The Cytoplasmic Tails of Uukuniemi Virus (Bunyaviridae) G_N and G_C Glycoproteins Are Important for Intracellular Targeting and the Budding of Virus-Like Particles

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Most enveloped viruses acquire their envelope from the plasma membrane; however, members of the bunyavirus (34), flavivirus, and coronavirus families assemble and bud at intracellular membranes (reviewed in reference 33). Not much is known about the budding mechanism of bunyaviruses, and most studies on bunyaviruses have focused on the processing of the two glycoproteins G_N and G_C and their transport to and retention in the Golgi complex (23). The budding process of UUK virus (30), a member of the Phlebovirus genus, one of five genera in the Bunyaviridae family, and a model virus for this family of viruses (7). These VLPs were shown to be able to package an artificial RNA segment, a minigenome containing a reporter gene flanked by the noncoding regions from one of the three viral segments (8, 30). These VLPs can infect new cells and transfer the minigenome that can be replicated and transcribed in these newly infected cells if the required UUK proteins (the polymerase and nucleoprotein, open reading frames of the L and the S segment, respectively) are coexpressed (30). The VLPs display morphology and cellular tropism identical to those of wild-type (wt) UUK virus (30) and are therefore a useful system for studying packaging and bud-
ding mechanisms of this family of viruses. This system has been successfully used to map amino acids in the GN cytoplasmic tail, which is important for the packaging of RNPs into VLPs for UUK virus (29). In addition to the four amino acids located in the end of the GC cytoplasmic tail demonstrated to be important for packaging, two other regions were shown to be important for the generation and release of VLPs. These two regions were shown to span residues 21 to 25 and 46 to 50 in the GN cytoplasmic tail (29). In the present study, we have analyzed and identified specific amino acids within these two regions in the GC cytoplasmic tail that are responsible for the generation and release of VLPs. Moreover, a functional motif within the GC cytoplasmic tail containing a well-conserved lysine residue was shown to be important for proper targeting and retention of both glycoproteins to the Golgi complex and subsequent particle release.

MATERIALS AND METHODS

Plasmids. pUUK-GN/GC, pUUK-L, and pUUK-N are cytomegalovirus-driven plasmids expressing the UUK viral glycoprotein precursor, RNA-dependent RNA polymerase, and nucleoprotein, respectively (9). M-CAT is the Pol I-driven UUK virus minigenome plasmid containing the noncoding regions from the M segment, flanking the reporter gene for chloramphenicol acetyltransferase (CAT) (8). The construction of all alanine mutant plasmids used in the alanine scan was performed by standard PCR cloning methods, such as overlap PCR and two- and three-fragment ligation, as described previously (29). All derivatives of pUUK-GN/GC were sequenced to verify the correct introduction of mutations in the absence of undesirable mutations. Primer sequences are available upon request.

Cell culture and transfection. BHK-21 (ATCC) cells were grown in plastic dishes in minimum essential medium with Earle’s salt supplemented with 5% fetal calf serum, 5% tryptose phosphate broth, 2 mM l-glutamine, 50 IU penicillin/ml, and 50 μg streptomycin/ml (Invitrogen). For the VLP reporter gene system (30), BHK-21 cells were transfected with pUUK-L, pUUK-N, pUUK-GN/GC, and M-CAT using Lipofectamine 2000 reagent (Invitrogen). Transfections were performed as described previously (29, 30). Cells were analyzed for reporter gene expression 24 h posttransfection, and the corresponding supernatants were used for VLP infection. Twenty-four hours prior to the passage of the supernatant, BHK-21 cells were transfected with L and N expression plasmids in order to support minigenome replication, transcription, and detection. After a 3-h incubation period, fresh medium was added to the VLP-infected cells, and cells were incubated for 24 h before reporter gene analysis.

CAT assays. Cells were resuspended in 50 μl of 0.25 M Tris-HCl (pH 7.4) and were lysed by two freeze-thaw cycles. The cell lysates were centrifuged for 10 min at 9,000 × g, and CAT activity was determined using a commercially available Fast cat kit (Invitrogen) as described previously (9). The reaction products were visualized by UV illumination and were documented by photography.

Harvesting and purification of UUK VLPs. The harvesting and purification of UUK VLPs were done as previously described (30). Briefly, supernatants from VLP-expressing cells were collected and clarified by centrifugation (4,000 × g for 10 min at 4°C). The particles were concentrated through a 20% (wt/vol) sucrose cushion and dissolved in TN buffer (0.05 M Tris-HCl, pH 7.4, and 0.1 M NaCl) by centrifugation at 100,000 × g for 1 h at 4°C. The pellet was dissolved for 10 min at 90°C, and the corresponding supernatants were used for VLP infection. Twenty-four hours prior to the passage of the supernatant, BHK-21 cells were transfected with L and N expression plasmids in order to support minigenome replication, transcription, and detection. After a 3-h incubation period, fresh medium was added to the VLP-infected cells, and cells were incubated for 24 h before reporter gene analysis.

