The Cytoplasmic Domain of Marburg Virus GP Modulates Early Steps of Viral Infection[▽]

Eva Mittler,¹ Larissa Kolesnikova,¹ Bettina Hartlieb,^{1†} Robert Davey,² and Stephan Becker^{1*}

Institut für Virologie, Philipps Universität Marburg, 35043 Marburg, Germany,¹ and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas²

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Marburg virus infection is mediated by the only viral surface protein, GP, a trimeric type I transmembrane protein. While its ectodomain mediates receptor binding and fusion of viral and cellular membranes and its transmembrane domain is essential for the recruitment of GP into budding particles by the matrix protein VP40, the role of the short cytoplasmic domain has remained enigmatic. Here we show that a missing cytoplasmic domain did not impair trimerization, intracellular transport, or incorporation of GP into infectious Marburg virus-like particles (iVLPs) but altered the glycosylation pattern as well as the recognition of GP by neutralizing antibodies. These results suggest that subtle conformational changes took place in the ectodomain. To investigate the function of the cytoplasmic domain during viral entry, a novel entry assay was established to monitor the uptake of filamentous VLPs by measuring the occurrence of luciferase-labeled viral nucleocapsids in the cytosol of target cells. This quantitative assay showed that the entry process of VLPs incorporating GP missing its cytoplasmic domain (GP Δ CD) was impaired. Supporting these results, iVLPs incorporating a mutant GP missing its cytoplasmic domain were significantly less infectious than iVLPs containing wild-type GP. Taken together, the data indicate that the absence of the short cytoplasmic domain of Marburg virus GP may induce conformational changes in the ectodomain which impact the filoviral entry process.

The *Filoviridae* family comprises Marburg virus (MARV) and Ebola virus (EBOV), the causative agents of fulminant hemorrhagic fevers in humans and nonhuman primates (24, 37). Dramatic outbreaks of filoviruses in sub-Saharan Africa and the importation of MARV into Europe and the United States emphasize their emerging potential (9, 54). While promising results have been obtained with different experimental vaccine approaches, to date, neither a vaccine nor effective antiviral treatment against MARV or EBOV hemorrhagic fevers is approved for human use (19, 32).

Filoviruses contain a single-stranded negative-sense RNA genome which is enwrapped by the nucleoprotein NP to form the helical nucleocapsid complex, which also contains the viral proteins VP35, VP30, VP24, and L (6). The nucleocapsid is surrounded by the viral matrix protein VP40, connecting the nucleocapsid with the viral envelope (6). Homotrimers of the single surface protein, GP, a type I transmembrane protein, are incorporated into the envelope (15). GP is composed of two subunits, GP₁ (170 kDa) and GP₂ (50 kDa), which are formed by proteolytic cleavage of the precursor, GP₀ (57). GP₂ is inserted into the viral envelope via its transmembrane domain and is linked to GP₁ by disulfide bridges (15). GP is highly N and O glycosylated, with approximately 50% of its apparent molecular mass being represented by sugar side chains (15, 20). Glycosylation of GP is composed of mannose-rich and complex-type N-glycans as well as mucin-type O-glycans; the

latter are concentrated in a mucin-like domain (MLD) spanning amino acid residues 289 to 501 (20). At the boundary between the transmembrane and cytoplasmic domains, GP is acylated at two cysteine residues, which may be necessary for a secure anchorage of the ectodomain in the viral envelope (5, 18, 27).

While it is well established that GP recognizes target cells by binding to cellular attachment factors, mainly lectins, several candidate receptors have been identified, and their roles are still debated (3, 7, 10, 21, 38, 49, 50, 53). The closely related EBOV probably enters target cells by using macropinocytosis-like mechanisms; the entry of MARV, on the other hand, has not yet been investigated in detail (41, 45). Virus-cell fusion mediated by EBOV GP has been shown to be dependent on the activity of endosomal cathepsins, especially cathepsins B and L (11, 47, 48). Fusion between the filoviral envelope and cellular membranes is presumably initiated by a conformational change in GP, resulting in the presentation of an otherwise hidden internal hydrophobic fusion loop at the protein surface. The fusion loop is then inserted into the target membrane, which finally induces fusion of viral and endosomal membranes (33, 60, 61).

While the function of the ectodomain and also, to some extent, the transmembrane domain of the filoviral GP has been addressed, the role of the four (EBOV)- or eight (MARV)-amino-acid long cytoplasmic domain is completely enigmatic. It has been described for other viral transmembrane proteins that the absence of the cytoplasmic domain affects multiple steps in virus assembly, egress, and infectivity. Using a recently developed infectious virus-like particle (iVLP) assay for MARV (62), we have investigated the role of the cytoplasmic domain of MARV GP. We present data showing that entry of

* Corresponding author. Mailing address: Institut für Virologie, Philipps Universität Marburg, Hans-Meerwein Str. 2, 35043 Marburg, Germany. Phone: 49 6421 2866253. Fax: 49 6421 2868962. E-mail: becker@staff.uni-marburg.de.

† Present address: Baxter Healthcare GmbH, 1020 Vienna, Austria.

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VLPs into target cells is strongly impaired when these are decorated with tailless GP (GP Δ CD). This is in line with a significantly reduced infectivity of iVLPs decorated with GP Δ CD. We further show that this occurs because deletion of the cytoplasmic domain results in conformational changes in the ectodomain, as demonstrated by an altered glycosylation pattern of GP and a reduced sensitivity of GP to neutralizing antibody.

MATERIALS AND METHODS

Cell lines. Human embryonic kidney cells (HEK 293) and a human hepatoma cell line (HUH-7) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2% L-glutamine, and a penicillin-streptomycin solution. The cells were cultivated in an incubator at 37°C under 5% CO₂.

