

Influences of Glycosylation on Antigenicity, Immunogenicity, and Protective Efficacy of Ebola Virus GP DNA Vaccines[∇]

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The Ebola virus (EBOV) envelope glycoprotein (GP) is the primary target of protective immunity. Mature GP consists of two disulfide-linked subunits, GP1 and membrane-bound GP2. GP is highly glycosylated with both N- and O-linked carbohydrates. We measured the influences of GP glycosylation on antigenicity, immunogenicity, and protection by testing DNA vaccines comprised of GP genes with deleted N-linked glycosylation sites or with deletions in the central hypervariable mucin region. We showed that mutation of one of the two N-linked GP2 glycosylation sites was highly detrimental to the antigenicity and immunogenicity of GP. Our data indicate that this is likely due to the inability of GP2 and GP1 to dimerize at the cell surface and suggest that glycosylation at this site is required for achieving the conformational integrity of GP2 and GP1. In contrast, mutation of two N-linked sites on GP1, which flank previously defined protective antibody epitopes on GP, may enhance immunogenicity, possibly by unmasking epitopes. We further showed that although deleting the mucin region apparently had no effect on antigenicity *in vitro*, it negatively impacted the elicitation of protective immunity in mice. In addition, we confirmed the presence of previously identified B-cell and T-cell epitopes in GP but show that when analyzed individually none of them were neither absolutely required nor sufficient for protective immunity to EBOV. Finally, we identified other potential regions of GP that may contain relevant antibody or T-cell epitopes.

Ebola virus (EBOV) belongs to the family *Filoviridae* and causes severe hemorrhagic fevers in humans and nonhuman primates. There are currently four recognized EBOV species: the Zaire (ZEBOV), Sudan, Cote d'Ivoire, and Reston Ebola viruses. The protective immune responses against filovirus infections appear to be complex, and multiple components of the immune system are likely required for a favorable outcome after infection. For example, alpha/beta interferon (IFN- α/β) receptor knockout mice or wild-type mice receiving anti-IFN- α/β polyclonal antiserum fail to survive EBOV challenge, whereas immunocompetent mice do, indicating the importance of innate immune responses to survival (1). In other mouse studies, the passive transfer of EBOV GP-specific monoclonal antibodies or polyclonal antiserum provided significant protection, suggesting that strong humoral immune responses specific for viral glycoproteins may protect against filovirus infection (6, 39). Consistent with this, B-cell-deficient mice vaccinated with

EBOV-like particles were not protected from EBOV challenge, whereas normal mice were (36). Furthermore, the survival of mice that received an adoptive transfer of EBOV-specific CD8⁺ effector T cells and death of vaccinated mice depleted of T cells suggests that a cell-mediated immune response is also crucial in protection (21, 36, 38). Clearly, additional studies are needed to identify both a correlate of protective immunity and the mechanism required for protection against EBOV infection.

EBOV has a nonsegmented, negative-strand RNA genome and encodes seven viral structural proteins with a gene order of nucleoprotein, virion structural protein 35 (VP35), VP40, glycoprotein (GP), VP30, VP24, and the RNA-dependent RNA polymerase. GP is not the primary product of the fourth gene but instead is generated through transcriptional editing, which causes the insertion of an extra adenine residue into a stretch of seven other adenine residues (26, 33). The nonedited gene encodes a secreted protein, sGP, which has the same amino-terminal 295 amino acids as GP but terminates after an additional 69 amino acids. Editing results in the continuation of GP for 381 amino acids beyond the divergence. GP is proteolytically cleaved to a large amino-terminal fragment (GP1) and a smaller carboxy-terminal fragment (GP2) in the trans-Golgi network by a furin-like enzyme (27, 34). GP1 and GP2 reassociate by disulfide bonding, and the mature GP1,2 complex is anchored in the membrane by a transmembrane domain near the carboxy terminus of GP2 (reviewed in reference 4).

EBOV GP is highly glycosylated, with approximately half of

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14. ABSTRACT The Ebola virus (EBOV) envelope glycoprotein (GP) is the primary target of protective immunity. Mature GP consists of two disulfide-linked subunits, GP1 and membrane-bound GP2. GP is highly glycosylated with both N- and O-linked carbohydrates. We measured the influences of GP glycosylation on antigenicity, immunogenicity, and protection by testing DNA vaccines comprised of GP genes with deleted N-linked glycosylation sites or with deletions in the central hypervariable mucin region. We showed that mutation of one of the two N-linked GP2 glycosylation sites was highly detrimental to the antigenicity and immunogenicity of GP. Our data indicate that this is likely due to the inability of GP2 and GP1 to dimerize at the cell surface and suggest that glycosylation at this site is required for achieving the conformational integrity of GP2 and GP1. In contrast, mutation of two N-linked sites on GP1, which flank previously defined protective antibody epitopes on GP, may enhance immunogenicity, possibly by unmasking epitopes. We further showed that although deleting the mucin region apparently had no effect on antigenicity in vitro, it negatively impacted the elicitation of protective immunity in mice. In addition, we confirmed the presence of previously identified B-cell and T-cell epitopes in GP but show that when analyzed individually none of them were neither absolutely required nor sufficient for protective immunity to EBOV. Finally, we identified other potential regions of GP that may contain relevant antibody or T-cell epitopes.		
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its apparent molecular weight attributed to the attached N-linked and O-linked carbohydrates (3). The majority of the N- and O-linked glycans are found in a variable, extremely hydrophilic central region of GP1,2 (reviewed in reference 4). This "mucin domain" is implicated in cell rounding in cultured cells and has been suggested to have a role in filovirus pathogenesis (29, 31, 41).