RESULTS

To evaluate and identify functional motifs in the cytoplasmic tails of GN and GC of UUK virus, we used the newly developed VLP system for UUK virus (30). The GC cytoplasmic tail of 81 residues has previously been shown to contain a Golgi complex targeting and retention signal (amino acids 10 to 40) (3), two palmitylation sites (amino acids 25 and 28) (2), and four amino acids important for packaging (amino acids 76 and 79 to 81) (29) (Fig. 1A). We recently used the VLP system to identify the packaging interactions between the GN cytoplasmic tail and the RNPs (Fig. 1A, residues indicated by black boxes) (29). In addition, two other regions in the GC cytoplasmic tail, namely residues 21 to 25 and 46 to 50 (Fig. 1A, underlined), were shown to be important for particle formation and release but not for packaging of RNPs. No UUK virus-specific proteins could be detected in the supernatant of BHK-21 cells transfected with the minigenome system and these glycoprotein mutants, suggesting that no VLPs were generated and released by the cells expressing these mutants (29).

Mutagenesis screen of regions 21 to 25 and 46 to 50 in the GN cytoplasmic tail. In the present study, we analyzed the regions encompassing amino acids 21 to 25 and 46 to 50 in more detail by a mutational screen, changing 1, 2, or 3 amino acids at a time to alanine. The mutants were tested in our VLP system for their ability to generate and release VLPs containing RNPs, as monitored by the transfer of CAT reporter activity to newly VLP-infected cells (Fig. 1B and C). BHK-21 cells transfected with the minigenome system of M-CAT, pUUK-L, and pUUK-N and either the wt or the mutated pUUK-GN/GC expression plasmids were analyzed for CAT activity 24 h posttransfection (Fig. 1B and C, upper panels). Both wt and all mutant glycoprotein transfected cells displayed strong CAT reporter activity in primary transfected cells, indicating efficient replication and expression of the minigenome M-CAT. Supernatants were collected 24 h posttransfection and were used to infect fresh BHK-21 cells, which were pre-transfected with the L and the N expression plasmids to allow amplification of the UUK virus RNA segments and expression of the reporter protein (30). The VLP-infected cells were harvested 24 h after VLP infection, and reporter activity was determined (Fig. 1B and C, lower panels). No CAT activity
could be detected in the negative control (Fig. 1B and C, lower panels, lanes 1), indicating that no minigenome can be transferred to new cells in the absence of the glycoprotein precursor expression plasmid. Maximum activity was detected in the positive control with wt GN/GC (Fig. 1B and C, lower panels, lanes 2). The mutant GN48-50 showed a reduced level of VLP-mediated transfer of CAT activity to new cells compared to that of wt GN/GC (Fig. 1C, lower panel, compare lanes 2 and 4), and two mutants with alanine substitutions for residues 23 to 24 and 46 to 47 in the cytoplasmic tail of GN showed only background levels (Fig. 1B, lower panel, compare lanes 1 and 4, and C, lower panel, compare lanes 1 and 3). Mutations of residues 21 to 22 and 25 did not have an effect on VLP-mediated transfer of CAT (Fig. 1B, lower panel, lanes 3 and 5).

We have previously shown that mutating residues 26 to 30 did not affect VLP-mediated transfer of CAT activity or intracellular localization (29), suggesting that the palmitylation of residue 25 or 28 is not critical for the generation of infectious VLPs. To verify protein expression of the wt and mutated glycoproteins in the transfected cells, as well as the protein composition of wt and mutant VLPs in the supernatant, Western blot analysis was performed (Fig. 1D). No difference in glycoprotein or nucleoprotein expression levels could be detected between the wt and mutated glycoproteins in primary transfected cells (Fig. 1D, upper panels). The corresponding supernatants from these transfected cells were collected, concentrated through a sucrose cushion (lower panels), and analyzed by Western blotting (Fig. 1D, lower panels). No UUK virus proteins were released from cells transfected with only the minigenome and the L and N expression plasmids (Fig. 1D, lane 1), and both glycoproteins and the nucleoprotein were detected when the wt GN/GC expression plasmid was present (lane 2). No UUK-virus-specific proteins were detected in the supernatants obtained from GN23-24-, GN46-47-, and GN48-50-transfected cells, although all these mutated glycoproteins were expressed at the same level as wt glycoproteins, as was observed for the cell lysates (Fig. 1D). Even though the GN48-50 mutant was able to transfer reduced amounts of CAT activity to VLP-infected cells (Fig. 1C, lane 4), no UUK virus proteins could be detected in the supernatant by Western blotting (Fig. 1D, lane 5), indicating that Western blotting is not sensitive enough to detect small amounts of secreted VLPs. These results indicate