Molecular cloning. Plasmids encoding MARV and EBOV NP, VP35, VP30, L, VP40, VP24, and GP under the control of a chicken β -actin promoter (vector pCAGGS) were constructed as described elsewhere (4, 22, 40, 62). The construction of a MARV-specific artificial minigenome encoding *Renilla* luciferase as a reporter protein under the control of a T7 promoter was described by Wenigenrath et al. (62). The plasmid pCAGGS-T7, encoding the T7 DNA-dependent RNA polymerase, was kindly provided by Y. Kawaoka (University of Tokyo, Japan, and University of Wisconsin, Madison, WI). Generation of pCAGGS-MARV GP Δ CD, in which the cytoplasmic domain coding sequence is completely deleted, was described previously by Mittler et al. (40). For MARV GP Δ MLD, the MLD, comprising amino acids 289 to 501, was removed by recombinant PCR (23), followed by ligation of the amplification product into pCAGGS by use of SmaI and SacI. Cloning of EBOV GP_F535R was performed with a QuikChange Multi site-directed mutagenesis kit (Stratagene, Heidelberg, Germany), using pCAGGS-EBOV GP as the template. EBOV VP30-Luc, composed of amino acids 142 to 272 of VP30 C-terminally linked to firefly luciferase, was constructed with the help of recombinant PCR. VP30 sequences encoding amino acids 142 to 272 were amplified using pCAGGS-EBOV VP30_{CTD} (22), and sequences encoding firefly luciferase were amplified using the vector pGL4.1 as a template (Promega, Mannheim, Germany). Subsequently, amplified products were linked via recombinant PCR and subcloned into pCAGGS by use of EcoRI and NotI. All constructs were verified by DNA sequencing. Detailed cloning strategies as well as primer sequences are available upon request.

Antibodies. Mouse monoclonal antibodies were used for the detection of MARV VP40 and NP (dilution for Western blotting [WB], 1:200), MARV GP (dilution for WB, 1:100; dilution for immuno-electron microscopic analysis [IEM], 1:50), EBOV GP (dilution for WB, 1:200), EBOV VP40 (dilution for WB, 1:200), and EBOV NP (dilution for immunofluorescence analysis [IF], 1:20). The presence of EBOV VP30 was verified with a guinea pig anti-VP30 IgG (kindly provided by V. Volchkov, Lyon, France) (dilution for IF, 1:50), and VP30-Luc was detected using a monoclonal anti-firefly luciferase antibody (Sigma Aldrich, Munich, Germany) (dilution for WB, 1:150). For neutralization assays, a goat anti-MARV serum raised against gamma-irradiated MARV was used (kindly provided by M. Niedrig, Berlin, Germany). Secondary antibodies conjugated with Texas Red or fluorescein isothiocyanate (FITC) (Dianova, Hamburg, Germany) were used for IF analysis (dilution, 1:200). Secondary antibodies conjugated to Alexa Fluor 680 (Molecular Probes, Karlsruhe, Germany) and to horseradish peroxidase (HRP; Dako, Glostrup, Denmark) were used for WB (dilutions, 1:5,000 and 1:30,000, respectively). Secondary antibodies conjugated to 5-nm colloidal gold particles were used for IEM (BB International, Cardiff, United Kingdom) (dilution, 1:30).

Infectious VLP assay. Production of MARV-specific iVLPs was carried out as described previously (62). As indicated in the figure legends, wild-type MARV GP was replaced by MARV GP Δ MLD or MARV GP Δ CD.

Neutralization assay. Neutralization assays were performed using a neutralizing goat anti-MARV serum as described previously (62).

Purification of filamentous particles from cell supernatants. HEK 293 cells in T75 cell culture flasks were cotransfected with plasmids encoding EBOV NP (1 μ g), VP35 (1 μ g), L (8.3 μ g), VP40 (2 μ g), VP30-Luc (12.5 μ g), and GP (2 μ g). As indicated in the figure legends, EBOV GP was replaced by EBOV GP_F535R, MARV GP, or MARV GP Δ CD. At 48 h posttransfection (p.t.), supernatants from HEK 293 cells were harvested, and filamentous particles were purified as described previously (40). Briefly, the supernatants were pelleted through a 20% sucrose cushion at 30,000 rpm for 2.5 h at 4°C, using an SW32 rotor (Beckman Coulter, Palo Alto, CA). After resuspension of the pellet in

TNE buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8]), samples were overlaid on a Nycodenz step gradient (Axis-Shield, Oslo, Norway) and centrifuged in a SW60 rotor (Beckman Coulter, Palo Alto, CA) at 16,000 rpm for 15 min at 4°C. The Nycodenz gradient was composed of seven steps, containing 2.5%, 5%, 7.5%, 10%, 15%, 20%, and 30% Nycodenz diluted in TNE (top to bottom). Fractions (500 μ l) were collected from the top: fractions 1 to 3 were discarded, and fractions 4 to 6 (filamentous VLPs) were pooled. To concentrate filamentous VLPs, pooled fractions 4 to 6 were centrifuged in a TLA45 rotor (Beckman Coulter, Palo Alto, CA) at 45,000 rpm for 1.5 h at 4°C. The resulting pellets were resuspended in 60 μ l TNE buffer and subjected to Western blot analysis and entry assays. The luciferase activity associated with purified filamentous VLPs was determined by incubating 10 μ l of VLPs with passive lysis buffer, followed by addition of the cell-permeating luciferase substrate luciferin (both from Promega, Mannheim, Germany) and subsequent measurement in a luminometer (Berthold, Bad Wildbad, Germany). To verify the integrity of filamentous VLPs, resuspended VLPs were incubated with luciferase assay buffer (Promega, Mannheim, Germany) to which luciferin was added, allowing the measurement of luciferase activity.

