JOURNAL OF VIROLOGY, Dec. 2002, p. 12463–12472 Vol. 76, No. 24
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.24.12463–12472.2002

Covalent Modifications of the Ebola Virus Glycoprotein

Scott A. Jeffers,[1] David Avram Sanders,[1]* and Anthony Sanchez[2]

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907,[1] and Special Pathogens Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[2]

Received 8 July 2002/Accepted 6 September 2002

The role of covalent modifications of the Ebola virus glycoprotein (GP) and the significance of the sequence identity between filovirus and avian retrovirus GPs were investigated through biochemical and functional analyses of mutant GPs. The expression and processing of mutant GPs with altered N-linked glycosylation, substitutions for conserved cysteine residues, or a deletion in the region of O-linked glycosylation were analyzed, and virus entry capacities were assayed through the use of pseudotyped retroviruses. Cys-53 was the only GP_1 (~130 kDa) cysteine residue whose replacement resulted in the efficient secretion of GP_1, and it is therefore proposed that it participates in the formation of the only disulfide bond linking GP_1 to GP_2 (~24 kDa). We propose a complete cystine bridge map for the filovirus GPs based upon our analysis of mutant Ebola virus GPs. The effect of replacement of the conserved cysteines in the membrane-spanning region of GP_2 was found to depend on the nature of the substitution. Mutations in conserved N-linked glycosylation sites proved generally, with a few exceptions, innocuous. Deletion of the O-linked glycosylation region increased GP processing, incorporation into retrovirus particles, and viral transduction. Our data support a common evolutionary origin for the GPs of Ebola virus and avian retroviruses and have implications for gene transfer mediated by Ebola virus GP-pseudotyped retroviruses.

Ebola viruses are a group of enveloped, single-stranded RNA viruses that, together with Marburg virus, are classified in the order *Mononegavirales* and the family *Filoviridae*. Filoviruses cause a severe hemorrhagic fever disease in human and/or nonhuman primates and are designated biosafety level 4 agents. These viruses contain a single structural glycoprotein (GP) that forms the peplomers that project from the surface of the enveloped, rod-shaped virion (7, 22, 23). The GPs of filoviruses are expressed from the GP gene, but the organization of this gene differs dramatically between Ebola and Marburg viruses. The GPs of all Marburg virus isolates are encoded in a single open reading frame (ORF), whereas the GPs of Ebola viruses are encoded in two frames (0 and −1) that are connected by transcriptional editing that results in an insertion of a single base (22, 30). The primary gene product of the Ebola virus GP gene is a secreted GP (cleaved to generate secreted GP [SGP] and delta peptide) (35), whereas the structurally important GP is a product of the edited mRNA. The functions of SGP and delta peptide are not well defined, but biochemical, immunological, and structural studies have provided clearer insights into the role of GP in virus entry and pathogenesis (2, 7, 8, 12, 13, 16, 20, 22, 23, 32, 36, 39). The peplomers covering the surface of Ebola virions are composed of GP trimers anchored in the lipid bilayer by a transmembrane (TM) sequence in a type I orientation (21–23). These structures mediate the entry of the virion into cells through a process involving (i) binding to receptor molecules, (ii) endocytosis of the virion, (iii) acidification of the endocytic vesicle, and (iv) membrane fusion brought about by acid-induced conformational changes in GP (1, 12, 28, 38, 40).

The processing of GP in cells leads to the production of various forms as it travels through the endoplasmic reticulum (ER) and Golgi apparatus to the plasma membrane (32). An N-glycosylated precursor form of GP (GP_{pre}), which is found in the ER, is further processed to a fully glycosylated uncleaved form in the Golgi apparatus (GP_0); trafficking to the Golgi apparatus also leads to the addition of O-linked glycans (7, 32). In the trans-Golgi apparatus, GP_0 is cleaved by the convertase furin to generate GP_1 (~130 kDa), whose role appears to involve receptor binding (21), and transmembrane GP_2 (~24 kDa); these two subunits are linked by disulfide bonding (22, 23, 32). Figure 1 shows a diagrammatic view of the Zaire species of Ebola virus GP. GP_1 is highly glycosylated with N-linked and O-linked glycans (7). Glycosylation contributes approximately half of the mass of GP_1, and O-linked glycans confer a mucin-like property to its C terminus. GP_2 also contains N-linked glycans (22, 23, 32) with two predicted N-linked sites but does not appear to contain O-linked glycans.

The disulfide bonding that holds the GP_1-GP_2 heterodimer together is predicted to involve the first cysteine of GP_1 (Cys-53) and the fifth cysteine from the amino terminus of GP_2 (Cys-609). This prediction is based on sequence and structural similarities of Ebola virus GP to the GPs of avian sarcoma and leukosis viruses (ASLVs) (10) and other retroviruses (6, 16, 19, 24, 31) and the fact that the Cys-53 residue of SGP is involved in forming the SGP homodimer (23, 34). The intramolecular disulfide bonding of GP_2 has also been predicted based on sequence similarity to the ASLV GPs (10). A putative fusion peptide of 16 hydrophobic and uncharged residues has been identified near the amino terminus of GP_2, and a synthetic version of this peptide was shown to penetrate and induce the fusion of membranes containing phosphatidylinositol (20). The

* Corresponding author. Mailing address: Department of Biological Sciences, 1392 Lilly Hall, Purdue University, West Lafayette, IN 47907. Phone: (765) 494-6453. Fax: (765) 496-1189. E-mail: retrovir@bragg.bio.purdue.edu.

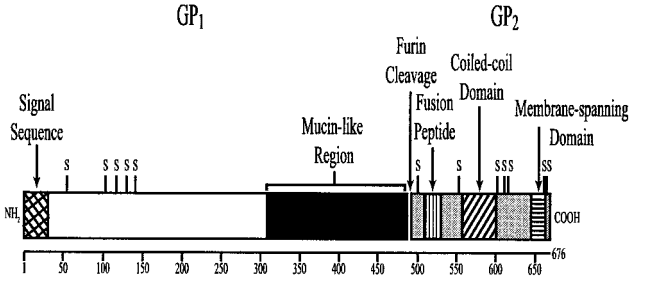

FIG. 1. Schematic representation of Ebola virus GP. The GP_1 and GP_2 subunits of GP are drawn to scale (residue numbers are indicated below the diagram). The positions of the signal sequence (cross-hatching), conserved cysteine residues (S), the mucin-like region (region of O-linked glycosylation; black), the furin cleavage site, the fusion peptide (vertical lines), the coiled-coil domain (diagonal lines), and the membrane-spanning domain (horizontal line) are indicated.

membrane-spanning anchor sequence near the C terminus of Ebola virus GP_2 contains two conserved cysteine residues that are palmitoylated (13). X-ray crystallography of recombinant-expressed portions of GP_2 have shown that alpha helices in the sequence form coiled coils and that these structures are remarkably similar to those of the transmembrane (TM) envelope protein of retroviruses and influenza viruses as well as SNAREs (cellular proteins involved in fusion of transport vesicles) (16, 36).

Recombinant DNA techniques have also been used to safely perform functional studies of the Ebola virus GP peplomer through pseudotyping of engineered vesicular stomatitis virus (12, 28) and retroviruses (1, 38–40). Pseudotyped retrovirus particles have been used to demonstrate the permissiveness to virus entry of endothelial cells and the relative lack of susceptibility of lymphocyte cell lines to transduction (1, 38, 40). In addition, it has been shown that mutations of the fusion peptide sequence block virus entry (12) and that elimination of furin cleavage during processing of the Ebola virus GP does not prevent virus entry (13, 39).

To further define the effects of covalent modifications of GP_1 and GP_2 on their functions, we performed site-directed mutagenesis of plasmid DNA to change specific residues of the encoded proteins. These mutated sequences were then used to study the effects of these changes on processing, disulfide bonding, and virus entry through the use of pseudotyped retrovirus particles. We specifically wished to determine the functional significance of the residues that are conserved between the filovirus and ASLV GPs and the role of the O-linked glycosylation region of the Ebola virus GP.

MATERIALS AND METHODS

Cell lines and culture conditions. The human kidney cell line 293 (ATCC CRL-1573), the mouse embryo cell line NIH 3T3 (CRL-1658), and the 293T cell-derived ϕNX cell line (second-generation retroviral packaging cells) (11, 18, 27) and gpnlslacZ cell line were cultured in Dulbecco's minimal essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U of penicillin G, and 100 μg of streptomycin sulfate/ml, with or without 0.25 μg of amphotericin B/ml (growth medium). gpnlslacZ cells produce envelope protein-deficient replication-incompetent Moloney murine leukemia virus (MuLV) particles carrying MFG.S-nlslacZ, a retroviral vector encoding β-galactosidase localized to the nucleus (25).

