of GP in VLPs influences infectivity. Increased intracellular levels of GP slightly inhibited the expression level of tubulin, while reporter gene activity seemed not to be significantly altered (Fig. 6a, b). Whether the observed inhibition of tubulin expression reflects a discussed cytotoxic effect of the filovirus glycoprotein or has another origin needs to be investigated (Alazard-Dany et al., 2006; Volchkov et al., 2001). Further experiments revealed that, as expected, incorporation of GP is essential for infectivity of released VLPs (Fig. 6c, d). Even very low amounts of GP in infectious VLPs, derived from cells that were transfected with 0.01 µg pCAGGS-GP, were sufficient to increase reporter gene activity in indicator cells by 10-fold over background (Fig. 6d). Those tiny amounts of GP could not be detected with the anti-GP antibody used. Increased intracellular expression of GP enhanced the amount of incorporated GP in released VLPs and their infectivity (Fig. 6c, d). Maximum infectivity was reached with VLPs

Fig. 4. Titration of VP40. HEK 293 cells were transfected with plasmids encoding NP, VP35, GP, VP30, VP24 and L, together with plasmids encoding the T7 polymerase and a MARV-specific minigenome containing the Renilla luciferase reporter gene. The amount of transfected pCAGGS-VP40 was titrated (0, 0.01, 0.05, 0.1, 0.2, 0.5 and 1 µg per well). At 65 h post-transfection, supernatants were harvested, cells were lysed and aliquots of both cell lysates and supernatant were subjected to SDS-PAGE and Western blot analysis (a, c). Cell lysates were also tested for Renilla luciferase activity, indicating viral transcription and replication (b). (d) Aliquots of the supernatant were also used to infect Huh-7 cells that were previously transfected with plasmids encoding the MARV nucleocapsid proteins. At 65 h post-infection, indicator cells were lysed and Renilla luciferase activity in the lysates was determined. (e) Indicator cells were infected with identical amounts of VLPs from the supernatant of (a) produced after transfection of different amounts of VP40. Reporter activity was determined in indicator cells at 65 h post-infection.
purified from cells transfected with 0.5 μg pCAGGS-GP. Further doubling the amount of transfected pCAGGS-GP to 1 μg slightly increased the amount of GP per particle, but neither enhanced nor inhibited the infectivity of VLPs (Fig. 6c, d). Taken together, incorporation of GP into VLPs is saturable and controls dose dependently their infectivity. This finding is in line with results published by Marzi et al. (2006), showing that while titrating the amount of Ebola virus GP incorporated into lentivirus pseudotypes, the concentration of incorporated GP influenced binding affinity to cellular receptors.

The composition of expression plasmids that gave optimal results in the MARV-specific infectious VLP system is presented in Supplementary Table S1 (available in JGV Online).

**Application of the infectious VLP system**

In order to demonstrate that the established infectious VLP system is suitable as a screening tool, we tested whether it could be used to determine neutralization titres of anti-MARV sera. For this purpose, we prepared VLPs from a 75 cm² cell culture dish. To determine the infectivity of the preparation, we serially diluted the sample and used the dilutions to infect indicator cells. At a dilution of 1:128, VLPs induced reporter signals that were 25 times above background level (not shown). This dilution was then used to test the neutralizing activity of a goat anti-MARV serum raised against gamma-irradiated MARV. The diluted infectious VLP preparation was incubated for 1 h with serial dilutions of the goat serum at room temperature. The incubated samples were then transferred to pre-transfected indicator cells in 96-well plates. Tests were performed in quadruplicate. At 48 h post-infection, cells were incubated with a cell permeable luciferase substrate and reporter gene activity was determined (Fig. 7). The anti-MARV serum was able to suppress reporter gene activity completely up to a dilution of 1:32. An inhibition of 50% reporter gene activity was observed at dilutions of 1:128. At a dilution of 1:512, the serum conferred no inhibition of infectious VLPs. The same serum was tested for neutralization of infectious MARV under BSL-4 conditions. Serial serum dilutions were incubated with 10 TCID₅₀ units of MARV for 1 h, and subsequently VeroE6 cells were infected with the serum-treated virus. Plates were evaluated after 10 days, when cytopathic effects were clearly visible.
Evaluation of the plates showed that the neutralizing titre of the serum for infectious MARV particles was in the same range as has been determined with the infectious VLP system (Supplementary Table S2, available in JGV Online). This experiment shows that the MARV infectious VLP system can be used to partially substitute experiments under BSL-4 conditions. This is especially valuable for screening tasks, such as the testing of sera or antiviral compounds.