Entry assay. Confluent HEK 293 cells were removed from the bottom of T75 cell culture flasks by use of cell dissociation buffer (Gibco, Karlsruhe, Germany), pelleted by centrifugation, and resuspended in 2 ml fresh DMEM without supplements. Subsequently, 200 μ l of the cell suspension was mixed with 10 μ l purified filamentous VLPs (see above) and incubated at 37°C on a rotating wheel at 10 rpm for the indicated intervals. To remove unbound filamentous VLPs, cells were pelleted by centrifugation at 200 $\times$ g for 4 min at 4°C, the supernatant was discarded, and pellets were washed twice with ice-cold DMEM without supplements and once with phosphate-buffered saline (PBS_{def}; 136 mM NaCl, 27 mM KCl, 605 mM Na₂HPO₄, 1.5 mM KH₂PO₄). The final cell pellet was resuspended in 50 μ l of luciferase assay buffer, followed by addition of luciferin (both from Promega, Mannheim, Germany) and measurement of luciferase activity in a luminometer (Berthold, Bad Wildbad, Germany). For each entry assay and each time point, intracellular luciferase activity was correlated to the total luciferase activity of the respective filamentous VLP preparation (see above).

Electrophoresis and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were carried out as described previously (30). The antibodies used are listed in the figure legends.

IEM analysis. For quantification of the number of gold particles bound to GP incorporated into the iVLP envelope, we used the method of microscopic analysis described by Mittler et al. (40).

Chemical cross-linking. Purified iVLPs were incubated with increasing concentrations of ethylene glycol bis(succinimidylsuccinate) (EGS; Thermo Fischer, Waltham, MA). The concentrations used were 0.01, 0.05, 0.1, 0.5, and 1 mM EGS. After 30 min at room temperature, the reaction was quenched with 50 mM Tris, pH 8.0. The cross-linked samples were separated by SDS-PAGE, and GP-specific bands were visualized by Western blotting.

Indirect immunofluorescence analysis. Immunofluorescence analysis was carried out as described elsewhere (40).

Lectin binding assay. Purified iVLPs were subjected to SDS-PAGE and blotted onto a nitrocellulose membrane. The glycosylation pattern of GP was characterized and quantified using a DIG glycan differentiation kit (Roche, Mannheim, Germany). In parallel, the amount of NP incorporated into iVLPs was determined by performing a Western blot analysis. NP was detected by using a mouse monoclonal anti-NP antibody and a secondary anti-mouse IgG linked to Alexa Fluor 680. Quantification was carried out with a LiCor Odyssey infrared imaging system. The binding of lectins was normalized to the amount of NP incorporated into the iVLPs.

In vitro proteolysis of GP with cathepsin L. Purified filamentous VLPs were treated with 20 ng/ μ l recombinant cathepsin L (CatL; Calbiochem, Darmstadt, Germany) at pH 5.5 in 40 mM HEPES, 40 mM morpholineethanesulfonic acid (MES), 50 mM NaCl, and 4 mM dithiothreitol (DTT) at 37°C. Control treatments were conducted in the same buffer without enzyme. CatL proteolysis was terminated at the indicated time points by the addition of E-64 (10 μ M; Calbiochem, Darmstadt, Germany). Cleavage of GP by CatL was detected by Western blot analysis using two GP₁-specific mouse monoclonal antibodies and a secondary HRP-conjugated goat anti-mouse antibody.

RESULTS

Cytoplasmic domain of MARV GP has no impact on its incorporation into iVLPs. To address the function of the cy-

FIG. 1. Incorporation of MARV GP into iVLPs is independent of the presence of its cytoplasmic domain. (A) Schematic representation of MARV GP deletion mutant. Filoviral glycoproteins are composed of the N-terminal ectodomain (ED), the hydrophobic membrane-spanning transmembrane domain (TMD), and the C-terminal cytoplasmic domain (CD). The deletion mutant of MARV GP lacking the cytoplasmic tail is designated MARV GPΔCD. (B) Cellular expression and incorporation of MARV wild-type and truncated glycoproteins into iVLPs. HEK 293 cells were transfected with plasmids carrying a MARV-specific minigenome containing the *Renilla* luciferase reporter gene and with plasmids encoding the T7 DNA-dependent RNA polymerase and all structural MARV proteins, including MARV GP or MARV GPΔCD. At 60 h p.t., particulate material in the cellular supernatant was pelleted through a 20% sucrose cushion. Purified iVLPs (lanes 5 to 8) and lysed cells (lanes 1 to 4) were subjected to SDS gel electrophoresis and blotted onto a nitrocellulose membrane. Immunostaining was performed with mouse monoclonal anti-MARV GP and anti-MARV NP, as well as anti-MARV VP40 IgG. Bound antibodies were detected by incubation with a secondary Alexa Fluor 680- or HRP-coupled anti-mouse antibody. (C) Analysis of MARV GPΔCD incorporation into iVLPs via immuno-electron microscopy. iVLPs were purified from the supernatant of HEK 293 cells as described for panel B, followed by fixation with paraformaldehyde. (Left) Glycoproteins were detected using a mouse monoclonal anti-MARV GP IgG and a goat anti-mouse antibody coupled with colloidal gold (5-nm gold beads). (Right) To evaluate the incorporation of GP into iVLPs, the density of gold particles was quantified (number of analyzed iVLPs, ≥ 10). Data represent the mean values, and error bars indicate standard deviations.