Plasmids and site-directed mutagenesis. A modified version of plasmid pTM1 was used in transient expression studies of Ebola virus Zaire GP sequences with a vaccinia virus-T7 RNA polymerase (VV-T7) system (5). The pTM1 vector was modified to remove an ATG codon (within an *Nco*I site) at the beginning of the multiple cloning site by *Nco*I digestion, mung bean nuclease treatment, and ligation of the blunt-ended DNA. The resulting vector, pTM1(Δ*Nco*I), was used to subclone the entire Ebola virus GP ORF. The GP ORF was cleaved from plasmid pGEM-EMGP1 (21) by digestion with *Bam*HI and *Dra*I, and the fragment was isolated and directionally ligated into the pTM1(Δ*Nco*I) vector cleaved with *Bam*HI and *Stu*I. The resulting clone, pTM1(Δ*Nco*I)-GP, was used as the target DNA for all site-directed mutagenesis reactions. This clone encodes a GP sequence that differs from the wild-type amino acid sequence in a single residue within the membrane-spanning sequence (I662V) and, for comparative purposes, will be referred to as the wild-type sequence. This mutation is present in the original pGEM-EMGP1 clone but does not appear to affect the processing or function of the GP. GP residue numbering commences with the methionine of the signal sequence and is continuous through the GP_1 and GP_2 sequences.

Site-directed mutagenesis targeted conserved cysteines and asparagines in conserved N-linked glycosylation sites. Table 1 shows 21 mutant GPs that were used for the analysis of GP_1 and GP_2 in the VV-T7 system. Additional mutants (T42D; double substitutions for the GP_1 cysteines [C108G/C135S,C108G/C147S,C121G/C135S, and C121G/C147S], C670A, C672A, C670A/C672A, and Δ309–489) were also generated but were used only in the pseudotyping experiments. Mutagenesis of plasmid DNA sequences was performed by using commercial kits, either the GeneEditor in vitro system (Promega Corp.) or the MORPH system (5 Prime → 3 Prime, Inc.), according to the manufacturer's instructions. Briefly, 5′-phophorylated mutagenic primers ranging in length from 25 to 36 nucleotides (mismatches centered in the sequence) were annealed to denatured pTM1(Δ*Nco*I)-GP DNA and extended with T4 DNA polymerase, and ends were ligated with T4 DNA ligase. Plasmid DNAs with mutations were enriched by specific antibiotic selection (GeneEditor) or digestion with *Dpn*I prior to transformation (MORPH). Mutant DNAs were used to transform *Escherichia coli* mismatch repair mutants (BMH 71-18 or MORPH *mutS* cells), and miniprep DNA was isolated (5 Prime → 3 Prime) and used in the second-round transformation of *E. coli* JM109 to isolate mutated DNA strands. The GP clone in which the mucin region was deleted (Δ309–489) was generated from two PCR clones linked by an *Xba*I restriction site, which resulted in the replacement of the mucin sequence with two residues (serine and arginine). Mutations in isolated plasmid clones were identified by direct sequencing of miniprep DNA with dye terminator cycle sequencing reactions (ABI) analyzed with either an ABI 373 or an ABI 377 sequencer. Large-scale preparations for each type of mutated plasmid DNA were made by using commercial kits (Promega or 5 Prime → 3 Prime). The DNA was quantified by UV absorbance readings at 260 nm and then stored at −70°C until needed. The coding region (*Bam*HI/*Sal*I fragments) from plasmid pTM1(Δ*Nco*I)-GP and mutated versions of this DNA were separately ligated into the *Bam*HI/*Xho*I polylinker sites of vector pcDNA3 (Invitrogen) and cloned in *E. coli*, and plasmid DNA was isolated for use in pseudotyping studies.

TABLE 1. Mutant Ebola virus GPs expressed in the VV-T7 system

Mutant GP	Change[a]
C53G	GP_1 Cys 1
C108G	GP_1 Cys 2
C121G	GP_1 Cys 3
C135S	GP_1 Cys 4
C147S	GP_1 Cys 5
N40D	GP_1 ΔN-linked glycan 1
N204D	GP_1 ΔN-linked glycan 2
N238Y	GP_1 ΔN-linked glycan 4
N257D	GP_1 ΔN-linked glycan 5
N277D	GP_1 ΔN-linked glycan 6
N296D	GP_1 ΔN-linked glycan 7
C511G	GP_2 Cys 1
C556S	GP_2 Cys 2
C601S	GP_2 Cys 3
C608G	GP_2 Cys 4
C609G	GP_2 Cys 5
C670F	GP_2 Cys 6
C672F	GP_2 Cys 7
C670F/C672F	GP_2 Cys 6 + Cys 7
N563D	GP_2 ΔN-linked glycan 1
N618D	GP_2 ΔN-linked glycan 2

[a] Δ, deletion.

VV-T7 expression of GP sequences. Plasmid pTM1(Δ*Nco*I)-GP, mutated versions of this clone, and the pTM1(Δ*Nco*I) vector (negative control) were introduced into 293 cells infected with a recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase (5). Cells were cultured in 12-well panels to 80% confluence and then infected with vTF7-3 for 1.5 h at a multiplicity of infection of ≥10 by using a purified virus preparation diluted in growth medium. Plasmid DNA was then introduced into infected cells by transfection. Transfection was performed by incubating a mixture of 300 μl of DMEM (minus antibiotics or serum), 1.5 μg of plasmid DNA, and 9.0 μl of Transfast (Promega) for 15 min at room temperature and then adding the mixture to naked monolayers of vTF7-3-infected 293 cells that had been gently washed twice with DMEM. Cells were cultured for 1 h, and then 1 ml of growth medium was added. Cells were cultured for an additional 5 h, and then the medium was replaced with 250 μl of Eagle's minimal essential medium minus cysteine (plus antibiotics and 2% dialyzed fetal bovine serum) and containing 150 μCi of [^{35}S]cysteine/ml. After 3 h of culturing, 300 μl of growth medium was added to each well and culturing was continued for 14 h. At that time, supernatant fluids were removed and mixed with 66 μl of 10× TNE buffer (0.1 M Tris-HCl [pH 7.4], 1.5 M NaCl, 0.02 M EDTA) containing 10% Triton X-100 (TX-100) and 10 mM phenylmethylsulfonyl fluoride. Cell monolayers were lysed by adding 1 ml of 1× TNE buffer containing 1% TX-100 and 1 mM phenylmethylsulfonyl fluoride to each well and incubating the mixtures at room temperature for 5 min. After transfer to 1.5-ml Eppendorf tubes, the lysates were subjected to brief centrifugation in a microcentrifuge (9,300 × *g*) to pellet the nuclei. Supernatant fluids were then transferred to new tubes. GP molecules were immunoprecipitated from culture supernatant fluids and cell lysates by the addition of 100 μl of a 10% staphylococcal protein A bacterial absorbent (Boehringer Mannheim) that had been preincubated for 15 min (with constant mixing) with rabbit anti-Ebola virus SGP-GP serum (20); immunoglobulin G from 4.0 μl of serum was bound to each 100-μl volume of bacterial absorbent. Reaction mixtures were incubated at room temperature (with constant mixing) for 1 h, and then bacterial cells were washed by three rounds of centrifugation (9,300 × *g*) and suspension in 1 ml of 1× TNE buffer containing 0.5% sodium deoxycholate and 0.5% Nonidet P-40. Pelleted cells were suspended in 50 μl of 0.125 M Tris-HCl (pH 6.8) containing 2.5% sodium dodecyl sulfate (SDS), 12.5% sucrose, and 0.01% bromophenol blue. Cell suspensions were boiled for 2 min and pelleted for 1 min at 16,000 × *g* in a microcentrifuge, and equal volumes of supernatant fluids were transferred to duplicate sets of 1.5-ml Eppendorf tubes. One set of fluids was reduced by adding 2-mercaptoethanol to a concentration of 1% (vol/vol), whereas the other was left untreated (nonreduced). Equal amounts of proteins radioimmunoprecipitated from the medium and from the cell monolayers were separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 10% gels and visualized by autoradiography.

