

# N-linked glycosylation of Gn (but not Gc) is important for Crimean Congo hemorrhagic fever virus glycoprotein localization and transport

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## Abstract

The mature Gn glycoprotein of Crimean Congo hemorrhagic fever (CCHF) virus contains two predicted glycosylation sites (557N and 755N). Of these, N-glycans are added only at 557N, as evidenced by abrogation of Gn-glycosylation by mutation of 557N but not 755N site. Mutational block of Gn-glycosylation at 557N did not significantly affect Gn proteolytic processing but did result in mislocalization and retention of Gn and other proteins synthesized from the virus M segment ORF (GP160, GP85, GP38 and Gc) in the endoplasmic reticulum. In contrast to Gn, similar mutational analysis demonstrated that, while N-glycosylation occurs at the two predicted sites in Gc, abrogation of their glycosylation did not alter localization of any of the CCHF virus glycoproteins. Studies of Gn expressed in the absence of Gc demonstrate that, while Gn processing and localization are independent of Gc, all the CCHF virus glycoproteins appear dependent on N-glycosylation of Gn for correct folding, localization and transport.

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## Introduction

Enveloped viruses usually contain one or more surface glycoproteins and the majority of these are N-glycosylated. The glycosylation machinery operating in the lumen of the endoplasmic reticulum of the cells recognizes a specific conformation of the glycosylation sequence Asn–X–Ser/Thr (NXS/T) and attaches the glycans through the formation of an N-glycosidic bond to the side chain of the asparagine residues (Helenius and Aebi, 2004). N-linked glycosylation is known to influence protein function in many ways, including protein folding (Helenius and Aebi, 2001, 2004), chaperone association (Molinari and Helenius, 2000); transport, cellular localization of glycoproteins and virus infectivity (Hauri et al., 2000; Hannah et al., 2005; Losman et al., 1999; Luftenegger et al., 2005; Shi and Elliott, 2004; Shi et al., 2005). Among members of the *Bunya-*

*viridae* family, the functional significance of N-glycosylation of the glycoproteins of Hantaan and Bunyamwera viruses has been well documented (Shi and Elliott, 2004; Shi et al., 2005). In both cases, mutation of the single N-glycosylation site utilized within Gn resulted in misfolding and mislocalization, suggesting N-glycosylation as a major determinant in the biogenesis of the glycoproteins. Furthermore, a Gn-glycosylation mutant was unable to rescue Bunyamwera virus in a reverse genetics system, confirming that N-glycosylation of Gn was essential for virus viability (Shi et al., 2005).

Crimean Congo hemorrhagic fever (CCHF) virus is a tick-borne virus belonging to the genus *Nairovirus* of the family *Bunyaviridae*. CCHF virus causes hemorrhagic manifestations in humans with up to 30% case fatality. In contrast to viruses in other genera of the family *Bunyaviridae* (such as Hantaan and Bunyamwera viruses), the M segment of CCHF virus is approximately twice the size and encodes a polyprotein which undergoes a complex proteolytic cascade to generate several precursor and possible non-structural proteins (GP85, GP160, GP38, mucin and NSm) in addition to the mature structural glycopro-

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teins Gn and Gc (Fig. 1A) (Bertolotti-Ciarlet et al., 2005; Garry and Garry, 2004; Sanchez et al., 2002, 2006; Vincent et al., 2003). The proteolytic processing of precursors involves multiple signalase cleavage events in the ER, SKI-1 and SKI-1 like protease cleavage events in the *cis*-Golgi network and furin/PC cleavage in the *trans*-Golgi network (Sanchez et al., 2002, 2006; Vincent et al., 2003). Given the increased processing complexity relative to Hantaan and Bunyamwera viruses, we chose to examine the functional role of N-glycosylation of the CCHF virus Gn and Gc in processing and localization and to examine how this compared with results obtained with these simpler members of the family *Bunyaviridae*. Results of our mutagenesis and expression studies demonstrate that, despite the increased complexity, similar results were found for CCHF virus in that N-glycosylation of both Gn and Gc occurs, but only Gn-glycosylation is important for the proper processing and trafficking of CCHF virus glycoproteins.