toplasmic domain of MARV GP, we made use of a deletion mutant in which the cytoplasmic domain was completely removed (GPΔCD) (Fig. 1A) (40). Analysis of transiently expressed GPΔCD revealed that its intracellular expression rate and transport were not distinguishable from those of wild-type GP (data not shown). We then employed GPΔCD in a recently established iVLP assay for MARV which serves as a model system for filoviral infection, allowing work under biosafety level 2 conditions, and analyzed the incorporation of GPΔCD into released iVLPs (62). HEK 293 cells were transfected with plasmids encoding GPΔCD or GP and all other MARV structural proteins, as well as the T7 polymerase, and with a

MARV-specific minigenome encoding the reporter protein *Renilla* luciferase. At 60 h p.t., cells were harvested and released iVLPs were purified from the supernatant. Western blot analysis of the cell lysates and iVLPs revealed that despite removal of the cytoplasmic domain, GPΔCD was efficiently expressed and incorporated into MARV-specific iVLPs in amounts comparable to those for wild-type GP (Fig. 1B). In addition, the protein composition of iVLPs (exemplified by the amounts of NP and VP40) was not altered by the incorporation of GPΔCD. To confirm these data, we evaluated the amount of incorporated GP in the specific filamentous iVLPs by IEM (34, 40). Quantification revealed that incorporation of GPΔCD was not significantly changed in comparison to that of wild-type GP (Fig. 1C) (22.5 ± 8.9 versus 24.9 ± 4.5 gold particles per μm). These results support the Western blot analysis of the released particles, on the one hand, and also support previously published data based on VLPs formed exclusively by VP40 and GP or GPΔCD (Fig. 1B) (40). All experimental approaches indicated that the cytoplasmic tail had no significant impact on the incorporation of GP into iVLPs or on the secure anchorage of GP to the lipid envelope.

Deletion of the cytoplasmic domain induces changes in the glycosylation pattern of MARV GP. To further characterize the deletion mutant GPΔCD, we then analyzed co- and posttranslational modifications of GPΔCD incorporated into iVLPs in comparison to those of GP. Using chemical cross-linking, we determined whether deletion of the cytoplasmic tail affected trimerization of GP and found wild-type GP as well as GPΔCD in monomeric, dimeric, and trimeric forms, suggesting that oligomerization was not significantly disturbed by removal of the cytoplasmic domain (Fig. 2A, compare columns 1 to 6 and 7 to 12).

Next, we analyzed the glycosylation patterns of GP and GPΔCD incorporated into purified iVLPs by performing a lectin binding study. Quantification of lectins bound to GPΔCD in comparison to GP revealed similar signals for high-mannose N-glycosylation (Fig. 2B) ($101\% \pm 9.9\%$ relative binding of the lectin *Galanthus nivalis* agglutinin [GNA]) and a slightly diminished signal for complex N-glycosylation (Fig. 2B) ($82.8\% \pm 3.2\%$ relative binding of the lectin *Datura stramonium* agglutinin [DSA]). Absence of the cytoplasmic tail of GP caused a significant decrease in binding of the lectin peanut agglutinin (PNA), suggesting that the loss of the cytoplasmic domain interfered with O-glycosylation of GP (Fig. 2B) ($50.3\% \pm 9.4\%$ relative lectin binding).

MARV GP cytoplasmic domain significantly influences the infectivity of iVLPs. To further determine the function of the MARV GP cytoplasmic tail during the infection process, purified iVLPs incorporating GP or GPΔCD were used to infect HUH-7 cells, which were pretransfected with plasmids encoding the MARV nucleocapsid proteins. Reporter protein activity in cell lysates was determined at 60 h postinfection (p.i.) (Fig. 3A) (62). Interestingly, iVLPs containing GPΔCD showed a significantly reduced infectivity in comparison to iVLPs containing wild-type GP (Fig. 3A) (23% infectivity). The same result was obtained when HEK 293 cells were used as target cells for infection with iVLPs (data not shown).

The data obtained so far led to two hypotheses: (i) the alterations in O-glycosylation are causative for the reduced infectivity of iVLPs containing GPΔCD; and (ii) the altered

FIG. 2. Influence of cytoplasmic tail on posttranslational modifications of MARV GP. (A) Oligomerization of MARV GP and MARV GPΔCD. HEK 293 cells were transfected and iVLPs were purified as described in the legend to Fig. 1. Glycoproteins incorporated into iVLPs were subjected to chemical cross-linking using increasing concentrations (0, 0.01, 0.05, 0.1, 0.5, and 1 mM) of the cross-linker EGS, followed by Western blot analysis. Immunostaining of cross-linked glycoproteins was performed by using a monoclonal anti-MARV GP IgG. Bound antibodies were detected by a secondary HRP-coupled anti-mouse antibody. Monomers, dimers, and trimers are indicated. (B) Quantification of N- and O-glycosylation of GP and GPΔCD incorporated into iVLPs. HEK 293 cells were subjected to transfection and iVLPs were pelleted through a 20% sucrose cushion as described in the legend to Fig. 1. Filamentous iVLPs containing MARV GP or MARV GPΔCD were subjected to a lectin binding assay. Binding of specific lectins to MARV GP or MARV GPΔCD was normalized against the amount of NP associated with released iVLPs. Data represent the mean values and standard deviations for three independent experiments. Asterisks indicate statistically significant differences (*, $P < 0.05$; ***, $P < 0.001$) in comparison to filamentous iVLPs containing MARV GP (set to 100%).

O-glycosylation reflects a conformational change in the ectodomain, which causes the reduced ability of GPΔCD to mediate infection.