Taken together, we have established and characterized a MARV-specific infectious VLP system. Infectious VLPs with a very high efficiency in establishing infection in target cells are derived from cells that express low levels of VP40, which reflects early stages of viral infection. The established infectious VLP system can be used to investigate in detail the complete viral replication cycle and opens the field for high-throughput screening of MARV-specific antisera or antivirals.

**METHODS**

**Cell lines, viruses.** HEK 293 and Huh-7 cells were maintained at 37 °C and 5 % CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 50 U penicillin ml⁻¹, 50 µg streptomycin ml⁻¹ as well as 2 mM glutamine. MARV strain Musoke was propagated and analysed in Vero cells as described previously (Kolesnikova et al., 2009). All experiments with infectious MARV were performed in the BSL-4 laboratory at the Institute of Virology in Marburg, Germany.

**Plasmids.** Plasmids encoding the MARV structural proteins NP, VP35, VP30 and L were subcloned from the expression vector pTM1 (Mühlberger et al., 1998) into the vector pCAGGS by using unique restriction sites. Construction of the plasmids pCAGGS-GP and pCAGGS-VP40 has been described by Mittler et al. (2007). Construction of pCAGGS-VP24 has been described by Bamberg et al. (2005). The plasmid 3M-5M (Mühlberger et al., 1999), containing the CAT gene flanked by the leader and trailer regions of the MARV genome (strain Musoke, GenBank accession no. Z12132), and the Ebola virus-specific minigenome plasmid 3E-5E, containing the Renilla luciferase (Hoennen et al., 2006a), were used for the construction of the artificial minigenome 3M-5M-Luc. Recombinant PCR was used to generate a fragment containing the MARV leader and the Renilla luciferase gene. The amplified product was subcloned into the 3M-5M vector using the restriction enzymes RsHl and NotI, thereby replacing the leader and the CAT gene. Detailed cloning strategies are available upon request. The plasmid pCAGGS-T7, encoding the T7 DNA-dependent RNA polymerase, was kindly provided by Dr Yoshihiro Kawaoka (University of Tokyo & University of Wisconsin – Madison).

**Antibodies.** Mouse monoclonal antibodies specific for MARV GP, VP40, NP, VP30 and α-tubulin (Sigma Aldrich) were used. The detection of VP24 and VP35 was performed by using affinity purified rabbit and guinea pig sera, respectively. For neutralization assays, a goat anti-MARV serum was used that was raised against gamma-irradiated (60 kGy) MARV. Secondary antibodies against mouse, rabbit or guinea pig conjugated to peroxidase were used for Western blot (DAKO).

**Transfection of cells.** Producer cells: HEK 293 cells grown in a six-well plate were transfected with plasmids encoding all MARV structural proteins (per one dish: 1000 ng pCAGGS-L, 500 ng pCAGGS-NP, 100 ng pCAGGS-VP35, 100 ng pCAGGS-VP30, 70 ng pCAGGS-VP24, 500 ng pCAGGS-VP40 and 500 ng pCAGGS-VP40 without otherwise stated) and the T7-driven minigenome 3M-5M-Luc (1000 ng) using FUGENE 6 (Roche) according to the manufacturer’s instructions. Transfected cells were incubated at 37 °C under 5% CO₂. At 12 h post-transfection, medium was removed and fresh medium was added. At approximately 65 h post-transfection, supernatant was harvested and cells were washed once with ice-cold PBS, scraped into 1 ml PBS, transferred to a centrifuge tube and spun down. The pellet was lysed in 500 µl passive lysis buffer (Promega) and reporter activity was determined using Promega’s Renilla luciferase reporter assay according to the manufacturer’s instructions and a Berthold LB 960 Centro luminometer. Indicator cells: Huh-7 cells were transfected at 70% confluence with pCAGGS-L, pCAGGS-NP, pCAGGS-VP35 and pCAGGS-VP30 as described above.