FIG. 1. Transfer of reporter gene activity and protein analysis of VLPs generated by GN glycoprotein mutants. (A) Schematic representation of the glycoprotein precursor open reading frame. The amino acid sequences of GN and GC cytoplasmic tails are shown, and functional motifs that were previously identified are highlighted. Underlined residues are analyzed in the present study using a mutagenesis screen. TM, transmembrane domain; SS, signal sequence. (B and C) Transfer of CAT activity by VLPs generated with mutated and wt glycoproteins. BHK-21 cells were transfected with the minigenome pUUK-N and pUUK-L were left untransfected (lane 1), were transfected with wt GN/GC (lane 2), or were transfected with mutated pUUK-GN/wt GC (lanes 3 to 5). The amino acid residues in the GN tail that were mutated to alanines are indicated by their numbers according to the GN sequence shown in panel A. Cells were harvested and analyzed for CAT activity 24 h posttransfection (upper panel). The corresponding supernatants, containing VLPs, were used to infect new cells pretransfected with pUUK-N and pUUK-L, and reporter activity was determined 24 h after VLP infection (lower panels). (D) Western blot analysis of the VLPs. Transfected cells (upper panels) and the corresponding supernatants containing VLPs, after concentration through a sucrose cushion (lower panels), were analyzed for glycoprotein (GN/GC) and nucleoprotein (N) expression. BHK-21 cells were transfected with M-CAT, pUUK-N, and pUUK-L (lane 1); with M-CAT, pUUK-N, pUUK-L, and wt GN/GC (lane 2); or with mutated pUUK-GN/wt GC (lanes 3 to 5). The two glycoproteins are detected with a polyclonal antibody recognizing both GN and GC, and the nucleoprotein was detected with polyclonal antibody recognizing the N protein. Data represent results from three independent experiments.
that these two regions in the GN cytoplasmic tail between residues 23 to 24 and 46 to 50 are important for generating and releasing particles into the supernatant.

**Intracellular localization of the GN cytoplasmic tail mutants.** UUK virus buds into the Golgi complex and is transported in large vesicles to the plasma membrane, where they are released into the media (23). We wondered if the defect in VLP release of these three glycoprotein mutants is caused by mislocalization of the mutant glycoproteins, thereby preventing budding into the Golgi complex. BHK-21 cells transfected with wt G\textsubscript{N}/G\textsubscript{C} and the mutants G\textsubscript{N}23-24, G\textsubscript{N}46-47, and G\textsubscript{N}48-50 were fixed 24 h posttransfection and were costained with a polyclonal antibody recognizing both G\textsubscript{N} and G\textsubscript{C} glycoproteins and the Golgi marker GM130, a cis-Golgi matrix protein (27) (Fig. 2A). In wt G\textsubscript{N}/G\textsubscript{C}-transfected and G\textsubscript{N}23-24 mutant-transfected cells, the glycoproteins colocalized with the Golgi marker, demonstrating that the lack of reporter gene transfer and release of particles for this mutant was not caused by a defect in intracellular targeting (Fig. 2A). In contrast, the G\textsubscript{N}46-47 and G\textsubscript{N}48-50 mutants displayed more reticular staining patterns typically observed for proteins localized to the ER (Fig. 2A, lower panels).

The wt G\textsubscript{N} protein has been reported to be partially Endo H resistant, a modification that occurs in the Golgi complex (22, 32). We therefore analyzed whether the G\textsubscript{N} mutants are processed in a manner similar to that of the wt G\textsubscript{N} protein, an indication that the glycoprotein is able to reach the Golgi complex. Cell lysates from BHK-21 cells transfected with wt or mutant glycoproteins were left untreated or were treated with Endo H, and the glycosylation pattern of G\textsubscript{N} was examined by Western blotting (Fig. 2B). We observed that the wt and G\textsubscript{N}23-24 are more Endo H resistant than G\textsubscript{N}46-47 and G\textsubscript{N}48-50 (Fig. 2B, compare lanes 2 and 4 with 6 and 8). Treatment of wt G\textsubscript{N} with PNGase F, a glycosidase that is able to remove both Endo H-sensitive and -resistant glycans, reveals that the lower band (Fig. 2B, indicated by an arrowhead) is the deglycosylated form of G\textsubscript{N}. This demonstrates that, unlike the wt G\textsubscript{N}, G\textsubscript{N}46-47 and G\textsubscript{N}48-50 are not as efficiently modified by the glycoprotein-modifying enzymes in the Golgi complex and, together with their intracellular distribution, suggests that these mutants are trafficking less efficiently to the Golgi complex. Together, these results suggest that the defect in VLP release observed for these two mutants is caused by mislocalization of the mutant glycoproteins.