To investigate whether reduction of O-glycosylation in GP directly impaired infectivity of iVLPs (the first hypothesis), we constructed a GP mutant lacking the mucin-like domain, and therefore all O-glycosylation sites (GPΔMLD) (Fig. 3B). Lectin analysis of GPΔMLD revealed that this mutant was indeed devoid of O-glycans (data not shown). Examination of GPΔMLD incorporation into the MARV-specific iVLP system showed no differences from wild-type GP (Fig. 3C, upper panel). In addition, iVLPs that contained GPΔMLD were released in the same amount as iVLPs with wild-type GP (Fig. 3C, lower panel). Likewise, infectivity of iVLPs containing GPΔMLD was not reduced significantly in comparison to that of iVLPs incorporating wild-type GP (Fig. 3D). These results indicate that O-glycosylation of MARV GP is dispensable for efficient infection of target cells and that altered O-glycosyla-

FIG. 3. Role of MARV GP cytoplasmic tail and GP O-glycosylation in infection of target cells. (A) Infectivity of iVLPs containing MARV GP deletion mutant. HEK 293 cells were transfected and supernatants and cell lysates were harvested at 60 h p.t. as described in the legend to Fig. 1. iVLPs purified from the supernatant were used to infect target cells which had been pretransfected with plasmids encoding MARV NP, VP35, VP30, and L. Cells were harvested at 60 h p.i., and cell lysates were assayed for *Renilla* luciferase reporter gene activity. Data represent the mean values and standard deviations for five independent experiments. Asterisks indicate statistically significant differences (***, $P < 0.001$) in comparison to iVLPs containing wild-type MARV GP (set to 100%). (B) Schematic representation of MARV GP lacking the mucin-like domain. The MARV surface protein is composed of the N-terminal ectodomain (ED), the hydrophobic membrane-spanning transmembrane domain (TMD), and the C-terminal cytoplasmic tail (CD). A deletion mutant of MARV GP lacking the mucin-like domain (MLD) is designated GPΔMLD. (C) Incorporation of GPΔMLD into iVLPs. HEK 293 cells were transfected and iVLPs were purified as described in the legend to Fig. 1. Purified iVLPs containing either wild-type MARV GP (lane 1) or MARV GPΔMLD (lane 2) were subjected to SDS gel electrophoresis and blotted onto a nitrocellulose membrane. Immunostaining was performed with monoclonal mouse anti-MARV GP or anti-MARV NP IgG, followed by incubation with a secondary HRP-coupled anti-mouse antibody. Released iVLPs showed comparable incorporation of MARV GP and MARV GPΔMLD. (D) Infectivity of iVLPs containing MARV GPΔMLD. HEK 293 cells were transfected as described in the legend to Fig. 1. Purified iVLPs were then used to infect HUH-7 cells, which were pretransfected with plasmids encoding MARV NP, VP35, VP30, and L. At 60 h p.i., cells were harvested and cell lysates assayed for *Renilla* luciferase reporter activity. The data represent the mean values and standard deviations for four independent experiments. Asterisks indicate statistically significant differences (*, $P < 0.05$) in comparison to iVLPs containing MARV GP (set to 100%).

tion most likely did not cause the observed reduced infectivity of iVLPs incorporating GPΔCD.

Modifications in O-glycosylation of GP indicate structural changes in its ectodomain. We presumed that the observed modifications in the O-glycosylation of GPΔCD were caused by slight conformational changes in its ectodomain which led

FIG. 4. Neutralization assay of iVLPs. HEK 293 cells were transfected with plasmids encoding all structural filoviral proteins, including MARV GP or MARV GP Δ CD, with a MARV-specific minigenome containing the *Renilla* luciferase reporter gene, and with a plasmid encoding the T7 DNA-dependent RNA polymerase. At 60 h p.t., iVLPs were harvested from the supernatant and pelleted through a 20% sucrose cushion. iVLPs were titrated, and aliquots that induced luciferase activity of approximately 10^5 relative light units in infected cells were incubated with serial dilutions of a goat anti-MARV serum for 1.5 h. Suspensions were inoculated onto HUH-7 cells pretransfected with plasmids encoding the MARV nucleocapsid proteins NP, VP30, VP35, and L. Incubation of iVLPs with undiluted antibody served as a positive control (inhibition of iVLP infectivity set to 100%), and iVLPs treated with no antibody were used as a negative control (data not shown). At 48 h p.i., *Renilla* luciferase reporter activity was determined by incubating the cells with the live cell substrate EnduRen. The serum dilution that reduced the luciferase activity in infected cells by 50% was determined (dashed line).

to an impaired accessibility of O-glycan attachment sites for glycosyltransferases. Conformational changes in mutant proteins can be verified by quantifying binding and neutralizing activities of specific antibodies. Changes in comparison to the wild-type protein indicate increased exposure and masking of protein epitopes, respectively (2, 14, 51, 58).

We performed a neutralization assay with iVLPs incorporating GP or GP Δ CD (60) and determined the dilution of MARV neutralizing serum that inhibited infectivity of iVLPs by 50%. While iVLPs incorporating GP Δ CD were inhibited at a serum dilution of approximately 1:32, neutralization of iVLPs containing wild-type GP was already accomplished at serum dilutions of 1:256 to 1:512 (Fig. 4). Since there were equal amounts of GP and GP Δ CD incorporated into iVLPs (Fig. 1), these results suggest that the absence of the cytoplasmic domain of GP does indeed influence the conformation of the ectodomain and leads to structural modifications of immunodominant epitopes and to partial inaccessibility of O-glycan attachment sites.