Retrovirus pseudotyping and viral transduction assays. Pseudotyped retrovirus particles consisting of MuLV cores and the Ebola GP in their envelopes were produced by transfecting wild-type or mutated plasmid DNA into gpnlslacZ cells as previously described (25). Viral transduction of β-galactosidase activity into NIH 3T3 cells was determined as previously described (25). All data presented are the average of the results of at least three experiments.

Immunoblot analysis of Ebola virus GP expression, processing, and incorporation into pseudotyped retroviruses. Medium from transfected ϕNX cells (11, 18, 27) containing recombinant retroviruses was passed through a 0.45-μm-pore-size filter and centrifuged through a 30% sucrose cushion at 25,000 rpm with a Beckman 50.2-Ti rotor in a Beckman SS-71 centrifuge. The fluid was aspirated from centrifuge tubes and discarded, and the virus pellet was suspended in 100 μl of radioimmunoprecipitation assay (RIPA) buffer (140 mM NaCl, 10 mM Tris HCl [pH 8.0], 5 mM EDTA, 1% sodium deoxycholate, 1% TX-100, 0.1% SDS). Cells were treated with lysis buffer (50 mM Tris HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 1% TX-100), and cell lysates were centrifuged in a microcentrifuge at 16,100 × *g* for 10 min. The proteins in the cell lysates and the suspended virus pellet were each precipitated with a final concentration of 4% trichloroacetic acid for 2 min. The precipitated proteins were centrifuged in a microcentrifuge at 16,100 × *g* for 10 min. The supernatant fluid was aspirated and discarded. The pellet was suspended in an equal volume of 1 M Tris and vortexed vigorously. Proteins whose glycosylation was analyzed were treated with peptide *N*-glycosidase F from *Chryseobacterium meningosepticum* (PNGase F), which removes N-linked glycosylation, and additionally with sialidase A and endo-*O*-glycosidase, β(1-4)-galactosidase, and glucosaminidase (ProZyme, Inc.), which together remove O-linked glycosylation, by following protocols provided by the supplier. The suspended pellet was mixed with a 1/6 volume of 300 mM Tris (pH 6.8)–60% (wt/vol) glycerol–4% (wt/vol) SDS–0.0012% (wt/vol) bromophenol blue–6% (vol/vol) 2-mercaptoethanol, and the mixture was boiled for 5 min.

Equal amounts of proteins, as determined by the Bradford assay, were separated by SDS-PAGE (8.5% acrylamide) and electrophoretically blotted onto nitrocellulose membranes. Membranes were immersed in reaction buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20 [pH 7.6]) containing 1% bovine serum albumin and incubated overnight at 4°C. Blots were incubated in reaction buffer containing rabbit anti-Ebola virus SGP-GP serum (diluted 1:1,000) for 1 h at room temperature, washed three times in reaction buffer, and reacted with a goat anti-rabbit serum– horseradish peroxidase conjugate (diluted 1:20,000 in reaction buffer) for 30 min at room temperature. Membranes were washed as described above and then treated with a commercial chemiluminescent substrate solution (Amersham Pharmacia Biotech), according to protocols provided by the manufacturer. Specific reactivity to GP was visualized by exposing treated blots to X-ray film.

RESULTS

Biochemical studies of mutant Ebola virus GPs. The role of covalent modifications of the Ebola virus GP was investigated through examination of the effects of mutations that would eliminate the modifications (Fig. 1). The rationale for the experiments involving substitutions for the cysteine residues in the extracellular domains of the GP is based on the fact that cysteines in extracellular domains are conserved exclusively for thiol-disulfide chemistry (24). Pairs of conserved cysteines form disulfide bonds. Elimination of one of a pair of half-cystines should have the same structural effects as elimination of the other. It follows that substitution of one should produce effects on GP processing similar to those produced by substitution of the other. Finally, elimination of both cysteines of a disulfide-bonded pair should have no additional effects on GP processing or function relative to elimination of either one alone. Indeed, the absence of a free cysteine in GPs with double substitutions may actually improve processing or function over that observed with GPs that have been mutated so that they possess an unpaired cysteine and are therefore likely to be recognized and retained intracellularly by the secretory pathway quality control system (24).

The processing of mutant Ebola virus GPs was examined through RIPAs performed with GPs whose expression was induced by the production of T7 RNA polymerase by a recombinant vaccinia virus (Fig. 2). In cell lysates, the wild-type protein is predominantly found in the form in which it is processed to GP_1 and GP_2, although some precursor protein that is not proteolytically processed (GP_{pre}) is detected. GP_1 molecules are shed into the medium at a level equal to that of cell-associated GP_1. Mutation of the first cysteine in GP_1 (Cys-53) resulted in the secretion of most of GP_1 into the medium and a higher electrophoretic mobility for GP_2 (Fig. 2, first autoradiograph). Mutation of each of the remaining cysteines in GP_1 reduced the levels of expression of GP_1 and GP_2, and the predominant form was the GP_{pre} molecule. Mutation of the second or fourth cysteine (Cys-108 and Cys-135) resulted in little or no GP_1 or GP_2 production, whereas plasmids with changes in the third or fifth cysteine (Cys-121 and Cys-147) generated small amounts of mature GP_1 and GP_2. Mutations in most of the conserved N-linked sites in GP_1 produced few changes in expression levels and patterns (Fig. 2, second autoradiograph). Elimination of the most amino-terminal site through substitution of an aspartate residue for Asn-40 caused more GP_1 to be secreted into the medium than was associated with cells. These changes to the N-linked sites in GP_1 caused no apparent changes in the migration of GP_1 or GP_2.

Mutation of any of the first through the fifth cysteines of GP_2 led to markedly increased levels of GP_1 in the medium com-

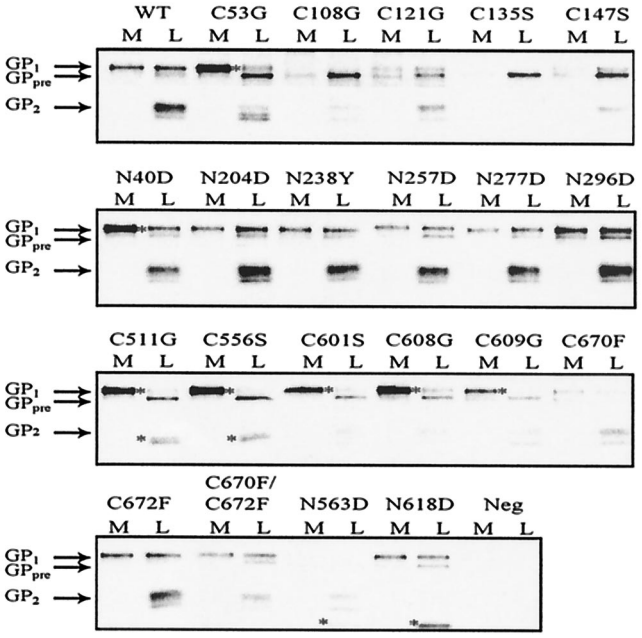

FIG. 2. RIPA of GP in 293 cells. The GPs listed in Table 1 were expressed in 293 cells by using a VV-T7 system and were radiolabeled with [^{35}S]cysteine. They were then immunoprecipitated with a rabbit anti-Ebola virus SGP-GP serum, the reduced proteins were analyzed by SDS–10% PAGE under reducing conditions, and autoradiography was performed. Immunoprecipitated GPs secreted or released into the medium (M) or associated with the cell monolayer (L) were run side by side; only the relevant portions of the gels are shown. Detection of GP_2 is shown only in monolayer lanes. The migration positions of GP_1, GP_2, and GP_{pre} are indicated on the left; GP_{pre} is an uncleaved immature or precursor form of GP that is primarily associated with the ER (27). Asterisks in the GP_1 region identify increased levels of this GP in the medium relative to cell-associated GP_1, compared to the levels in the wild type (WT). Asterisks in the GP_2 region identify faster-migrating forms of GP_2. There are cross-reactive species migrating just slower and somewhat faster than wild-type GP_2. Neg, transfection of pTM1(ΔNcoI) vector into 293 cells infected with a recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase.

pared to those in cell lysates. GP_{pre} predominated in cell lysates, and little or no normally processed GP_2 was produced (Fig. 2, third autoradiograph). Only the C511G and C556S GP_2 molecules were easily detected, and they displayed a higher electrophoretic mobility that was similar to that for GP_2 resulting from the GP_1 C53G substitution. Substitution of the Cys-672 residue in the GP_2 membrane-spanning region with phenylalanine resulted in only a slight diminution in the expression of GP_1 and GP_2, whereas substitution of the nearby Cys-670 residue or both Cys-670 and Cys-672 with phenylalanine produced more marked reductions in expression (Fig. 2, fourth autoradiograph).