## Results

### *The only predicted N-glycosylation site (557N) in the Gn extracellular domain is glycosylated*

Using the existing CCHF virus glycoprotein processing model as a starting point (Sanchez et al., 2006) and

N-glycosylation (NetNGly 1.0), transmembrane and protein topology (TMHMM server v.2.0) prediction software, four potential N-glycosylation sites within the mature Gn and Gc glycoprotein sequences were identified for experimental investigation. These included two potential glycosylation sites (NXT/S motif) within the mature Gn of CCHF virus (Fig. 1A). The motif at 557N is completely conserved among CCHF virus strains for which sequences are known (32/32 strains, Deyde et al., 2006), while the motif at 755N is conserved in 30/32 of the virus strains. 557N is likely glycosylated as it is strongly predicted to be located in the extracellular domain. In contrast, 755N is likely not glycosylated due to its cytoplasmic orientation as per membrane topology predictions. These NXT/S sites were individually mutated to investigate their potential utilization and function (Fig. 1A). The intracellular processing of mature Gn and protein mobility differences were analyzed by expressing the glycoproteins using a recombinant vaccinia virus expression system (Fuerst et al., 1986) and comparing wild type (FL-WT) and mutant (FL-557N→K and FL-755N→K) proteins (Fig. 1A). Radiolabeling and immunoprecipitation of FL-WT-expressing cell lysates using a polyclonal hyperimmune mouse ascitic fluid (HMAF) against CCHF virus proteins indicated the presence of four prominent proteins with approximate molecular masses, 140 kDa, 85 kDa, 75 kDa and 37 kDa (Fig. 1B, lane 1). These proteins have

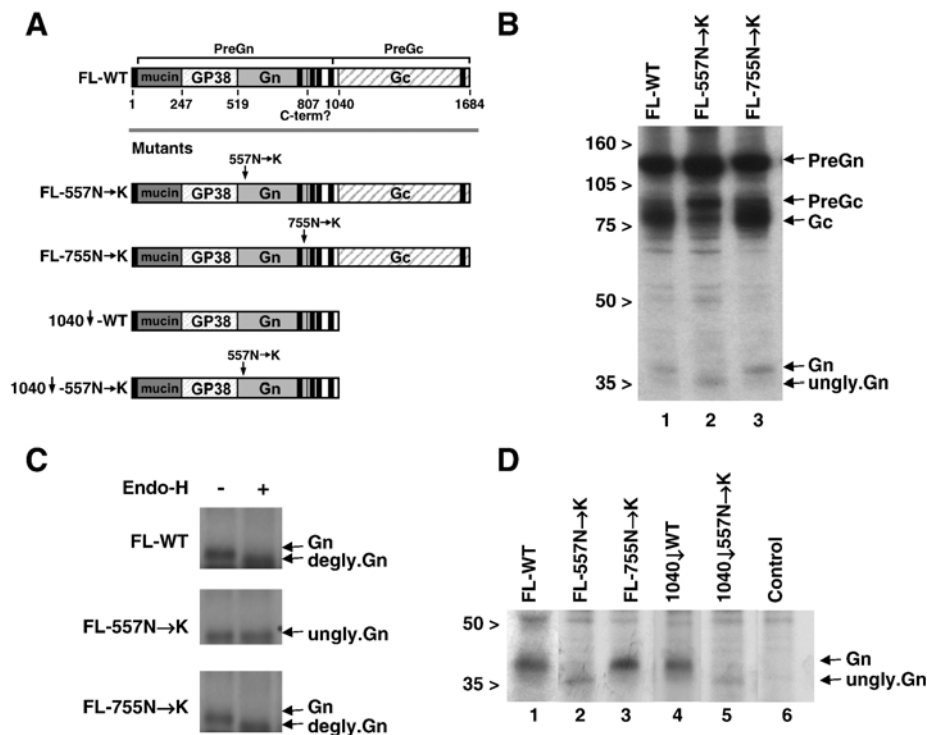


Fig. 1. Glycosylation of Crimean Congo hemorrhagic fever virus Gn. (A) Diagrammatic representation of CCHF virus glycoproteins and the mutants examined. N→K denotes the mutation of asparagine to lysine and the numbers preceding N→K denote the amino acid positions. (B) SW13 cells expressing WT, FL-557N→K or FL-755N→K were labeled for 30 min with  $^{35}\text{S}$  cysteine and chased for 3 h. Cells were then lysed and the clarified supernatants were immunoprecipitated using CCHF virus-HMAF and proteins resolved in 3–8% NuPAGE gel. PreGn and PreGc refer to the precursors of Gn and Gc, respectively and ungly.Gn refers to the unglycosylated, but cleaved Gn. The numbers in the left denote molecular weight marker locations in the gel. (C) Experiment was performed as described for panel B. The immunoprecipitates were subjected to endoglycosidase digestion (+) or left untreated (–) before resolving in 3–8% NuPAGE gel. Gn and degly.Gn refer to mature Gn and deglycosylated Gn, respectively. (D) SW13 cells expressing WT or mutants indicated on the top of the panel were immunoprecipitated using an antibody against Gn epitopes and proteins resolved in 10% NuPAGE gel.

previously been verified as corresponding to the CCHF virus PreGn, PreGc, Gc and Gn proteins, respectively (Sanchez et al., 2002). Abrogation of the 557N site (FL-557N→K, Fig. 1B, lane 2) but not the 755N site (FL-755N→K, lane 3) resulted in the faster mobility of the mature Gn. This mobility shift confirms the identity of the protein bands as Gn and demonstrates that N-glycosylation normally occurs at the 557N site but not the 755N site. The 557N alteration appeared to have no effect on PreGn production or its proteolytic processing to generate Gn, suggesting that these events occur independent of the glycosylation at 557N. This is in contrast to the FL-557N→K effect on PreGc to Gc conversion, in that significant impairment of Gc processing is seen with the mutant (compare PreGc to Gc in Fig. 1B, lanes 1 and 2).