We therefore presumed that the conformational changes in GP Δ CD altered the functional properties of the molecule, which became apparent by the lower infectivity of iVLPs containing GP Δ CD. We suggested that this conformational change in GP especially impaired entry of iVLPs, since mediation of the fusion process requires a complex structural reorganization that might be inhibited by even slight conformational changes.

Establishment of an assay to monitor entry of filamentous VLPs. To date, no reliable fusion assay for MARV is available, and several of our own attempts to establish a fusion assay failed. Thus, it was not possible to determine the fusion activity of GP directly. It was therefore investigated whether the miss-

ing cytoplasmic domain of GP influenced the entry of VLPs into target cells. To this end, we established a novel entry assay using filamentous VLPs that contained an enzymatically active luciferase. We constructed a fusion protein composed of the C terminus of EBOV VP30 (amino acids 142 to 272) linked C-terminally to firefly luciferase (EBOV VP30-Luc) (Fig. 5A). It has been published previously that the C terminus of VP30 is sufficient to mediate binding to the major nucleocapsid protein NP (22). To test whether VP30-Luc was also able to interact with NP, we investigated its cellular distribution upon coexpression with EBOV NP in HUH-7 cells. Immunofluorescence analysis of singly expressed VP30-Luc revealed a homogenous distribution throughout the cytoplasm which was indistinguishable from that of wild-type VP30, while NP was concentrated in NP-induced inclusions, which have previously been shown to contain nucleocapsid-like structures (Fig. 5B, left panel) (31). Upon coexpression, both wild-type VP30 and VP30-Luc were recruited to and concentrated in NP-induced inclusions, indicating an interaction of VP30 and VP30-Luc with nucleocapsids (Fig. 5B, right panel). We were then interested in whether VP30-Luc was recruited into filamentous VLPs. To address this question, EBOV NP, VP35, L, VP40, and VP30-Luc were coexpressed together with different wild-type and mutant filoviral glycoproteins. EBOV GP and MARV GP served as positive controls, while the fusion-inactive mutant EBOV GP_F535R served as a negative control (59). Purification of released filamentous VLPs followed by an analysis via Western blotting revealed that both VP30-Luc and the different GP molecules were incorporated efficiently into filamentous VLPs (Fig. 5C). An important prerequisite for the establishment of the novel entry assay was the integrity of purified filamentous VLPs, which guaranteed that nucleocapsid-associated luciferase was detectable only after uncoating of the nucleocapsid following membrane fusion. To analyze membrane integrity, we incubated purified filamentous VLPs with the firefly luciferase substrate luciferin in the presence or absence of detergent to mimic the opening of the viral envelope in the endosomal compartment (26). When filamentous VLPs were treated with detergent and subsequently incubated with luciferin, a high level of luciferase activity was detected, which was set to 100%. In the absence of detergent, the luciferase activity in resuspended filamentous VLPs dropped to 20%, indicating the integrity of most filamentous VLPs (Fig. 5D) ($16.4\% \pm 9\%$ luciferase activity). Taken together, the data showed that the constructed filamentous VLPs were morphologically similar to filoviruses and that the majority of incorporated firefly luciferase acquired access to its substrate only after the envelope of the particles was removed, as this takes place during uncoating of the nucleocapsid. Thus, the established assay was suitable for investigating the role of the MARV GP cytoplasmic tail in the entry of VLPs.

Efficiency of VLP entry is modulated by the cytoplasmic tail of MARV GP. In previous studies, it was illustrated that the endosomal/lysosomal cysteine proteases cathepsin B and cathepsin L (CatL) play essential and accessory roles in the entry of authentic EBOV as well as HIV or vesicular stomatitis virus (VSV) particles pseudotyped with EBOV GP into target cells (11, 29, 47, 63). It was suggested that cleavage of GP₁ by cathepsins removes a highly glycosylated part of the protein and allows efficient binding of GP to an endosomal receptor by

FIG. 5. Establishment of a filovirus entry assay based on filamentous VLPs. (A) Schematic presentation of EBOV VP30 and EBOV VP30-Luc. The fusion protein VP30-Luc is composed of the C-terminal amino acids 142 to 272 of EBOV VP30 C-terminally linked to firefly luciferase. (B) Intracellular distributions of EBOV NP, EBOV VP30, and EBOV VP30-Luc upon single and coexpression. Subconfluent HUH-7 cells were transfected with plasmids encoding EBOV NP, EBOV VP30, or EBOV VP30-Luc (left) or cotransfected with plasmids encoding EBOV NP and EBOV VP30 or EBOV NP and EBOV VP30-Luc (right). At 24 h p.t., cells were fixed with 4% paraformaldehyde and subjected to IF analysis using a guinea pig anti-EBOV VP30 IgG and a mouse anti-EBOV NP IgG. Bound antibodies were detected with a FITC-labeled goat anti-mouse IgG serum and a Texas Red-labeled goat anti-guinea pig antibody. (C) Incorporation of EBOV VP30-Luc into filamentous VLPs. HEK 293 cells were subjected to transfection with plasmids encoding EBOV NP, VP35, L, VP40, GP, and VP30-Luc. Where indicated, the plasmid encoding EBOV GP was replaced by a plasmid encoding MARV GP, MARV GPΔCD, or EBOV GP_F535R. At 48 h p.t., VLPs were harvested and pelleted through a 20% sucrose cushion, and filamentous particles were separated using a Nycodenz step gradient. Filamentous VLPs were subjected to Western blot analysis using monoclonal anti-EBOV VP40, anti-firefly luciferase, anti-MARV GP, and anti-EBOV GP IgGs. Bound antibodies were detected using a secondary HRP-coupled anti-mouse antibody. Quantification of protein incorporation into VLPs was done using Raytest TINA software. (D) Determination of the integrity of filamentous particles. Transfection of HEK 293 cells and purification of filamentous particles were performed as described for panel C. Particles incorporating MARV GP were resuspended in buffer with or without detergent (as indicated) and mixed with the luciferase substrate luciferin, and luciferase activity was measured. Luciferase activity in the absence of detergent represents VLPs that were penetrated by luciferin. The data represent the mean values and standard deviations for three independent experiments. Asterisks indicate statistically significant differences (***, $P < 0.001$) in comparison to VLPs resuspended in detergent-containing buffer (set to 100%).