The effect of mutation of each of the two conserved N-linked glycan sites of GP_2 depended on the site that was eliminated. When the first site was changed (Asn-563), little GP_1, GP_2, or GP_{pre} was detected in the medium or cell lysates (Fig. 2, fourth autoradiograph). Mutation of the second site (Asn-618) appeared to cause only a small reduction in expression, but the migration of GP_2 appeared significantly faster, presumably due to the loss in mass normally contributed by glycosylation at the site.

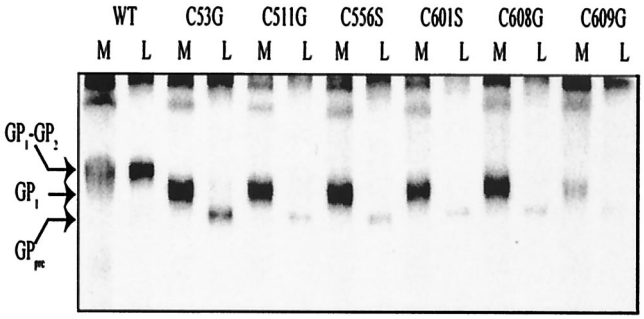

FIG. 3. Migration of GP under nonreducing conditions. Shown is an autoradiogram of SDS-PAGE analysis (under nonreducing conditions) of wild-type (WT) GP and of the proteins analyzed in Fig. 2 that showed increased release of GP_1 into the medium. Immunoprecipitated GPs secreted or released into the medium (M) or associated with the cell monolayer (L) were run side by side. The migration positions of GP_1, GP_2, and GP_{pre} are indicated on the left.

To determine whether there was any disulfide bonding between GP_1 and GP_2 for GPs in which mutated cysteines led to increased GP_1 release into the culture medium, SDS-PAGE analysis of nonreduced GP preparations was performed (Fig. 3). Whereas the mobilities of wild-type GP_1 and mutant GP_1 were identical under reducing conditions (Fig. 2), under nonreducing conditions, only wild-type GP_1 had a lower mobility that was consistent with the formation of a GP_1-GP_2 covalent heterodimer. These data indicate that the mutations affect disulfide bonding between GP_1 and GP_2 and that the most N-terminal cysteine residue of GP_1 forms the cystine bridge with GP_2.

Pseudotyped retroviruses bearing GPs containing substitutions for conserved cysteines. In order to assay the functional consequences of the GP mutations, we examined the association of mutant GPs with recombinant retroviruses that were concentrated by ultracentrifugation and with gene transduction by the pseudotyped viruses (Fig. 4 and Table 2). Mutation of Cys-53, which we identified as being involved in GP_1-GP_2 cystine bridge formation, completely abolished the association of GP_1 with retrovirus particles as well as transduction. Viruses bearing the C108G, C121G, C135S, and C147S GPs all conveyed lower levels of transduction than did the virus bearing the wild-type GP. Each of these four mutant GPs exhibited decreased processing and association with virus particles. We also examined the result of substituting two GP_1 cysteines simultaneously. Remarkably, the virus bearing the C121G/C147S GP had only a moderately reduced capacity to transduce cells compared to the virus bearing the wild-type GP (Table 2), despite the fact that the C147S GP conferred a very low transduction capacity on pseudotyped viruses. The C108G/C135S GP possessed very modest function.

Mutation of each of the ectodomain cysteines in GP_2 (Cys-511, Cys-556, Cys-601, Cys-608, and Cys-609) resulted in a reduction in the ratio of cell-associated mature GP_1 to GP_{pre}, a minimal association of GP_1 with retrovirus particles (Fig. 4), and the complete abolition of transduction (Table 2). It is worth noting that similar levels of the processed forms of the C601S and C608G GPs were detected and that these levels were higher than those of the processed forms of the C511G, C556S, and C609G GPs.

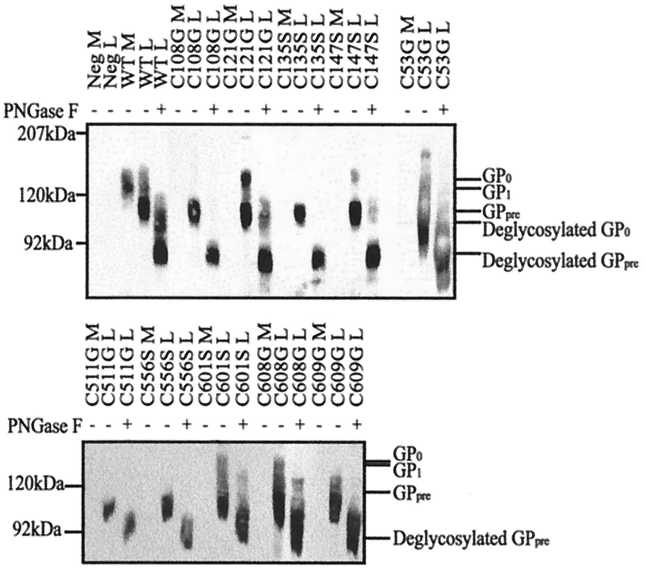

FIG. 4. Analysis of the expression and incorporation into pseudotyped retroviruses of Ebola virus GPs with substitutions of ectodomain cysteine residues. ϕNX cells were transfected with plasmids encoding Ebola virus GPs. The cell lysates (L) and viral particles collected from the culture medium (M) were analyzed by SDS-PAGE (8.5% acrylamide) and immunoblotting with anti-Ebola virus SGP-GP antibody. Analysis of a cell lysate aliquot that was treated with PNGase F (+), which removes N-linked glycosylation, is also shown. The migration positions of mature GP_1, GP_0 (the glycosylated but uncleaved form), GP_{pre} (the N-glycosylated but not O-glycosylated uncleaved form), and deglycosylated GP_0 and GP_{pre} are indicated. WT, wild type; Neg, ΦNX cells transfected with pcDNA3 vector.

The two cysteines within the membrane-spanning sequence of GP_2 (Cys-670 and Cys-672) are palmitoylated (13). Substitution of either Cys-670 or Cys-672 or both with alanine residues did not have major effects on GP processing and association with retrovirus particles (Fig. 5), nor did it affect function in the transduction assay (Table 2). Substitution of Cys-670 with phenylalanine decreased transduction by 43% and greatly reduced but did not eliminate GP processing and association with virus particles. The C672F mutation led to a 24% decrease in transduction, and the GP was processed and incorporated into virus particles at nearly wild-type levels. The double mutant C670F/C672F was expressed and associated with virus particles at greatly diminished levels and showed a complete loss of transduction capacity.

TABLE 2. Transduction of NIH 3T3 cells by virus pseudotyped with mutant Ebola virus GPs with substitutions for cysteine residues

Mutant GP	% Transduction[a]
C53G	<0.1
C108G	8.2 ± 4.3
C121G	59 ± 8
C135S	<0.1
C147S	2.3 ± 2.0
C108G/C135S	1.1 ± 1.0
C108G/C147S	<0.1
C121G/C135S	0.5 ± 0.5
C121G/C147S	72 ± 30
C511G	<0.1
C556S	<0.1
C601S	<0.1
C608G	<0.1
C609G	<0.1
C670F	57 ± 7
C672F	76 ± 4.0
C670F/C672F	<0.1
C670A	113 ± 21
C672A	98 ± 28
C670A/C672A	82 ± 15

[a] Relative to that for the wild type. The average transduction by virus bearing wild-type GP was 1.5×10^4 transducing units/ml.

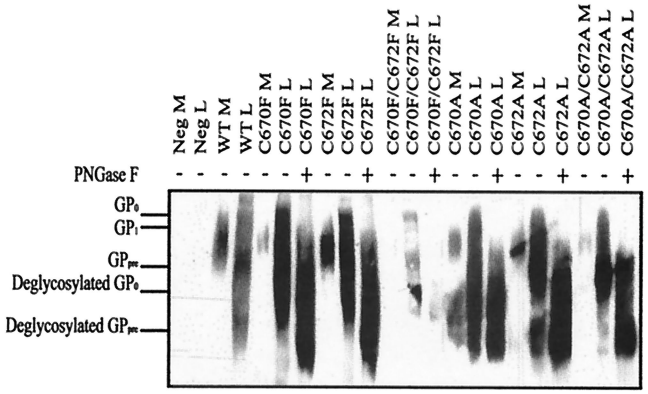

FIG. 5. Analysis of the expression and incorporation into pseudotyped retroviruses of Ebola virus GPs with substitutions of membrane-spanning domain cysteine residues. Analysis was conducted as described in the legend to Fig. 4. The migration positions of mature GP_1, GP_0 (the glycosylated but uncleaved form), GP_{pre} (the N-glycosylated but not O-glycosylated uncleaved form), and deglycosylated GP_0 and GP_{pre} are indicated. WT, wild type; Neg, ΦNX cells transfected with pcDNA3 vector.