In order to more precisely identify the glycosylated and unglycosylated Gn proteins, immunoprecipitated CCHF virus FL-WT or mutant glycoproteins were subjected to endo-H digestion. From the untreated FL-WT samples, mature Gn resolved as a 37 kDa protein and endo-H digestion resulted in Gn with faster mobility (35 kDa), consistent with removal of glycosidic modifications (Fig. 1C, top panel). However, in the case of Gn from FL-557N→K, both untreated and endo-H treated proteins showed no size shift and resolved at 35 kDa. These data provide further evidence that 557N is glycosylated and the mutation effectively abrogated the glycosylation. In contrast, the pattern of endo-H sensitivity seen with FL-755N→K was very similar to that of the Wt protein (Fig. 1C, bottom panel), indicating that 755N is not an N-glycosylation site.

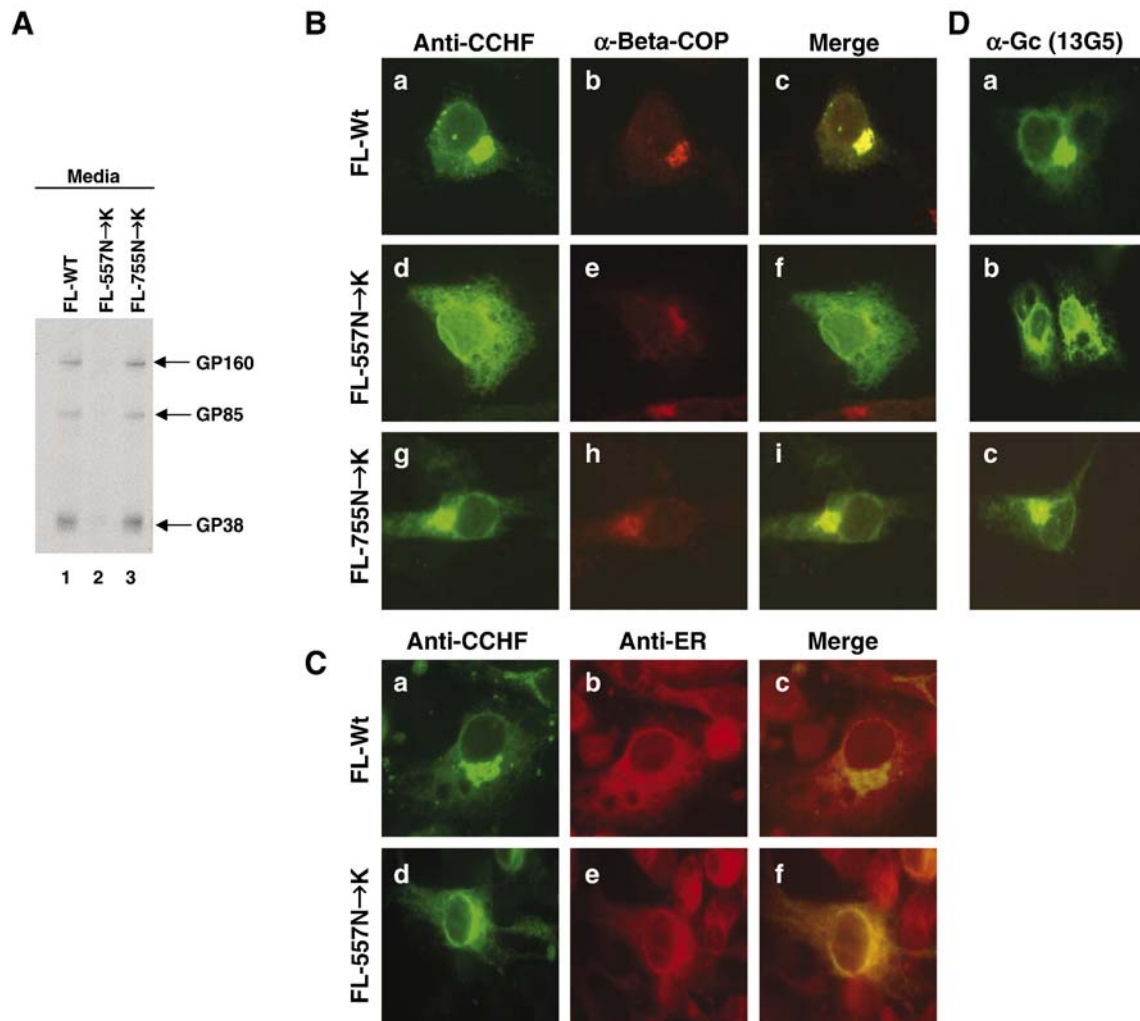


Fig. 2. Mutation in the N-glycosylation site of Gn results in mislocalization of CCHF virus glycoproteins. (A) The supernatants of cells from Fig. 1B were immunoprecipitated using CCHF virus-HMAF, and the proteins were resolved in 3–8% NuPAGE gel. GP160, GP85 and GP38 are the three proteins found in the media during chase periods. Note the dramatic absence of those proteins in cells expressing FL-557N→K. (B) BHK-21 cells were transfected with plasmids indicated at the left side, permeabilized and dual stained with CCHF virus polyclonal antibody (Anti-CCHF) and  $\beta$ -COP. Images were captured with both labels independently and merged. a, b and c are patterns observed with FL-WT. d, e and f are patterns observed for FL-557N→K. g, h and i are patterns observed for FL-755N→K. Note the change in localization of the proteins in FL-557N→K compared to FL-WT or FL-755N→K. (C) Localization of FL-WT and FL-557N→K using anti-CCHF and anti-ERp72 antibodies. a, b, and c are FL-WT localization patterns. d, e and f are FL-557N→K localization patterns. Note that FL-557N→K is predominantly colocalized with the ER marker in contrast to FL-WT. (D) Analysis of the localization of FL-WT and Gn-glycosylation mutants using Gc monoclonal antibody (13G5). a, FL-WT; b, FL-557N→K; and c, FL-755N→K.