**Identification of residues important for the formation of VLPs.** In order to identify which of the two amino acid residues, leucine 23 or leucine 24, is important for the generation and release of VLPs, single-amino-acid mutants G\textsubscript{N}L23A and G\textsubscript{N}L24A were generated. Both G\textsubscript{N}L23A and G\textsubscript{N}L24A displayed a clear Golgi staining pattern identical to that of wt G\textsubscript{N}/G\textsubscript{C} (data not shown), verifying their correct intracellular targeting. The mutants G\textsubscript{N}L23A and G\textsubscript{N}L24A were analyzed for their ability to generate and release VLPs and transfer CAT activity to new cells. Supernatants from wt and mutant glycoprotein-transfected cells were collected 24 h posttransfection, an aliquot was used for VLP infection, and the remainder of the supernatant was concentrated and used for Western blot analysis (Fig. 3). The primary transfected cells were harvested, and reporter protein expression was determined using the CAT assay (Fig. 3A, upper panel). All transfected cells showed strong reporter activity, verifying efficient transcription and replication of the UUK virus minigenome. Strong CAT activity also was detected in cells infected with wt VLPs (Fig. 3A, lower panel, lane 1). Transfer of CAT activity was greatly reduced compared to that of the wt for the G\textsubscript{N}L24A mutant (compare lane 1 with lane 3), while only background levels could be observed with the G\textsubscript{N}L23A mutant (lane 2). Both glycoproteins and the nucleoprotein were detected in VLPs generated from wt G\textsubscript{N}/G\textsubscript{C}-transfected cells. However, no glycoproteins...
or nucleoproteins were detected in the supernatant obtained from GNL23A- or GNL24A-transfected cells (Fig. 3B). These results show that both residues L23 and L24 are important for the generation and release of VLPs into the medium.

**GNL23A has a defect in budding into the Golgi membrane.**

In a previous report, it was shown by using TEM that a temperature-sensitive UUK virus mutant was unable to bud into the Golgi membrane and form particles at the restrictive temperature, while particles were observed to bud into the Golgi membrane at the permissive temperature (12). We explored the possibility that the GNL23A glycoprotein mutant, as well as the GNL24A glycoprotein mutant, which correctly localized to the Golgi complex, is defective in budding into the Golgi membrane and therefore cannot secrete VLPs into the supernatant. To address this, we examined the morphology of wt GNC/GC- and GNL23A-transfected cells by TEM. The GNL23A mutant was chosen, since that mutant showed the strongest phenotype in VLP-mediated transfer of CAT activity. BHK-21 cells were cotransfected with a GFP-expressing plasmid together with a wt GNC/GC- or mutant GNL23A-expressing plasmid and were trypsinized 24 h posttransfection, and GFP-expressing cells were sorted and collected by FACS analysis. This procedure enriched the population of cells expressing the glycoproteins from 16% (primary transfected cells) to almost 100% (FACS-sorted cells), as shown by immunofluorescence microscopy performed on a small aliquot of the sorted cells (Fig. 4A). Both wt and mutant transfected cells showed fibroblast-specific morphology after sorting, and most of the sorted cells expressed the transfected glycoprotein, although the expression levels varied somewhat between cells (Fig. 4A). The
majority of the FACS-sorted cells were fixed by modified Ito’s fixative (35) and subsequently were prepared for thin-section TEM (Fig. 4B). Cells expressing wt Gs/Gc clearly contained VLPs inside Golgi vesicles (Fig. 4B, indicated by arrows), as was also shown previously (30). No visible particles or budding activity could be detected in the Golgi vesicles of cells expressing the GsL23A mutant glycoprotein, strongly indicating that L23 in the Gs cytoplasmic tail is important for the initiation of VLP budding. Interestingly, although no particles were formed with GsL23A, we observed a vacuolization of the Golgi complex similar to what was seen in wt Gs/Gc-transfected cells and UUK virus-infected cells (23, 30), indicating that the formation of particles is not driving this process. We conclude that the inability of GsL23A, and most likely GsL24A, to release VLPs is caused by a defect in budding into the Golgi membranes.