Pseudotyped retroviruses bearing GPs with altered glycosylation. The effects of mutating conserved N-linked glycosylation sites (N-X-T/S) on pseudotyping and transduction were measured (Fig. 6 and Table 3). It was found that processing and incorporation of the mutant GPs were similar to those of

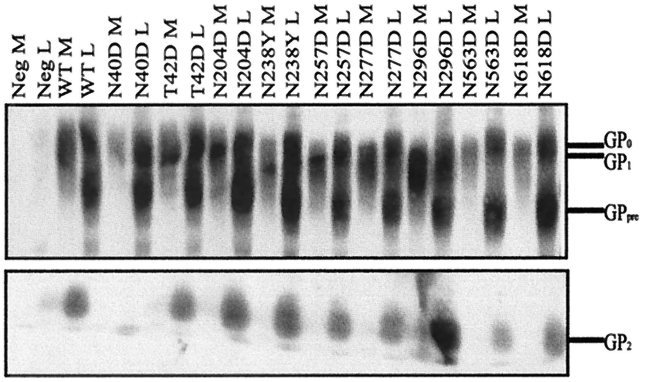

FIG. 6. Analysis of the expression and incorporation into pseudotyped retroviruses of Ebola virus GPs with substitutions eliminating sites of N-linked glycosylation. Analysis was conducted as described in the legend to Fig. 4, except that no PNGase F was used. The migration positions of mature GP_1, GP_0 (the glycosylated but uncleaved form), GP_{pre} (the N-glycosylated but not O-glycosylated uncleaved form), and GP_2 are indicated. WT, wild type; Neg, ΦNX cells transfected with pcDNA3 vector.

TABLE 3. Transduction of NIH 3T3 cells by virus pseudotyped with mutant Ebola virus GPs with altered glycosylation

Mutant GP	% Transduction[a]
N40D	<0.1
T42D	113 ± 17
N204D	102 ± 14
N238Y	88 ± 4
N257D	88 ± 9
N277D	84 ± 10
N296D	62 ± 10
N563D	80 ± 4
N618D	102 ± 3
Δ309–489	696 ± 142

[a] Relative to that for the wild type. The average transduction by virus bearing wild-type GP was 1.4×10^4 transducing units/ml.

the wild-type GP at six of the eight sites mutated. The mutant GP_1 molecules that had substitutions at Asn-238, Asn-257, Asn-277, and Asn-296 and that were incorporated into viruses all had increased mobilities, indicating that they had lower levels of glycoslation and therefore that these modification sites are utilized in the cell (Fig. 6). Mutation of Asn-40 (first N-linked glycan), which is near the cysteine that forms the GP_1-GP_2 cystine bridge, and mutation of Asn-296, which is on the cusp of the variable, mucin-like region, each resulted in a reduction in viral transduction. The N40D mutation completely abolished transduction (Table 3) and greatly reduced GP processing and association with virus particles. In order to investigate further the role of glycan attachment at this site, a T42D mutation was engineered into the wild-type sequence to prevent a glycan addition at Asn-40. This mutation had no effect on the transduction capacity of pseudotyped particles or on the level of expression and electrophoretic mobility of the proteins. These results suggest that the negative effects of the N40D mutation resulted not from the loss of glycosylation but rather from conformational disruption produced by the substituted residue.

The role of O-linked glycosylation of the Ebola virus GP was examined through analysis of the effect of deletion of the region of the protein that is O glycosylated. Remarkably, the processing and viral incorporation of the Δ309–489 GP were greatly enhanced (Fig. 7), and there was a corresponding sevenfold increase in transduction by the Δ309–489 GP-pseudotyped viruses (Table 3). The absence of an increase in the mobility of the Δ309–489 GP with sialidase A and endo-*O*-glycosidase treatment provides confirmation that the region of GP that is O glycosylated was removed (Fig. 8).

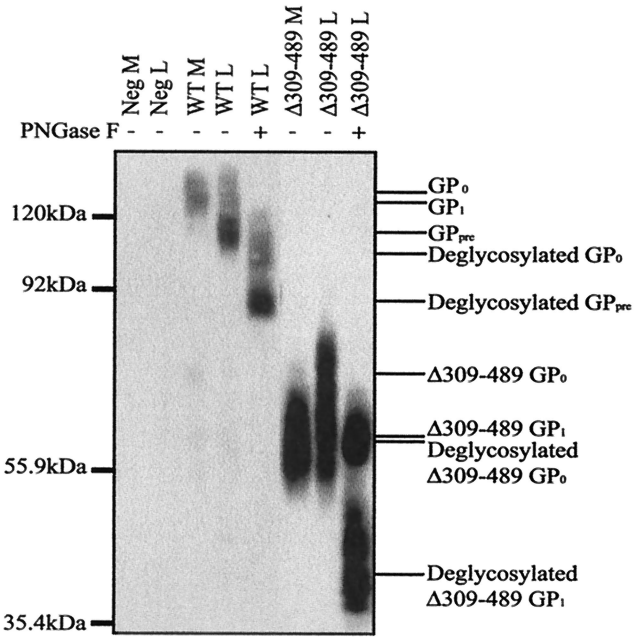

FIG. 7. Analysis of the expression and incorporation into pseudotyped retroviruses of the Δ309–489 Ebola virus GP. Analysis was conducted as described in the legend to Fig. 4. The migration positions of the mature GP_1, GP_0 (the glycosylated but uncleaved form), GP_{pre} (the N-glycosylated but not O-glycosylated uncleaved form), and deglycosylated GP_0 and GP_{pre} forms of wild-type (WT) GP and of the GP_1, GP_0, and deglycosylated GP_1 and GP_0 forms of Δ309–489 GP are indicated. Neg, ΦNX cells transfected with pcDNA3 vector.

DISCUSSION

Our protein expression and recombinant virus studies have enabled us to study the role of covalent modifications of the Ebola virus GP complex. The primary and quaternary structures of the Ebola virus GP have remarkable similarities to those of retrovirus GPs (6, 10, 16, 36), and the results presented here support the hypothesis that additional remarkable common properties are shared.

The combination of sequence analysis, structure determination, and the results presented in this article lead us to propose the model of a cystine bridge map for the Ebola virus GP (Fig. 9A). The extracellular domains of the Ebola virus GP contain 10 conserved cysteine residues, 5 in GP_1 and 5 in GP_2. The five cysteines in Ebola virus GP_2 are conserved not only in Marburg virus GP_2 but also in ASLV TM, which are known to be linked by a stable disulfide bond to their envelope protein surface (SU) components (Fig. 9B). On the basis of sequence analysis and X-ray diffraction studies of Ebola virus GP_2, a putative structure for the linkage of Ebola virus GP_1 and GP_2 has taken shape. As we noted earlier, the first cysteine in GP_1 (Cys-53) had been predicted to be linked to the last cysteine in the extracellular domain of GP_2 (Cys-609) (10, 23). We have confirmed the involvement of the first cysteine in GP_1 in the disulfide linkage to GP_2. A substitution of a glycine for Cys-53 led to the release of most of GP_1 into the medium in the VV-T7 expression system (Fig. 2), with no evidence of C53G GP_1 being disulfide linked to GP_2 in either the medium or the cell (Fig. 3). This amino-terminal cysteine is conserved in the GPs of filoviruses, and it is anticipated that it also links Marburg virus GP_1 to GP_2. It has been proposed, through analogy to the thiol-disulfide exchange reactions that take place in MuLV GPs (19, 24), that the disulfide bond between GP_1 and GP_2 is reduced by a cellular enzyme on entry of the Ebola virus (24).