*Gn processing occurs independent of Gc*

Our previous studies indicated that PreGn and PreGc are generated from the full-length precursor in a co-translational manner (Fig. 1A) (Sanchez et al., 2002; Vincent et al., 2003). However, the potential role of Gc in regulating or modulating Gn processing or N-glycosylation is unknown. To address this question, we created plasmid 1040 ↓ WT by PCR amplification of the region of the M segment which encodes the amino acids 1–1040 followed by an introduced stop codon (using the reverse primer) to prevent addition of plasmid backbone encoded protein tags (Fig. 1A). In addition, the 557N→K mutation was introduced in the background of 1040 ↓ WT to produce the expression plasmid 1040 ↓ 557N→K. Proteins expressed from these and full-length constructs were analyzed by immunoprecipitation using an antipeptide antibody specific to Gn sequences (Fig. 1D). In cells expressing Gn in the absence of Gc (i.e. 1040 ↓ WT), mature glycosylated Gn (37 kDa) was found as seen

for FL-WT (Fig. 1D, compare lanes 1 and 4), indicating that Gn processing and N-glycosylation were independent of Gc. These results were further confirmed by showing that the mature but unglycosylated Gn (35 kDa protein) was synthesized by both FL-557N→K and 1040 ↓ 557N→K (lanes 2 and 5). The fact that the Gn-antipeptide antibody reacts with the 37 kDa and 35 kDa proteins further confirms the identity of glycosylated and unglycosylated Gn, respectively. Taken together, these results indicate that, in addition to Gn cleavage being independent of N-glycosylation, Gc does not play a critical role in facilitating Gn processing or N-glycosylation.

*Mutation of 557N alters the localization of CCHF virus M segment-encoded proteins*

In order to examine if the mutation of the N-glycosylation site has altered the protein transport properties, we analyzed the cell supernatants for CCHF virus-specific glycoproteins