uncovering receptor-binding sequences in the GP₁ head subdomain recessed in the intact trimer (12, 25, 29). The functions of these enzymes can be recapitulated *in vitro*: incubation of pseudotypes bearing EBOV GP with purified human CatL resulted in cleavage and removal of GP₁ MLD and glycan cap sequences, leaving a stable N-terminal GP₁ intermediate linked to intact GP₂ (29, 47, 63).

To investigate the susceptibility of wild-type and mutant MARV GP to cathepsins and the suggested role of proteolytic

cleavage in the entry of VLPs into target cells, we incubated filamentous VLPs from the supernatant of cells expressing EBOV NP, VP40, VP35, L, and VP30-Luc and MARV GP or GPΔCD (see above) with recombinant human CatL *in vitro*. Western blot analysis revealed that CatL treatment resulted in truncation of wild-type GP₁ to an approximately 25-kDa fragment which also appeared after proteolysis of GPΔCD (Fig. 6A, compare columns 1 to 4 and 5 to 8). Extended proteolytic processing (>20 min) led to conversion of GP₁ into fragments

FIG. 6. Entry of filamentous VLPs into susceptible cells. (A) Cleavage of MARV GPs by cathepsin L. Filamentous VLPs were purified from HEK 293 cells as described in the legend to Fig. 5 and were treated with 20 ng/μl CatL at pH 5.5 for 10, 20, or 30 min at 37°C. Mock treatment was performed in the same buffer containing no enzyme. Proteolytic reactions were terminated at the indicated time points by chilling the reaction to 4°C and incubating VLPs with the protease inhibitor E-64. VLP preparations were subjected to Western blot analysis using two monoclonal anti-MARV GP IgGs, both detecting GP₁, and a secondary HRP-coupled anti-mouse antibody. (B) Entry of VLPs incorporating mutant or wild-type GP into target HEK 293 cells. Fresh HEK 293 cells were incubated with filamentous VLPs, and at the indicated time points, cells were washed to remove unbound particles and resuspended in detergent-free buffer, after which luciferase activity was measured. The cellular luciferase activity representing the entry of VLPs was correlated with the total VLP-associated luciferase signal measured by incubating the same amount of VLPs used in the entry assay with luciferin in the presence of detergent. The data represent the mean values and standard deviations for three independent experiments. Asterisks indicate statistically significant differences (*, $P < 0.05$; ***, $P < 0.001$).

no longer detectable by the anti-GP₁ antibodies used in this study. These results indicated an efficient cleavage of MARV GP and GPΔCD by CatL to intermediates that are presumed to trigger the fusion process.

To further determine the role of the GP cytoplasmic tail in the VLP entry process, filamentous particles from the supernatant of cells expressing EBOV NP, VP40, VP35, L, and VP30-Luc and one of the filoviral wild-type or mutant glycoproteins (see above) were gradient purified and allowed to enter susceptible cells. The intracellular luciferase activity was then determined at different time points after infection (Fig. 6B). For the positive control, we observed that luciferase signals could be detected after a lag phase of 60 min and continued to rise until 180 min, when approximately 40% of the input luciferase activity was detectable intracellularly (Fig. 6B, squares). As expected, the fusion-inactive glycoprotein EBOV

GP_F535R did not induce a significant luciferase signal (Fig. 6B, diamonds). Next, we compared the entry of filamentous VLPs mediated by MARV GP and MARV GPΔCD 180 min after incubation. MARV GP induced intracellular luciferase signals that were not significantly different from the signals produced by EBOV GP (Fig. 6B) ($32.2\% \pm 1.2\%$ versus $39.1\% \pm 10.5\%$). In contrast, employing VLPs with MARV GPΔCD, a significant reduction in luciferase signal was observed (Fig. 6B) ($13.8\% \pm 7.9\%$), which correlated well with the infectivity data presented above (Fig. 3). In addition, the entry process mediated by GPΔCD was considerably slower than the wild-type GP-mediated process (Fig. 6B). Taken together, these results suggest that the cytoplasmic domain of filoviral glycoproteins plays a crucial role in stabilizing the conformation of the ectodomain, which influences efficient entry into target cells.