Conformational changes resulting from the elimination of the GP_1-GP_2 cystine bridge probably produce the changes in processing that alter the mobility of GP_2 in polyacrylamide gels (Fig. 2). Substitutions for any of the GP_2 cysteines (Cys-511, Cys-556, Cys-601, Cys-608, and Cys-609) all led to higher levels of secretion of GP_1 in the VV-T7 expression experiments (Fig. 2) and to inefficient processing and incorporation into recom-

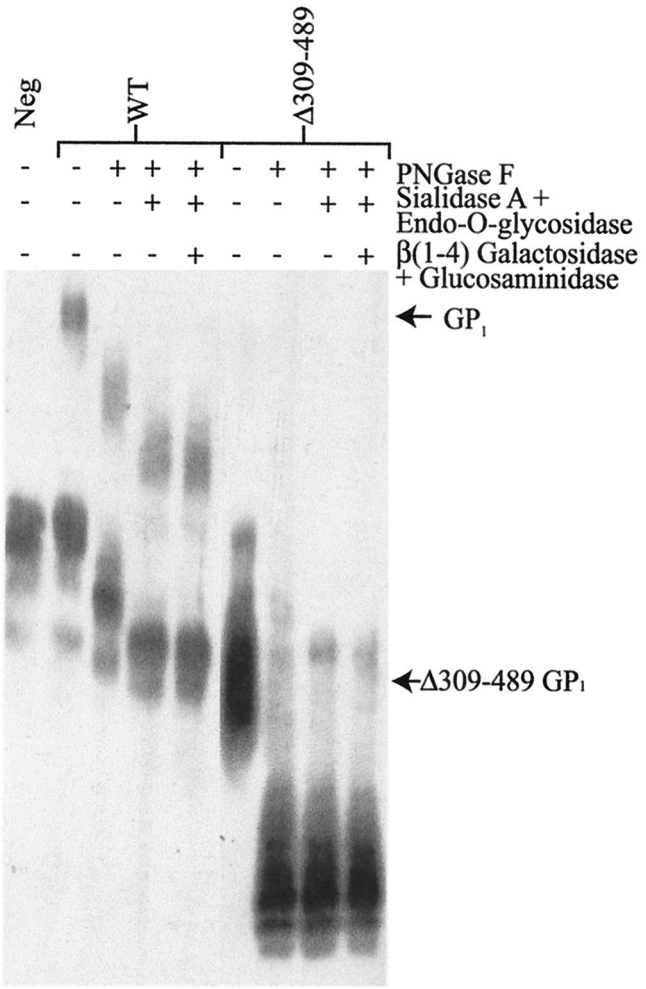

FIG. 8. Analysis of the glycosylation of the Δ309–489 Ebola virus GP incorporated into pseudotyped retroviruses. Analysis was conducted as described in the legend to Fig. 4, except that aliquots of the samples were treated with PNGase F, with a combination of PNGase F, sialidase A, and endo-*O*-glycosidase, or with a combination of the previous three enzymes and β(1-4)-galactosidase and glucosaminidase. The migration positions of the mature GP_1 forms of the wild-type (WT) and Δ309–489 GPs are indicated. In this experiment, a glycosylated serum protein showing a mobility intermediate between those of wild-type GP_1 and Δ309–489 GP_1 was detected. The heterogeneous mobility of the PNGase F-treated proteins was indicative of the incomplete removal of N glycosylation. Neg, ΦNX cells transfected with pcDNA3 vector.

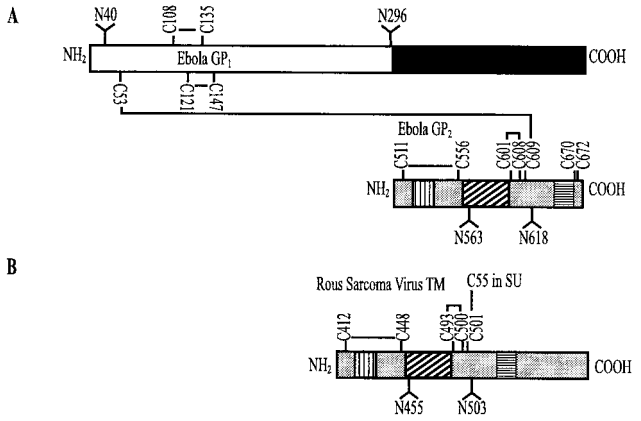

FIG. 9. Cystine bridge model for Ebola virus GP and comparison of GP_2 to the Rous sarcoma virus GP TM subunit. Representational elements for the mucin-like region, the fusion peptides, the coiled-coil domains, and the membrane-spanning domains are identical to those used in Fig. 1. (A) The cystine bridge arrangement in the Ebola virus GP deduced from the results presented here and elsewhere (10, 16, 36) and the critical N-glycosylation sites (Y) discussed in the text are depicted. (B) A proposed cystine bridge model for the TM protein of Rous sarcoma virus (an ASLV) (3, 10) is presented for comparison with that for Ebola virus GP_2.

binant Moloney MuLV (Fig. 4) particles and consequent abolition of transduction capacity. We conclude from these data that when any of the GP_2 cysteines are replaced with another residue, a disruption in the structure of GP_2 occurs that prevents the GP_1-GP_2 linkage from forming. These changes in GP_2 did not appear to affect the migration or level of production of GP_1 in the VV-T7 expression system (Fig. 2), suggesting that disulfide bonding between Cys-53 and Cys-609 is not absolutely required for the transport and processing of highly expressed GP_1, but increased GP_{pre} production in the cell may indicate some reduction in trafficking of GP to the Golgi apparatus. In lysates of the pseudotyped retrovirus-producing cells, on the other hand, the processing of the five mutants was greatly reduced. The pronounced effects of the GP_2 C601S, C608G, and C609G substitutions on processing were similar to those obtained when the equivalent residues of the MuLV TM protein were altered (29).

It is noteworthy that in the pseudotyped virus-producing cells, similar levels of the processed forms of the C601S and C608G GPs were detected and that these levels were higher than those of the processed forms of the C511G, C556S, and C609G GPs (Fig. 4). The total level of C511G and C556S GP expression was lower than those of the other GPs. Interestingly, in the VV-T7 expression experiments, only the C511G and C556S GP_2 molecules were easily detected, and they displayed similar altered electrophoretic mobilities (Fig. 2). X-ray diffraction studies of the structure of a fragment of Ebola virus GP_2 had suggested that Cys-601 and Cys-608 form a cystine bridge (16, 36) and, given the premises of our experiments, our findings support this suggestion and the hypothesis that Cys-511 and Cys-556 also form a disulfide-bonded pair (Fig. 9A). The filovirus GP_2 subunits and ASLV TM GPs contain an internal fusion peptide (residues 524 to 539 in Ebola virus GP_2) that is flanked by cysteine residues that correspond to this latter pair (Cys-511 and Cys-556). These cysteines are absent from the TM GPs of other retroviruses, in which the fusion peptide is positioned very close to the furin cleavage site. The filovirus GP_2 and ASLV TM GP cystine bridge could help stabilize the stalk structure and fix the fusion peptide into a conformation that is favorable for membrane insertion. It should be noted that our results indicating processing defects for the C511G and C556S GPs in the pseudotyped virus-producing cells contrast somewhat with recently published data concerning ASLV TM GPs. It was demonstrated that substitutions for residues in ASLV TM GPs that are equivalent to Cys-511 and Cys-556 do not affect ASLV GP processing (3). Nevertheless, the entry of viruses bearing the altered GPs was dramatically reduced. It appears likely that, although there are

similar structural consequences of elimination of the cystine bridge in ASLV TM and Ebola virus GPs, they manifest themselves earlier in Ebola virus GP.

Four cysteine residues in the Ebola virus GP_1 molecule, Cys-108, Cys-121, Cys-135, and Cys-147, are likely to be linked in two intramolecular cystine bridges (Fig. 9A). The GP containing the C121G mutation showed the least impairment of function (Table 2), suggesting that the disulfide bonding partner of Cys-121 may not be exposed on the surface (4, 24). It is possible that the failure to detect C121G GP_1 associated with virus particles was due to instability of the protein during the process of concentration (Fig. 4). It is noteworthy that the processing of the C121G GP and the processing of the C147S GP were similar in each of the two expression systems (Fig. 2 and 4); given the premises of our experiments, this result would indicate that Cys-121 and Cys-147 form a disulfide bond in the wild-type protein. Further support for this prediction is provided by the finding that the C121G/C147S GP can convey transduction capacity on virus nearly equivalent to that conveyed by the wild-type GP despite the low level of function of the C147S GP (Table 2). Consistent with this proposal is the probability of a cystine bridge between Cys-108 and Cys-135 of Ebola virus GP_1. These residues are also conserved in the GP_1 molecules of all Marburg virus isolates. Together, these data provide strong support for our proposed cystine bridge map (Fig. 9A).