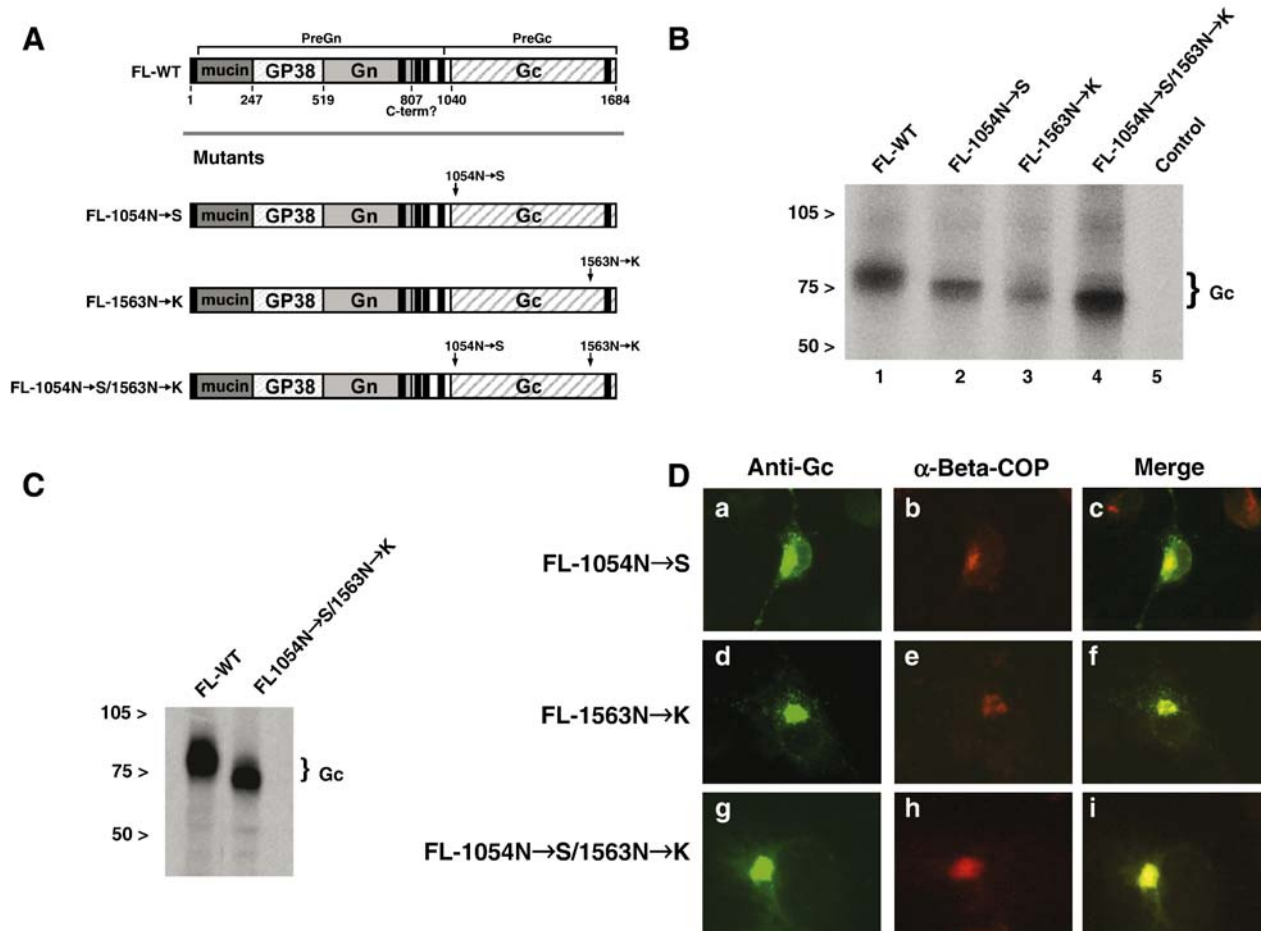


Fig. 3. N-glycans are added to Gc at aa 1054N and 1563N. (A) Diagrammatic representation of CCHF virus Gc mutants examined. N→S or N→K denotes the mutation of asparagine to serine or lysine and the numbers preceding denote the amino acid positions. (B) SW13 cells expressing WT, FL-1054N→S, FL-1054N→K or both mutations incorporated in the gene (FL-1054N→S/1054N→K) were labeled for 30 min with <sup>35</sup>S cysteine and chased for 3 h. Cells were then lysed and the clarified supernatants were immunoprecipitated using CCHF virus-HMAF and proteins resolved in 3–8% NuPAGE gel. Note the mobility shift based on individual and double mutations. (C) Cells expressing WT-FL or FL-1054N→S/1054N→K were lysed and immunoprecipitated using a Gc-specific monoclonal antibody (11E7). Note the change in mobility of Gc in the double mutant. (D) BHK-21 cells were transfected with plasmids indicated at the left side, permeabilized and dual stained with 13G5 (anti-Gc monoclonal) and β-COP. Images were captured with both labels independently and merged. a, b and c are patterns observed with FL-1054N→K. d, e and f are patterns observed for FL-1563N→K. g, h and i are patterns observed for FL 1054N→S/1054N→K. Note that no change in localization of the proteins was observed due to mutations.