Identification of single amino acids in the Gs cytoplasmic tail important for intracellular targeting. To evaluate which specific amino acid in the region of residues 46 to 50 of the Gs cytoplasmic tail is responsible for the observed mislocalization of the mutant glycoprotein and subsequent defect in VLP release, single-amino-acid mutants, in which individual amino acids were changed to alanines, were constructed. The mutant glycoproteins were first evaluated for their ability to generate VLPs and infect new cells (Fig. 5A). Both wt and the single-amino-acid-mutant Gs glycoproteins showed strong CAT activity in transfected cells (data not shown). GsE48A and GsG49A showed reporter activity in VLP-infected cells that was as strong as that of wt Gs/Gc (Fig. 5A, compare lanes 5 and 6 to lane 2). GsL46A and GsL50A (lanes 3 and 7) were unable to transfer any CAT activity to VLP-infected cells, while GsE47A (lane 4) displayed a reduced ability to transfer CAT activity. To evaluate whether this defect in transfer of CAT activity was caused by mislocalization of the mutant glycoproteins, we determined the intracellular localization of these five mutant glycoproteins by immunofluorescence microscopy (Fig. 5B). The GsE48A and GsG49A mutants that were able to transfer CAT activity as efficiently as wt Gs/Gc colocalized with the Golgi marker GM130 (Fig. 5B), indicating correct targeting of these mutant glycoproteins. However, GsL46A, GsE47A, and GsL50A, all being defective in transfer of CAT activity, displayed a more reticular staining pattern and only partially colocalized with the Golgi marker GM130 (Fig. 5B), suggesting that these mutants are partially localized to the ER. However, no difference in intracellular localization alanine, generating the Gs mutants GsL46A, GsE47A, GsE48A, GsG49A, and GsL50A. VLPs obtained from cells transfected without or with wt Gs/Gc served as negative (lane 1) and positive (lane 2) controls, respectively. (B) Intracellular localization of the Gs single-amino-acid glycoprotein mutants that were assayed for CAT activity in panel A. Cells were costained for glycoprotein mutant Gs and wt Gc (Gs/Gc; green) and the Golgi marker GM130 (red). (C) Colocalization of the wt Gs and GsL46A and GsE47A mutants with the wt Gc protein. Cells were transfected with wt Gs, GsL46A, and GsE47A in the presence of wt Gc and were costained with a polyclonal antibody specifically recognizing the cytoplasmic tail of the Gs glycoprotein (green) and a monoclonal antibody specifically recognizing the Gc protein (red). The panels to the right show the merged images.
could be detected between mutant GₘE47A, with reduced CAT activity, and mutants GₘL46A and GₘL50A, without any detectable CAT activity. These results suggest that amino acid residues L46, E47, and L50 are important for correct targeting of the glycoproteins to the Golgi complex and subsequent formation of VLPs.

Gₘ is known to be retained in the ER when expressed alone, while Gₘ when expressed alone, is transported to and retained in the Golgi complex due to the Golgi complex targeting and retention signal located in its cytoplasmic tail (1). When coexpressed, Gₘ and Gₜ form heterodimers that are targeted to the Golgi complex (see Fig. 2) (31). It is unknown which part or parts of the glycoproteins are mediating this dimerization, either the ectodomain, the transmembrane domain, the cytoplasmic tails, or a combination of all three domains. Since GₘL46A, GₘE47A, and GₘL50A show typical ER and only partial Golgi staining, we speculated that mutation of these residues in the Gₜ cytoplasmic tail prevented this heterodimerization. We therefore transfected BHK-21 cells with wt and mutant glycoproteins and costained them with a Gₘ polyclonal antibody (42) and a Gₜ monoclonal antibody (31) in order to detect both glycoproteins separately (Fig. 5C). In wt Gₘ/Gₜ-transfected cells, both glycoproteins colocalize well in the Golgi area (Fig. 5C, upper panels). In cells cotransfected with GₘL46A, GₘE47A, and GₘL50A together with wt Gₜ, the Gₘ mutant glycoproteins colocalized with the wt Gₜ protein, confirming that both glycoproteins are localized to the same compartment (Fig. 5C, lower panels, and data not shown). In addition, coinmunoprecipitation experiments also showed that mutants GₘL46A, GₘE47A, and GₘL50A interacted as efficiently as wt Gₘ with a Gₜ construct containing a truncated ectodomain and a wt transmembrane domain and cytoplasmic tail (data not shown). This demonstrates that the localization of both glycoproteins Gₘ and Gₜ is dependent on the three amino acids L46, E47, and L50 in the Gₜ cytoplasmic tail but that these residues were not required for heterodimerization. The heterodimer of wt Gₘ and Gₜ is transported from the ER to the Golgi complex 45 min after the synthesis of the Gₜ protein (31). To analyze trafficking and transport from the ER to the Golgi complex, wt Gₘ/Gₜ-transfected and mutant Gₘ/wt Gₜ-transfected BHK-21 cells were treated with cycloheximide to block new protein synthesis 5 h posttransfection. The intracellular localization of the transfected proteins was examined by immunofluorescent microscopy 0, 0.5, 1, 2, and 4 h after cycloheximide treatment (Fig. 6 and data not shown). The wt Gₘ/Gₜ heterodimer colocalized well with the Golgi marker at all time points examined, reflecting the efficient transport of the Gₘ/Gₜ heterodimer to the Golgi complex (Fig. 6). The mutants GₘL46A, GₘE47A, and GₘL50A showed partial ER and Golgi complex staining at the beginning of the cycloheximide chase, and the localization of the mutant glycoproteins did not change significantly during the 4-h chase (Fig. 6 and data not shown). These results demonstrate that these three glycoprotein mutants are less efficient in transport to the Golgi complex than the wt glycoprotein.