DISCUSSION

Using filoviral iVLP systems as model systems for native virus infection (62), we discovered that the cytoplasmic tail is important for efficient filoviral entry into target cells. To understand mechanistically why filoviral glycoproteins lacking the cytoplasmic domain mediate infection less efficiently, we investigated their intracellular expression levels and transport, as well as the incorporation of GPΔCD into iVLPs, and found that none of these parameters was altered significantly (Fig. 1B and C). Further investigation indicated that O-glycosylation was significantly modified in the absence of the cytoplasmic domain (Fig. 2B). While it has been suggested that O-glycosylation contributes to the characteristic cytopathology of EBOV GP, as well as to the steric shielding of GP epitopes and host proteins, the role of the O-glycosylation of filoviral GPs in the infection process is currently not well understood (16, 17, 44). However, filovirus infection of macrophages and dendritic cells was shown to be mediated effectively by interaction of O-glycans with the cellular attachment factor hMGL (53). Interestingly, infection of the hepatoma cell line HUH-7 was not impaired by removal of the MLD of GP, which harbors all of the O-glycans (Fig. 3D). These results are consistent with data published by Manicassamy et al. demonstrating that HIV particles pseudotyped with MARV GPΔMLD did not show a significant reduction in infectivity if the differences in incorporation of wild-type GP and GPΔMLD were taken into account (36). Taking these data together, we assumed that modifications in the O-glycosylation pattern seen with GPΔCD had no direct effect on the capability of iVLPs to infect target cells. It was presumed that the observed changes in the glycosylation pattern of GPΔCD rather indicated overall conformational changes in its ectodomain which altered the accessibility of some of the glycan attachment sites. Changes in the structure of the GPΔCD ectodomain as a consequence of the missing cytoplasmic domain were confirmed by a neutralization assay, which showed that GPΔCD was less susceptible than wild-type GP to neutralization by an anti-MARV serum (Fig. 4). Together, these results indicate that changes in the cytoplasmic domain are relayed to the structure of the GP ectodomain. Previous studies showed that deletion of the cytoplasmic tail of viral surface proteins can result in an aberrant virus phenotype. In the case of influenza virus neuraminidase (NA), removal of

the cytoplasmic domain led to reduced incorporation of NA into the viral envelope, the release of particles that had an aberrant pleomorphic instead of wild-type spherical morphology, and a diminished particle infectivity (8, 28, 39). The observed phenotypes were attributed to an impaired interaction of NA and the matrix protein M1 resulting in aberrant particle assembly. From these studies, it remained unclear whether the diminished infectivity of viruses incorporating tailless NA was caused by the altered virus shape or had another basis. An impaired interaction between the cytoplasmic domain of GP and VP40 as a reason for reduced infectivity of iVLPs can be disregarded; in previous studies, we were able to show that GPΔCD significantly colocalizes with VP40 in IF analysis, suggesting an interaction of both proteins enabled via a domain distinct of the cytoplasmic tail of GP (40).

Truncation of the cytoplasmic domain of the envelope proteins of several retroviruses, e.g., Moloney murine leukemia virus and HIV-1, led to altered recognition by conformation-specific monoclonal antibodies, suggesting a conformational change within the respective ectodomain (2, 14). Interestingly, elongation of the cytoplasmic domain of the F protein of simian virus 5 (SV5) also resulted in conformational alterations in the ectodomain (58).

To find out whether the decreased infectivity of iVLPs incorporating GPΔCD (Fig. 3A) was associated with impaired entry into target cells, we established a novel entry assay, based on filamentous VLPs, in which the uncoating of the nucleocapsid complex was monitored. In recent years, several assays to study the entry of filoviruses have been established. Most of them are based on recombinant viruses, e.g., VSV or HIV, pseudotyped with filoviral glycoproteins. The limitation inherent in these approaches is that the spherical or bullet-shaped morphology and/or small size of the particles may allow them to use different entry pathways from those used by filamentous filoviruses. In addition, these assays monitor particle entry at 16 h postentry at the earliest, as the reporter gene needs to be transcribed and translated before its activity can be measured. Recently, filovirus-specific entry assays were also established based on VP40-induced VLPs incorporating reporter proteins, whose release into the target cell was then monitored. The reporter proteins were either incorporated into released particles in the form of membrane-associated proteins (43, 46) or linked to the matrix protein VP40, allowing the measurement of reporter protein activity shortly after membrane fusion. In the case of VP40 linked to the reporter protein β-lactamase, GP-mediated fusion of viral and cellular membranes, and thus release of β-lactamase, can be measured by the cleavage of a fluorogenic substrate present in the cytoplasm (35, 56). In addition, real-time systems monitoring the endocytic uptake of either VP40-induced VLPs or native filoviruses were established; these systems are based on incorporation of fluorescently labeled lipophilic tracers or green fluorescent protein (GFP) into the viral particles (41, 45). We improved the available approaches, on the one hand, by using purified filamentous VP40-induced VLPs, which have the most morphological similarity to filoviruses and probably exploit an entry mechanism into target cells similar to that used by filoviruses (30, 40, 42, 52, 55). On the other hand, the employed reporter protein (VP30-Luc) is specifically recruited into the nucleocapsid and is enzymatically active only when the viral envelope is re-

moved. Therefore, we monitor the appearance of the nucleocapsid in the cytoplasm, which is possible only after the fusion process has been completed. It is believed that the introduction of the nucleocapsid into the cytoplasm represents a hallmark of a successful infection process.

By using this assay, we observed that particles containing GPΔCD were impaired in the ability to enter target cells (Fig. 6B). To date, it remains unclear which of the multiple steps necessary for viral entry is hampered by deletion of the cytoplasmic domain of GP. Often, modification of the cytoplasmic tails of viral fusion proteins results in reduced fusogenic activity, as observed for the surface protein Env of HIV-1 (1, 64). Similarly, when 19 of the 20 amino acids in the cytoplasmic domain of SV5 F were removed, the resulting mutant was still able to facilitate formation of the fusion pore, but its subsequent expansion, which is needed to release the large viral nucleocapsids into the cytoplasm, was inhibited (13). It was presumed that the cytoplasmic domain maintained the appropriate fusogenic conformation of the transmembrane domain and the connected ectodomain (13, 58). Investigations into the precise mechanism underlying the reduced efficiency of entry mediated by filoviral GP without the cytoplasmic domain are under way.

Taken together, the presented data indicate a functional link between the ectodomain and cytoplasmic domain of MARV GP that exceeds a simple physical stabilization of the protein in the lipid viral envelope.

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