recovered from the experiment depicted in Fig. 1B. As reported previously (Sanchez et al., 2006), three viral glycoproteins (GP160, GP85 and GP38) were detected in the supernatants from FL-WT and FL-755N→K expressing cells (Fig. 2A, lanes 1 and 3). Interestingly, although the intracellular levels of the PreGn were similar (refer Fig. 1B), the proteins shed into the supernatants of FL-557N→K expressing cells were dramatically reduced (Fig. 2A, lane 2). This result suggested that abrogation of Gn-glycosylation at 557N had impaired the secretion of M segment-encoded glycoproteins. To determine if block of Gn-glycosylation also affected glycoprotein localization, glycoproteins generated from plasmid FL-WT and the Gn-glycosylation mutant plasmids were examined by indirect immunofluorescence assay performed using the CCHF virus polyclonal antibody (Fig. 2B). FL-WT glycoproteins exhibited predominantly Golgi staining with a moderate amount of staining also detected in the endoplasmic reticulum (ER) (Fig. 2B, a). Double staining using  $\beta$ -COP (Golgi marker) antibody confirmed the colocalization of CCHF virus glycoproteins with the Golgi (panel B-b and c). These data are consistent with previous reports describing the intracellular localization of CCHF virus glycoproteins (Bertolotti-Ciarlet et al., 2005; Haferkamp et al., 2005). A similar staining pattern was observed for the FL-755N→K mutant (Figs. 2B, g, h and i) as would be expected based on the above results indicating lack of glycosylation at this site. In contrast, similar analysis with FL-557N→K indicated that almost all the proteins were localized in reticular structures of the ER with very little present in Golgi structures (Figs. 2B, d, e and f). Confirmation of the ER localization of FL-557N→K was obtained by colocalization experiments using antibodies against CCHF virus and an ER protein (anti-ERp72). CCHF virus polyclonal antibody stained the glycoproteins synthesized from FL-WT predominantly in the Golgi and to a limited extent in the ER and significant localization was not observed with the ER marker (Figs. 2C, a–c). On the other hand, FL-557N→K showed extensive ER staining and colocalization as evidenced by CCHF virus polyclonal antibody and ER marker, respectively (Figs. 2C, d–f). These data clearly indicated that abrogation of glycosylation at 557N had resulted in the retention and mislocalization of the glycoproteins predominantly in the ER. In addition, the specific localization of Gc was examined by using a Gc-specific monoclonal antibody (13G5, kindly provided by Dr. Jonathan Smith, USAMRIID). Gc synthesized from FL-WT or FL-755N→K stained both in the ER and Golgi structures (Figs. 2D, a and c), while Gc synthesized from FL-557N→K stained predominantly in the ER with no apparent colocalization with Golgi marker (Fig. 2D, b). Collectively the above data confirmed that N-linked glycosylation at 557N plays an important role in the correct transport of CCHF virus glycoproteins from the ER to subsequent cellular compartments.

*N-linked glycosylation occurs at site 1054N and 1563N in Gc but is not essential for correct protein processing or localization*

We next investigated the potential utilization and functional role of N-glycosylation sites within Gc (75 kDa), the other

major structural glycoprotein of CCHF virus. There are a total of four NXS/T motifs in Gc, and all of them are conserved among 32 characterized virus isolates (Deyde et al., 2006). However, of these four NXS/T sites, only 1054N and 1563N had a higher score as potential glycosylation sites, whereas 1194 is unlikely to be glycosylated due to the presence of a proline residue at the X position (Shakin-Eshleman et al., 1996; Kasturi et al., 1997) and 1345N had a negative score. In order to examine the role of glycosylation in Gc processing and transport, mutations were introduced in 1054N (FL-1054N→S) and 1563N (FL-1563N→K) individually or in combination (FL-1054N→S/1563N→K) and analyzed by immunoprecipitation using CCHF virus-HMAF (Fig. 3A). Our data indicated that both 1054N and 1563N are N-glycosylated in the FL-WT protein as evidenced by a shift in mobility of Gc in both mutants (Fig. 3B, lanes 1–4). Consistent with glycosylation at the individual sites, mutations at both 1054N and 1563N resulted in a protein resolving approximately 5 kDa smaller than that observed from FL-WT protein (Fig. 3B, lane 4). A similar Gc protein mobility shift was observed in a separate immunoprecipitation experiment utilizing a Gc-specific monoclonal antibody, thus confirming the identity of the Gc protein bands (Fig. 3C). Although the glycosylation was abrogated by mutation, Gc glycosylation negative mutants were found to exhibit Golgi and ER staining indistinguishable from that observed with FL-WT-expressing cells (compare Fig. 3D with Fig. 2B). Together, these data show that while Gc glycosylation does occur at the 1054N and 1563N sites, its abrogation did not affect the localization of the glycoproteins.

## Discussion

The synthesis of CCHF virus glycoproteins appears to involve a complex cascade of proteolytic cleavage events to generate the mature Gn and Gc glycoproteins and their trafficking from the ER to the Golgi where virus assembly takes place (Sanchez et al., 2002, 2006; Vincent et al., 2003; Garry and Garry, 2004; Bertolotti-Ciarlet et al., 2005; Haferkamp et al., 2005). Two primary precursor proteins, PreGn (140 kDa) and PreGc (85 kDa), are cleaved to generate mature Gn and Gc, respectively (Sanchez et al., 2002). The protease SKI-1 has been shown to cleave at an RRLL motif to generate the Gn amino terminus (Vincent et al., 2003). Similarly, the Gc amino terminus is preceded by an RKPL motif which is probably cleaved by an SKI-1 like protease (Vincent et al., 2003). SKI-1 protease, which is responsible for generating Gn from PreGn, is localized in early Golgi and ER structures and plays a key role in the proteolytic activation of few prohormones (brain derived neurotrophic factor, sterol regulatory element-binding proteins, activating transcription factor 6) (Pullikotil et al., 2004) and the glycoproteins of two arenaviruses (Lassa and lymphocytic choriomeningitis viruses) (Beyer et al., 2003). Experiments performed under BFA treatment conditions indicate that SKI-1 cleavage of CCHF virus glycoprotein occurs early in the ER or *cis*-Golgi structures (Vincent et al., 2003), similar to results seen with Lassa virus