Analysis of the Gₜ cytoplasmic tail. The intracellular tail of UUK virus Gₜ is not as well characterized as the Gₘ tail, but it has been suggested that the Gₜ cytoplasmic tail of the members of the Phlebovirus genus, being rich in lysines, contains a lysine-based ER retrieval signal (15). Indeed, an amino acid alignment of the extreme C terminus of Gₜ from viruses belonging to the genera Phlebovirus, Hantavirus, and Orthobunyavirus revealed that the lysine at position −3 is conserved across the genera in this family (Fig. 7A, indicated by a gray box). However, this amino acid was not conserved in the Nairovirus and Tospovirus genera (data not shown). UUK virus has a very short Gₜ cytoplasmic tail of only 5 amino acids (Fig. 1A), and a mutational screen was performed to analyze the importance of these residues in VLP generation and transfer of CAT activity. The last four residues were all changed to alanines (GₜL2-5), the cytoplasmic tail of Gₜ was inverted (GₜL1-5 invert), and finally the last four amino acids were changed to alanines, one residue at a time (GₜP2A, GₜK3A, GₜK4A, and GₜS5A) (Fig. 7B). These mutants were, in the presence of wt Gₘ, analyzed for their ability to generate and release infectious VLPs (Fig. 7C). All the Gₜ mutant proteins showed strong reporter activity in primary transfected cells, verifying minigene replication and transcription (data not shown). In VLP-infected cells, strong reporter activity could be detected with wt Gₘ/Gₜ (Fig. 7C, lane 2), and similar transfer of activity was seen with two of the mutants, GₜP2A and GₜK4A (Fig. 7C, lanes 5 and 7). The other Gₜ mutants, GₜL2-5, GₜL1-5 invert, GₜK3A, and GₜS5A (Fig. 7C, lanes 3, 4, 6, and 8), displayed reduced CAT activity compared to that of wt Gₜ. Next, the protein composition of the Gₜ mutant VLPs generated and released from the transfected cells was analyzed by Western blotting (Fig. 7D). Both glycoproteins, Gₘ and Gₜ, and the nucleoprotein were readily detected in the supernatants of wt Gₘ/Gₜ-transfected cells (Fig. 7D, lane 2) and in the supernatants of the two CAT-positive mutants GₜP2A and GₜK4A (Fig. 7D, lanes 5 and 7). Reduced amounts of both glycopro-
peptides and the nucleoprotein were detected in the supernatant from cells transfected with the mutants Gc2-5, Gc1-5 invert, GcK3A, and GcS5A (Fig. 7D, lanes 3, 4, 6, and 8), indicating that in these Gc cytoplasmic tail mutants efficient generation and release of infectious VLPs were compromised. The primary transfected cells also were analyzed for protein expression, and both the glycoprotein and nucleoprotein were expressed at similar levels (Fig. 7D, lower panel, and data not shown).

Localisation of the Gc cytoplasmic tail mutants. To determine the mechanism that caused the Gc mutants Gc2-5, Gc1-5 invert, GcK3A, and GcS5A to result in reduced particle formation and release, we decided first to analyze the intracellular localization of these glycoproteins by immunofluorescence microscopy (Fig. 8). The two glycprotein mutants GcV2A and GcK4A, which both displayed a wt phenotype as judged from VLP-mediated transfer of CAT activity as well as the protein composition of the VLPs, colocalized with the Golgi marker (Fig. 8A), a pattern identical to that observed with wt Gn/Gc-expressing cells (Fig. 2A). In cells transfected with the GcS5A mutant, the glycoproteins did not colocalize with the Golgi marker but instead displayed a reticular staining pattern, including staining of the nuclear rim, typically observed for proteins localized to the ER (Fig. 8A, bottom panel). It appears that this Gc mutant, together with the wt Gc protein, forms a heterodimer (Fig. 8B), which is effectively retained in the ER. For cells transfected with the three other mutants, Gc2-5, Gc1-5 invert, and GcK3A, a different staining pattern was observed (Fig. 8A). The glycoproteins in these cells only partially colocalized with the Golgi marker and, in addition, displayed staining at the plasma membrane. This suggests a defect in Golgi complex retention for the glycoprotein mutants Gc2-5, Gc1-5 invert, and GcK3A, resulting in the plasma membrane expression of both glycoproteins.

Colocalization of Gn and Gc in the plasma membrane. To study the cell surface staining of the Gc mutants in more detail, we performed immunofluorescence analysis on nonpermeabilized glycoprotein-transfected cells. Cells were transfected with wt Gn/Gc or the different Gc mutants in the presence of wt Gc and were analyzed 24 h posttransfection. Nonpermeabilized cells were stained with monoclonal antibodies that specifically recognize the Gc or the Gc protein (31). Neither Gc nor Gc could be detected at the cell surface in wt Gn/Gc-transfected cells, demonstrating that both glycoproteins are not present at the cell surface (Fig. 9, top panels). However, in cells transfected with the mutants Gc2-5, Gc1-5 invert, and GcK3A, both Gc and Gc were detected at the cell surface (Fig. 9, middle panels, and data not shown), while no glycoprotein was detected at the cell surface in GcV2A, GcK4A, and GcS5A mutant-transfected cells (Fig. 9, lower panels, and data not shown). These data suggest that the Gc tail and, more specifically, residue K3 is important for retention of the Gn/Gc heterodimer in the Golgi complex, allowing efficient formation of particles.