Virtually all of the N-linked glycosylation sites in the Ebola virus GP that we eliminated were individually dispensable (Fig. 6 and Table 3). These data are very similar to those obtained when the N-linked glycosylation sites of the MuLV Env proteins were mutated (9, 14, 15). Our results bode well for structural studies in which the reduction of protein glycosylation (and consequent heterogeneity) is advantageous to protein crystallization. In this context, it is worth noting the effects of eliminating the first site of GP_1 N-linked glycosylation, which precedes Cys-53 by 11 residues. The mutant GP bearing the N40D substitution is secreted at higher levels in the VV-T7 expression system, is poorly processed and incorporated into Moloney MuLV particles, and has no transduction capacity. However, it is not N-linked glycosylation per se that is required, since a T42D substitution (which would also eliminate the first site of N-linked glycosylation) did not impair transduction.

There are conserved N-linked glycosylation sites in similar relative positions near the intersubunit half-cystines in the SU proteins of MuLV and homologous virus GPs (14, 19), and it has been shown that an aspartate-for-asparagine substitution at this site leads to a complete loss of function (9, 14). Remarkably, substitution of a valine for the threonine residue at this N-linked glycosylation site, which is similar to our GP_1 T42D substitution, does not lead to such severe defects in function (15). It was concluded that although N-linked glycosylation at the conserved site was not required for infectivity, the conformation of this region of the polypeptide was critical for normal envelope protein processing and GP subunit association and that N-linked glycosylation played a role in these functions (15). It is likely that the conformational role of the first N-linked glycosylation site of Ebola virus GP_1 is similar. Although this site is conserved in all Ebola virus GP_1 molecules, an equivalent site in Marburg virus GP_1 molecules is absent.

Significant deleterious effects on GP_1 and GP_2 processing were observed when the N563D substitution mutant was expressed in the VV-T7 expression system. The two GP_2 N-linked glycosylation sites may together play an important role in GP folding. Intra- and intermolecular disulfide bond formation or reduction could be facilitated by the proximity of N-linked glycans to particular cysteine residues. In this context, it is noteworthy that ER-localized thiol-disulfide exchange enzymes that promote disulfide bond formation in secreted proteins appear to be found in a complex with the N-linked glycan-binding ER chaperones calnexin and calreticulin (17). It is interesting that the Rous sarcoma virus TM GP also has N-linked glycosylation sites near the ectodomain cysteine residues (Fig. 9B) and that some of the retrovirus TM GPs that have their fusion peptides positioned at the extreme amino terminus have only one predicted N-linked glycosylation site, which is located near the cysteine involved in forming the SU-TM protein linkage.

The effect of deleting the O-linked glycosylation region of GP_1 (Δ309–489) on expression and transduction was striking. This segment, which is rich in proline, serine, and threonine residues, is the most variable among the Ebola virus GPs. Elimination of this mucin-like domain results in enhanced GP processing and incorporation into retrovirus particles (Fig. 7) and consequently higher levels of transduction by the pseudotyped retroviruses (Table 3). Transduction by Ebola virus Δ309–489 GP-pseudotyped viruses is increased from the relatively mediocre titers achieved with wild-type GP to levels that are comparable to those achieved with standard vesicular stomatitis virus G protein-pseudotyped viruses in our system. It is possible that wild-type GP is retained in the Golgi apparatus until all of the serine and threonine residues in the mucin-like region are modified. Elimination of this segment may permit more rapid transit through the Golgi apparatus and higher levels of processing to GP_1 and GP_2 and of cell surface expression. Increased virus incorporation may also result from a diminution of GP toxicity. It has been reported that deletion of the O-linked glycosylation region reduces the cytopathic effects of Ebola virus GP expression (41) or reduces the loss of adherence of GP-expressing cells (26). It has also been suggested that the expression of high levels of wild-type Ebola virus GP may lead to exhaustion of the cellular glycosylation machinery (33), consistent with our results and our interpretation. In any case, the improved levels of transduction seen with the virus pseudotyped with Δ309–489 GP, combined with its potential safety advantages, should make such a recombinant virus the first choice for gene therapies with Ebola virus GP-pseudotyped retroviruses or lentiviruses.

The conservation of a mucin-like region and its variability between isolates indicate that it is likely to play a critical role in the ecology and pathogenesis of the virus, factors which cannot be assessed in the pseudotype system. The probable surface exposure of the charged sugar moieties in this region may make it a dominant target for the humoral immune system. Indeed, protective monoclonal antibodies recognizing epitopes that lie between sequences that are likely to be modified by O-linked glycosylation have been identified (37). The toleration of the mucin-like domain for variations could allow

the virus to readily escape immune recognition through mutations. Indeed, the protective monoclonal antibodies recognizing the Ebola virus Zaire GP *O*-linked glycosylation region have been shown to be specific for particular isolates (37).

The presence of cysteines in membrane-spanning regions appears to be a conserved feature of many virus GPs, including the TM GPs of retroviruses. An analysis of the effects of substitutions of the conserved cysteine residues of the membrane-spanning sequence of Ebola virus GP_2 indicates that the nature of the substituted residue is critical. Substitutions of alanine residues for either Cys-670 or Cys-672 or both led to no major consequences in our assays. These results are similar to those obtained in a system where vesicular stomatitis virus was pseudotyped with mutant Ebola virus GPs (13). Substitutions of phenylalanine residues for either Cys-670 or Cys-672 had only moderate effects on transduction titers, whereas mutant Ebola virus GPs bearing substitutions in both sites were poorly expressed and incapable of promoting transduction (Table 2).

Our biochemical data confirm the significance of the sequence similarity between filovirus GP_2 and TM GPs of oncogenic retroviruses, in particular, TM GPs of ASLVs (10). These findings support the hypothesis of a common evolutionary origin. The fact that the retrovirus TM GPs that show the greatest similarity to filovirus GP_2 come from birds could be an indication that avian species have or have had some role in the ecology and evolution of filoviruses.

In conclusion, this study has provided the basis for a greater understanding of the structure and function of Ebola virus GP and has enhanced our appreciation of the relationship between filovirus and retrovirus GPs. We have demonstrated the importance of the conserved cysteines of Ebola virus GP for its processing, assembly of the peplomer, and virus entry. Although individual conserved N-linked glycosylation sites were not found to be as important as conserved cysteines, it is expected that collectively they have a strong influence on protein folding and disulfide bond formation. Our finding that the elimination of the O-linked glycosylation region of Ebola virus GP enhances its processing, incorporation into retrovirus particles, and transduction by pseudotyped retroviruses could have major implications for gene therapy applications with these viruses. We anticipate that further analyses of Ebola virus GP will provide additional insights into the mechanisms of virus entry for filoviruses and other pathogenic viruses.

ACKNOWLEDGMENTS

This work was supported through grants given by the Purdue Research Foundation and the Cystic Fibrosis Foundation (ENGELH98S0) to D.A.S.