glycoproteins (Lenz et al., 2001). In addition, a furin-like protease further cleaves precursor proteins (at an RSKR motif) in the *trans*-Golgi network (Sanchez et al., 2006). Glycosylation can play a major role in viral glycoprotein folding and trafficking and can profoundly influence virus tropism, infectivity and antigenicity. For these reasons we chose to examine the role of Gn and Gc N-glycosylation in the processing and trafficking of these important proteins.

Our data demonstrated that one N-glycosylation site located in the extracellular domain of Gn (557N) and two sites located in the extracellular domain of Gc (1054N and 1563N) were glycosylated. The motif representing these glycosylation sites is conserved among all 32 CCHF virus M segments for which the sequences are known (Deyde et al., 2006) strongly suggestive of a functional role. Interestingly, mutational abrogation of N-glycosylation at 557N did not prevent the cleavage of mature Gn, but rather disrupted correct localization of Gn and the other major structural glycoprotein Gc predominantly in the ER. In contrast, mutational abrogation of Gc glycosylation did not negatively affect Gc proteolytic processing or localization of Gn, Gc or other virus glycoproteins. These findings are reminiscent of those obtained for Bunyamwera (BUN) virus glycoproteins, where it was found that the replacement of the only glycosylation site in Gn (N60 to Q) did not abolish the proteolytic processing of mature Gn, but instead generated a misfolded Gn protein which resulted in both Gn and Gc being retained in the ER (Shi et al., 2005). In addition, it was found that infectious BUN virus could not be rescued by reverse genetics using an M segment derived expression plasmid producing Gn with the N60 to Q mutation. In contrast, using the same approach, Gc mutations which abrogated Gc glycosylation did allow recovery of infectious BUN viruses (Shi et al., 2005). The authors hypothesized that the larger Gc protein depended on Gn for proper folding and heterodimerization. A similar interpretation may apply to the CCHF virus glycoprotein data presented here. We also observed that mutation of the only N-glycosylation site at the extracellular domain of mature Gn resulted in the retention of both Gn and Gc in the ER. Thus, N-linked glycosylation of Gn, but not Gc, appears to play an important role in the proper localization and transport of CCHF and other family *Bunyaviridae* virus glycoproteins. In spite of that similarity, CCHF virus appears to follow a very complex pathway utilizing many proteases in the proteolytic cleavage of its glycoprotein subunits.

A single mutation within CCHF virus mature Gn affecting the localization of PreGn and PreGc derived glycoproteins suggests that there is likely a Gn-glycosylation dependent folding event which facilitates the transport of Gn, Gc and other CCHF virus glycoproteins. Previous studies have indicated that Gc expressed in the absence of Gn is localized in the ER, that Gn has the signal for Golgi targeting, and Gn/Gc association results in the localization of Gc in the Golgi (Bertolotti-Ciarlet et al., 2005; Haferkamp et al., 2005). It follows then that the Gn 557N→K which results in abrogation of glycosylation and mislocalization of Gn in the ER should result in the retention of Gc and other associated proteins in the same compartment. Interestingly, although the 557N→K mutation did not affect

Gn processing, the cleavage of Gc from PreGc appears to be impaired as evidenced by the detection of less Gc and more PreGc than seen with FL-WT (Fig. 1B, lane 2). As earlier studies have shown that Gc cleavage occurs early in the secretory pathway (Vincent et al., 2003), it is unlikely that the reduction in Gc processing seen with unglycosylated Gn is due to retention of Gc in the ER. It is more plausible that the lack of Gc interaction with unglycosylated Gn and its misfolding results in the Gc cleavage site being less accessible to the protease.

Given the apparent equivalent importance of the Gn-glycosylation found in both CCHF and BUN viruses, it would be predicted that abrogation of Gn-glycosylation might be detrimental to the growth and infectivity of CCHF virus in a manner similar to that seen with BUN virus (Shi et al., 2005). Testing of this prediction will have to await successful development of a reverse genetics system for CCHF virus.

In summary, this study demonstrates the N-glycosylation of a conserved site in the CCHF virus Gn glycoprotein and documents its apparent importance for correct glycoprotein localization and transport. These data provide insight into the complex pathway involved in the biosynthesis of CCHF virus glycoproteins and have important implications for design of recombinant vaccines and antiviral strategies.