DISCUSSION

In a recent report, we identified two regions in the Gc cytoplasmic tail of the UUK virus that were important for VLP-mediated transfer of reporter activity (29). In the present

FIG. 7. Analysis of the Gc cytoplasmic tail. (A) Alignment of the last seven C-terminal amino acids of the Gc proteins from members in the Phlebovirus, Hantavirus, and Orthohantavirus genera. The conserved lysine is indicated by a gray box. (B) Schematic representation of the Gc cytoplasmic tail sequence of the wt Gc and the six Gc cytoplasmic tail mutants. Residues that were mutated or inverted are underlined. TM, transmembrane domain. (C) CAT activity in cells infected with the VLPs collected from cells transfected with Gc cytoplasmic tail mutants and wt Gc. (D) Western blot analysis of the supernatants used for VLP infection shown in panel B. VLPs were concentrated and analyzed for the presence of both glycoproteins (Gn and wt or mutant Gc) and for nucleoprotein (N) (upper panels). The lysates from the corresponding transfected cells also were analyzed for nucleoprotein expression (lower panel).
The study we have analyzed both regions in more detail and explored the mechanism behind this defect in generating and releasing VLPs. We found two different mechanisms responsible for this lack of VLP-mediated transfer of reporter protein activity. Two residues in the Gc cytoplasmic tail, L23 and L24, were demonstrated to be important for the generation and budding of VLPs into the Golgi membrane. Three other residues in the Gn tail, L46, E47, and L50, were found to be important for the proper intracellular localization of both glycoproteins, Gn as well as Gc, and mutation of these residues prevented efficient targeting of these two glycoproteins to the Golgi complex and subsequent formation of VLPs. In addition, we also revealed that the short cytoplasmic tail of GC harbors important information for the proper intracellular targeting of both glycoproteins and the release of VLPs.

Until now, little was known about the budding mechanism of bunyaviruses. Here we have identified two amino acids (L23 and L24) in the cytoplasmic tail of GN that, when changed to alanines, disrupt the release of VLPs. The fact that both glycoproteins, mutant GN as well as wt GC, were correctly targeted and located to the Golgi complex suggested to us that these GN mutants possibly interfered with either the budding or release of particles. Indeed, morphological analysis by high-resolution TEM revealed that the Golgi complex of cells expressing GNL23A were devoid of particles, while the Golgi complex of cells expressing wt GN/GC contained VLPs (Fig. 4B) (30). This was corroborated by the fact that we have been unable to extract any infectious UUK virus VLPs from GNL23A-transfected cells using freeze-thaw (data not shown), indicating the absence of infectious VLPs inside the cell. Previously, a temperature-sensitive mutant of UUK virus (ts12) was reported to be unable to generate and release virus particles at the restrictive temperature (39°C) (11, 12). Both glycoproteins accumulated in the Golgi membrane but were unable to initiate budding, resulting in large vacuolization of the Golgi complex. The function of the Golgi complex remained intact, and at a lower temperature (32°C) virus particles could be produced (13, 14). However, we did not observe an increase in VLP formation at lower temperatures, indicating that our
Gₙ mutants are not temperature sensitive. This indicates that although the GₙL23A mutant morphologically resembles the ts12 mutant, the mechanism by which particle formation in the Golgi complex is blocked appears to be different.

The cytoplasmic tail of UUK virus Gₙ was shown to be palmitoylated at its two cysteines (C25 and C28), but the functional significance of this modification has remained unknown (2). Palmitoylation was reported to be important for the budding process of other viruses, such as Sindbis virus (19), retroviruses (36), rubella virus (43), coronavirus (41), and influenza virus (5). We were unable to detect any changes in intracellular distribution, particle formation, and release when both cysteines in the Gₙ cytoplasmic tail of UUK virus were changed to alanines (reference 29 and the present study), demonstrating that palmitoylation of Gₙ is not important for the formation of infectious VLPs.