**Infectious VLP assay.** Harvested supernatant of producer cells was cleared from cellular debris by centrifugation at 971 g for 10 min and then pelleted through a 20% sucrose cushion at 163 659 g for 3 h at 4 °C. The pellet was resuspended in 50 µl DMEM.

Infection with VLPs: at 24 h post-transfection, Huh-7 cells expressing L, NP, VP35 and VP30 (indicator cells, transfection procedure see above) were incubated with VLPs in 50 µl DMEM for 1.5 h at 37 °C and 5% CO₂. Then, 3.5 ml DMEM medium supplemented with 2.5% FCS was added and the cells were incubated for 65 h before reporter gene analysis.

**Protease protection assay.** The assay was performed essentially as described previously (Bamberg et al., 2005).

**Determination of infectious titre and neutralization assay.** Infectivity of VLPs was determined by serially diluting VLPs in DMEM. These dilutions were then inoculated onto pre-transfected Huh-7 cells. At 65 h post-infection, reporter activity was detected by using affinity purified rabbit and guinea pig sera, respectively. For neutralization assays, a goat anti-MARV serum was used that was raised against gamma-irradiated MARV antigen, with VLPs for 1 h at room temperature before inoculating the suspension onto pre-transfected Huh-7 cells in 96-well plates (see above, indicator cells). VLPs incubated with a control goat antibody were used as a positive

![Fig. 7. Application of infectious VLPs to determine the neutralization titre of a goat anti-MARV serum. Infectious VLPs were purified as described under Fig. 1, serially diluted and the dilutions were used to infect pre-transfected Huh-7 cells. A dilution of infectious VLPs that gave a luciferase signal that was approximately 50-fold over background was used to determine the neutralization titre of a goat anti-MARV serum. (—), Without infectious VLPs; (+), without anti-MARV serum.](image-url)
control and mock infection served as a negative control. At 65 h post-infection, reporter activity was quantified.

Neutralization of MARV and TCID50 tests under BSL-4 conditions were performed as described by Kolesnikova et al. (2009).

Density gradient analysis of infectious VLPs. HEK 293 cells were transfected with the indicated plasmids as described above. Supernatants were harvested and pelleted through a 20% sucrose cushion at 163,659 g for 3 h at 4 °C. The pellet was resuspended in TNE [10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8)], loaded onto a Nycodenz step gradient and centrifuged at 26,292 g for 15 min at 4 °C. The gradient was composed of seven steps: 30 (730 μl), 20, 15, 10, 7.5, 5, 2.5% (490 μl each) Nycodenz (bottom to top). After centrifugation, six 0.5 ml fractions were harvested from the top, diluted in TNE and pelleted by centrifugation at 90,720 g for 2 h at 4 °C. Infectious VLPs were resuspended in 100 μl TNE and used for infection of Huh-7 cells, Western blot and electron microscopy analysis.

Electrophoresis and immunoblot analysis. Western blot analysis was carried out as described previously (Kolesnikova et al., 2004). The antibodies are listed in the figure legends. The intensities of the bands for cell- and VLP-associated proteins were quantified by using the Odyssey imaging system (Licor).

Electron microscopy. Ultrastructural analyses of MARV particles and VLPs, as well as MARV-infected cells and cells that produced VLPs were performed as described by Kolesnikova et al. (2000, 2002, 2007a).

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