## Materials and methods

### *Cells, viruses, plasmids and antibodies*

SW13 and BHK-21 cells were used in the present study. SW13 cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics as previously described (Sanchez et al., 2002). BHK-21 cells were grown in Glasgow Minimum Essential Medium (G-MEM), with 10% fetal bovine serum, penicillin–streptomycin, sodium pyruvate, tryptone broth and L-glutamine added. Stocks were grown and titered in SW13 cells in the biosafety level 4 laboratory of the Centers for Disease Control and Prevention, Atlanta, GA using IbAr10200 (from *Hyalomma excavatum* ticks from Sokoto, Nigeria) as the seed stock. The M segment WT-ORF cloned into pcDNA3.1 Directional-TOPO expression vector (Invitrogen) was described previously (Sanchez et al., 2002). Protein expression was performed using the recombinant vaccinia virus (vvTF7-3) expression system developed by Fuerst et al. (1986).

Polyclonal hyperimmune mouse ascitic fluid (HMAF) developed against total CCHF virus IbAr10200 strain proteins was kindly provided by Drs. T. Ksiazek and P. Rollin, CDC. Gn-specific rabbit antipeptide antibody was generated under contract (Research Genetics) and is referred to as Gn/540–551. Gc-specific monoclonal antibody (MAb) 11E7 originally from Jonathan Smith (formerly of the U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID), Fort Detrick, MD) was kindly provided by Dr. Robert Doms; rabbit anti-β-COP polyclonal antibody and rabbit anti-Erp72 were purchased from ABR-Affinity BioReagents (Golden, CO) and Calbiochem (La Jolla, CA), respectively.

### *Generation of CCHF virus glycoprotein expression constructs with mutations in the N-glycosylation sites*

CCHF virus strain IbAr10200 complete M segment wild type (WT) ORF (Sanchez et al., 2002) was used as templates to generate mutants. To introduce mutations at the predicted glycosylation sites, we employed the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene) following the manufacturer's recommendations using the following primers: (1) for FL-557N→K: forward primer: ATAACCATCTGCAAAGGCTCAACTATT and reverse primer: AATAGTTGAGCCTTTGCAGATGGTTAT; (2) for FL-755N→K: forward primer: ATGCATGACCTCAAATGCAGTTACAAC and reverse primer: GTTGTAAGTGCATTTGAGGTCATGCAT; (3) for FL-1054N→S: forward primer: AAAAATCTGCTAAGTTCACAAGTTTA and reverse primer: TAAACTTGTTGAACCTTAGCAGATTTTT; for FL-1563N→K: forward primer: ATCGGAAAGCAAAAAGCACTTGCACG and reverse primer: CGTGCAAGTGCTTTTTTGTCTTCCGAT. The presence of introduced changes in the gene was confirmed by complete sequencing of the ORF. Due to the larger plasmid size (more than 10 kb), we chose amino acid substitutions for N which can be generated with minimal nucleotide alterations.

### *Transfection, metabolic labeling and radioimmunoprecipitation of glycoproteins*

Sub-confluent monolayers of SW13 or BHK-21 cells ( $5 \times 10^5$ ) grown in 6 well plates were infected with recombinant vaccinia virus vTF7-3 at an MOI of 5 for 1 h at 37 °C. During virus adsorption, a mix containing plasmid DNA, Plus Reagent (Invitrogen) and Lipofectamine Reagent (Invitrogen) was made as recommended by the manufacturer. After removal of viral inoculum, DNA complexes were added to cell monolayers and incubated for 20 h. For experiments involving radioimmunoprecipitation, cells were starved in cysteine-free medium for 1 h followed by labeling with 100  $\mu$ Ci of [ $^{35}$ S]cysteine (Perkin Elmer Life Sciences) for 30 min. Label was then removed and replaced with normal growth media containing cysteine and cells chased for indicated times. At the end, cells were lysed and immunoprecipitated as described before (Sanchez et al., 2002), resolved on 3–8% NuPAGE precast gel systems (Invitrogen) and analyzed by autoradiography.

### *Treatment of immunoprecipitated CCHF virus glycoproteins with glycosidase enzymes*

In order to investigate the nature of glycosidic modifications on the CCHF virus glycoproteins, endo-H digestion of immunoprecipitated proteins was performed at 37 °C overnight. The digested proteins were resolved on NuPAGE gels under reducing conditions and analyzed by autoradiography.

### *Indirect immunofluorescence assay*

Cells grown on cover slips were infected with recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase at

an MOI of 5 and transfected with the various constructs following the same procedures described for immunoprecipitation assays. At 20 h post-transfection cells were rinsed with PBS and fixed with 3.6% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 10 min. After appropriate primary and secondary antibody treatments for 30 min at room temperature, cells were thoroughly washed and were mounted onto glass slides. Localization of glycoproteins was analyzed using a Zeiss Axioplan microscope and images captured with a Nikon CoolPix 9500 digital camera.

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The finding and conclusion in the report are those of the authors and do not necessarily represent the views of the funding agency.

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