Moreover, we found that the proper intracellular targeting of the two glycoproteins is critical for the budding and release of particles. Particles were not generated when the Gₙ/G₉ proteins were localized to the ER. It is known that the UUK virus already starts to bud in the ER-Golgi intermediate compartment, but no UUK virus budding was observed to occur in the ER (20). Three Gₙ glycoprotein mutants showing similar intracellular distributions located to both the ER and Golgi complex did not have the same ability to generate VLPs as the wt Golgi complex-located glycoproteins. Although we demonstrated that all three Gₙ mutants were less efficient in ER exit, only two mutants, GₙL46A and GₙL50A, could not generate and release VLPs at all, while the GₙE47A mutant could transfer some CAT activity. At the moment, we cannot rule out that the introduction of some of these mutations might have an additional effect on the formation or release of VLPs, resulting in the complete absence of VLPs. It was hypothesized that mutation of these residues in the Gₙ cytoplasmic tail disrupted heterodimer formation with the wt G₉, resulting in ER retention of G₉ while Gₙ was still able to exit the ER and was transported to the Golgi complex, as is known to occur when these proteins are expressed alone (26). Simultaneous detection of the mutated Gₙ and wt G₉ clearly showed that both glycoproteins are always colocalized, in the ER as well as in the Golgi membrane, implying that they still are able to form heterodimers. These results indicate that mutation of residues L46, E47, and L50 in the Gₙ cytoplasmic tail interferes with the correct targeting of the Gₙ/G₉ heterodimers to the Golgi complex, the site where budding occurs, resulting in no or strongly reduced release of infectious particles. The G₉ was proposed to contain an ER retention signal in its short cytoplasmic tail (15, 37), and masking of this signal through heterodimerization with the Gₙ might be a requirement for its exit out of the ER and entry into the secretory pathway. Our results suggest that the masking of this G₉ ER retention signal may be disrupted when residues L46, E47, and L50 are mutated, leading to the ER retention of the Gₙ/G₉ heterodimers. No conservation of amino acids 46 to 50 was observed between the cytoplasmic tail of UUK virus Gₙ and other phleboviruses (reference 17 and data not shown).

The five-amino-acid G₉ cytoplasmic tail postulated to contain an ER retention signal (15, 37) was also analyzed by a mutagenesis screen. We found that two residues, the third residue, a lysine, and the last residue, a serine, were important for the proper localization of both glycoproteins. Mutation of the residue K3 in G₉K3A resulted in the expression of both G₉ and Gₙ at the cell surface, in addition to its Golgi complex localization. Mutation of residue S5 effectively prevented both glycoproteins from exiting the ER, suggesting that mutation of this residue interferes with the ER-to-Golgi complex trafficking. Although we observed a similar phenotype for the G₉K3A and G₉ invert mutants, a lysine is present at position 3 in the G₉ invert mutant, indicating that the effect of this amino acid also is context dependent. This indicates that residue K3 plays a role in the retention of the glycoprotein heterodimer in the Golgi membrane, thereby allowing the efficient formation of infectious particles. In addition to its proposed role in ER retention, K3 also is involved in the retention of the glycoprotein heterodimers in the Golgi membrane. We hypothesize that mutation of this conserved lysine residue affects the interaction with the Gₙ cytoplasmic tail and possibly alters the secondary structure of the Golgi retention motif, previously found to be localized between residues 10 and 40. This would lead to inefficient Golgi retention of the heteromeric glycoprotein complex, leading to transport to the plasma membrane. Interestingly, this residue is absolutely conserved among the Phlebovirus, Hantavirus, and Orthobunyavirus genera. Most G₉ proteins of the different members in the Bunyaviridae family are retained in the ER when expressed alone, although Punta Toro virus G₉ has been shown to reach the plasma membrane despite the presence of the conserved lysine at position −3 (6).

In the present study we demonstrate, using a VLP system for UUK virus, that the cytoplasmic tails of both Gₙ and G₉ contain specific motifs essential for the virus life cycle. In a previous study, four amino acids in the Gₙ cytoplasmic tail were shown to be involved in the packaging through a direct interaction between the RNPs and the viral spike proteins (29). Here we continued our analysis of the glycoprotein cytoplasmic tails and showed that leucine at position 23 and, to a lesser extent, at position 24 in the Gₙ tail are important for the initiation of VLP budding in the Golgi membrane. For many viruses it has been established that the matrix protein is the major determinant for budding (16, 18, 21, 28, 40), and specific motifs in the matrix proteins, so-called late domains, have been identified that are known to interact with cellular factors to enable the budding of enveloped viruses (4, 10). We have not been able to identify a late domain in the Gₙ or G₉ cytoplasmic tail that could be involved in VLP formation. Also, bunyaviruses do not contain a matrix protein, and we have shown that the two glycoproteins alone can generate particles (29, 30). So far, the exact mechanism for how bunyaviruses bud into the Golgi membrane and if additional cellular factors are required for this process are not known. We also show that the cellular localization of the two glycoproteins Gₙ and G₉ is important for generation of VLPs and that specific residues, other than the previously identified Golgi complex retention motif (residues 10 to 40), play an important role in intracellular targeting of the viral spike proteins. These results, together with our identification of the packaging signal, give a better understanding of the biogenesis of UUK virus and other, more clinically relevant members of the Bunyaviridae family.
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REFERENCES


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