REFERENCES

1. **Chan, S. Y., R. F. Speck, M. C. Ma, and M. A. Goldsmith.** 2000. Distinct mechanisms of entry by envelope glycoproteins of Marburg and Ebola (Zaire) viruses. J. Virol. **74:**4933–4937.
2. **Chepurnov, A. A., M. N. Tuzova, V. A. Ternovoy, and I. V. Chernukhin.** 1999. Suppressive effect of Ebola virus on T cell proliferation in vitro is provided by a 125-kDa GP viral protein. Immunol. Lett. **68:**257–261.
3. **Delos, S. E., and J. M. White.** 2000. Critical role for the cysteines flanking the internal fusion peptide of avian sarcoma/leukosis virus envelope glycoprotein. J. Virol. **74:**9738–9741.
4. **Ellgaard, L., M. Molinari, and A. Helenius.** 1999. Setting the standards: quality control in the secretory pathway. Science **286:**1882–1888.
5. **Elroy-Stein, O., T. R. Fuerst, and B. Moss.** 1989. Cap-independent translation of mRNA conferred by encephalomyocarditis virus 5′ sequence improves the performance of the vaccinia virus/bacteriophage T7 hybrid expression system. Proc. Natl. Acad. Sci. USA **86:**6126–6130.
6. **Fass, D., S. C. Harrison, and P. S. Kim.** 1996. Retrovirus envelope domain at 1.7 angstrom resolution. Nat. Struct. Biol. **3:**465–469.
7. **Feldmann, H., S. T. Nichol, H. D. Klenk, C. J. Peters, and A. Sanchez.** 1994. Characterization of filoviruses based on differences in structure and antigenicity of the virion glycoprotein. Virology **199:**469–473.
8. **Feldmann, H., V. E. Volchkov, V. A. Volchkova, and H. D. Klenk.** 1999. The glycoproteins of Marburg and Ebola virus and their potential roles in pathogenesis. Arch. Virol. Suppl. **15:**159–169.
9. **Felkner, R. H., and M. J. Roth.** 1992. Mutational analysis of the N-linked glycosylation sites of the SU envelope protein of Moloney murine leukemia virus. J. Virol. **66:**4258–4264.
10. **Gallaher, W. R.** 1996. Similar structural models of the transmembrane proteins of Ebola and avian sarcoma viruses. Cell **85:**477–478.
11. **Grignani, F., T. Kinsella, A. Mencarelli, M. Valtieri, D. Riganelli, F. Grignani, L. Lanfrancone, C. Peschle, G. P. Nolan, and P. G. Pelicci.** 1998. High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res. **58:**14–19.
12. **Ito, H., S. Watanabe, A. Sanchez, M. A. Whitt, and Y. Kawaoka.** 1999. Mutational analysis of the putative fusion domain of Ebola virus glycoprotein. J. Virol. **73:**8907–8912.
13. **Ito, H., S. Watanabe, A. Takada, and Y. Kawaoka.** 2001. Ebola virus glycoprotein: proteolytic processing, acylation, cell tropism, and detection of neutralizing antibodies. J. Virol. **75:**1576–1580.
14. **Kayman, S. C., R. Kopelman, S. Projan, D. M. Kinney, and A. Pinter.** 1991. Mutational analysis of N-linked glycosylation sites of Friend murine leukemia virus envelope protein. J. Virol. **65:**5323–5332.
15. **Li, Z., A. Pinter, and S. C. Kayman.** 1997. The critical N-linked glycan of murine leukemia virus envelope protein promotes both folding of the C-terminal domains of the precursor polyprotein and stability of the postcleavage envelope complex. J. Virol. **71:**7012–7019.
16. **Malashkevich, V. N., B. J. Schneider, M. L. McNally, M. A. Milhollen, J. X. Pang, and P. S. Kim.** 1999. Core structure of the envelope glycoprotein GP2 from Ebola virus at 1.9-A resolution. Proc. Natl. Acad. Sci. USA **96:**2662–2667.
17. **Oliver, J. D., H. L. Roderick, D. H. Llewellyn, and S. High.** 1999. ERp57 functions as a subunit of specific complexes formed with the ER lectins calreticulin and calnexin. Mol. Biol. Cell **10:**2573–2582.
18. **Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore.** 1993. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA **90:**8392–8396.
19. **Pinter, A., R. Kopelman, Z. Li, S. C. Kayman, and D. A. Sanders.** 1997. Localization of the labile disulfide bond between SU and TM of the murine leukemia virus envelope protein complex to a highly conserved CWLC motif in SU that resembles the active-site sequence of thiol-disulfide exchange enzymes. J. Virol. **71:**8073–8077.
20. **Ruiz-Arguello, M. B., F. M. Goni, F. B. Pereira, and J. L. Nieva.** 1998. Phosphatidylinositol-dependent membrane fusion induced by a putative fusogenic sequence of Ebola virus. J. Virol. **72:**1775–1781.
21. **Sanchez, A., M. P. Kiley, B. P. Holloway, and D. D. Auperin.** 1993. Sequence analysis of the Ebola virus genome: organization, genetic elements, and comparison with the genome of Marburg virus. Virus Res. **29:**215–240.
22. **Sanchez, A., S. G. Trappier, B. W. Mahy, C. J. Peters, and S. T. Nichol.** 1996. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. Proc. Natl. Acad. Sci. USA **93:**3602–3607.
23. **Sanchez, A., Z. Y. Yang, L. Xu, G. J. Nabel, T. Crews, and C. J. Peters.** 1998. Biochemical analysis of the secreted and virion glycoproteins of Ebola virus. J. Virol. **72:**6442–6447.
24. **Sanders, D. A.** 2000. Sulfhydryl involvement in fusion mechanisms, p. 483–514. *In* H. Hilderson and S. Fuller (ed.), Fusion of biological membranes and related problems. Kluwer Academic/Plenum Publishers, New York, N.Y.
25. **Sharkey, C. M., C. L. North, R. J. Kuhn, and D. A. Sanders.** 2001. Ross River virus glycoprotein-pseudotyped retroviruses and stable cell lines for their production. J. Virol. **75:**2653–2659.
26. **Simmons, G., R. J. Wool-Lewis, F. Baribaud, R. C. Netter, and P. Bates.** 2002. Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence. J. Virol. **76:**2518–2528.
27. **Swift, S., J. Lorens, P. Achacoso, and G. P. Nolan.** 1999. Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems, p. 10.17.14–10.17.29. *In* R. Coico (ed.), Current protocols in immunology, Suppl. 31. John Wiley & Sons, Inc., New York, N.Y.
28. **Takada, A., C. Robison, H. Goto, A. Sanchez, K. G. Murti, M. A. Whitt, and Y. Kawaoka.** 1997. A system for functional analysis of Ebola virus glycoprotein. Proc. Natl. Acad. Sci. USA **94:**14764–14769.
29. **Thomas, A., and M. J. Roth.** 1995. Analysis of cysteine mutations on the transmembrane protein of Moloney murine leukemia virus. Virology **211:**285–289.
30. **Volchkov, V. E., S. Becker, V. A. Volchkova, V. A. Ternovoj, A. N. Kotov, S. V. Netesov, and H. D. Klenk.** 1995. GP mRNA of Ebola virus is edited by the Ebola virus polymerase and by T7 and vaccinia virus polymerases. Virology **214:**421–430.

31. **Volchkov, V. E., V. M. Blinov, and S. V. Netesov.** 1992. The envelope glycoprotein of Ebola virus contains an immunosuppressive-like domain similar to oncogenic retroviruses. FEBS Lett. **305:**181–184.
32. **Volchkov, V. E., H. Feldmann, V. A. Volchkova, and H. D. Klenk.** 1998. Processing of the Ebola virus glycoprotein by the proprotein convertase furin. Proc. Natl. Acad. Sci. USA **95:**5762–5767.
33. **Volchkov, V. E., V. A. Volchkova, E. Muhlberger, L. V. Kolesnikova, M. Weik, O. Dolnik, and H. D. Klenk.** 2001. Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. Science **291:**1965–1969.
34. **Volchkova, V. A., H. Feldmann, H. D. Klenk, and V. E. Volchkov.** 1998. The nonstructural small glycoprotein sGP of Ebola virus is secreted as an antiparallel-orientated homodimer. Virology **250:**408–414.
35. **Volchkova, V. A., H. D. Klenk, and V. E. Volchkov.** 1999. Delta-peptide is the carboxy-terminal cleavage fragment of the nonstructural small glycoprotein sGP of Ebola virus. Virology **265:**164–171.
36. **Weissenhorn, W., A. Carfi, K. H. Lee, J. J. Skehel, and D. C. Wiley.** 1998. Crystal structure of the Ebola virus membrane fusion subunit, GP2, from the envelope glycoprotein ectodomain. Mol. Cell **2:**605–616.
37. **Wilson, J. A., M. Hevey, R. Bakken, S. Guest, M. Bray, A. L. Schmaljohn, and M. K. Hart.** 2000. Epitopes involved in antibody-mediated protection from Ebola virus. Science **287:**1664–1666.
38. **Wool-Lewis, R. J., and P. Bates.** 1998. Characterization of Ebola virus entry by using pseudotyped viruses: identification of receptor-deficient cell lines. J. Virol. **72:**3155–3160.
39. **Wool-Lewis, R. J., and P. Bates.** 1999. Endoproteolytic processing of the Ebola virus envelope glycoprotein: cleavage is not required for function. J. Virol. **73:**1419–1426.
40. **Yang, Z., R. Delgado, L. Xu, R. F. Todd, E. G. Nabel, A. Sanchez, and G. J. Nabel.** 1998. Distinct cellular interactions of secreted and transmembrane Ebola virus glycoproteins. Science **279:**1034–1037.
41. **Yang, Z. Y., H. J. Duckers, N. J. Sullivan, A. Sanchez, E. G. Nabel, and G. J. Nabel.** 2000. Identification of the Ebola virus glycoprotein as the main viral determinant of vascular cell cytotoxicity and injury. Nat. Med. **6:**886–889.