Several studies have demonstrated that glycosylation can greatly influence the structure, function, antigenicity, and immunogenicity of various viral glycoproteins (5, 7, 18, 19, 35). In particular, numerous studies have shown that the extensive glycosylation found on envelope protein (gp120) of human immunodeficiency virus (HIV) is involved in the conformational integrity, antigenicity, and immunogenicity of the protein. The gp120 carbohydrates are clustered together on an outer exposed domain of the protein and have been suggested to form a "glycan shield" that prevents neutralizing antibodies from binding due to steric hindrance (37). In addition, comparing antibodies raised to glycosylated versus nonglycosylated gp120 or eliminating potential glycosylation sites in gp120 showed that glycosylation might also prevent the induction of antibodies that could block the initial stage of HIV infection involving binding to the primary virus receptor, CD4, or chemokine coreceptor molecules (12, 13). Further, eliminating potential N-linked glycosylation sites from gp120 of HIV or simian immunodeficiency virus (SIV) resulted in increased accessibility to previously hidden neutralizing or T-cell epitopes (2, 11, 17, 23).

Like HIV and SIV, EBOV elicits only low levels of neutralizing antibodies in humans and other animals (reviewed in reference 25). It is possible that glycosylation of EBOV GP might mask otherwise neutralizing or protective epitopes, as previously described for HIV. Alternatively, the removal of glycosylation sites could interfere with the correct folding or processing of EBOV GP1 or GP2. To address these issues and to determine whether specific glycosylation sites positively or negatively influenced EBOV antigenicity and immunogenicity, we generated several mutant GP genes in which one or more potential N-linked glycosylation sites were deleted, as well as mutants missing part or all of the mucin domain. We evaluated the expression products of these genes in cell culture expression studies and in vaccination and challenge studies in mice. In addition, we compared mouse B-cell and T-cell responses to overlapping peptides representing GP.

MATERIALS AND METHODS

ELISA. To prepare antigen for enzyme-linked immunosorbent assay (ELISA), ZEBOV (1976, isolate Mayinga) was purified and inactivated as described previously (8). Half-area, 96-well, high-binding enzyme immunoassay/radioimmunoassay flat-bottom plates were coated with irradiated, sucrose-purified antigen at a predetermined optimal dilution (1:1,000) in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Plates were washed four times with wash buffer (1× PBS plus 0.05% Tween 20). Nonspecific binding was blocked by adding blocking buffer, consisting of wash buffer plus 3% powdered nonfat milk, and 3% goat serum (Sigma, St. Louis, MO). Sera were prepared in duplicate at an initial dilution of 1:100 in blocking buffer and serially diluted in twofold increments. Diluted antibody was dispensed into the appropriate wells, followed by incubation at 37°C for 1 h. Plates were washed four times as described above, and a peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) secondary antibody (KPL, Gaithersburg, MD) was added to each well. After incubation for 1 h at 37°C, the plates were washed as described above, and the detector substrate [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] (ABTS; KPL) was added. The

reactions were stopped after 12 min at room temperature by adding ABTS stop solution (KPL). Values were read at 405 nm. For each serum dilution series performed in duplicate, optical density (OD) values from the negative-control antigen wells were subtracted from the averaged experimental values to give adjusted OD values. Endpoint titers were determined as the highest dilution with an absorbance value greater than the mean absorbance value from negative control plasmid (pWRG 7077)-vaccinated animals plus three standard deviations. Final results were reported as geometric mean titers.

PRNT. Plaque reduction-neutralization tests (PRNT) were performed as described earlier (16, 39). Briefly, twofold dilutions of test sera were incubated with 100 PFU of EBOV for 1 h at 37°C. The virus-antibody mixture was adsorbed on confluent monolayers of Vero E6 cells in six-well plates, incubated for 1 h at 37°C, and then overlaid with 2 ml of agarose containing overlay medium. Cells were stained after 6 to 7 days with neutral red, and plaques were counted the next day. The 50% PRNT (PRNT₅₀) was calculated as the reciprocal of the highest dilution of serum that caused a 50% reduction in the number of plaques compared to the medium-only control.

Peptide ELISA with overlapping peptide pools. An overlapping group of biotinylated peptides covering the entire GP sequence was purchased from Mimotopes (Raleigh, NC). This set consisted of 124 peptides synthesized on a 1 µM scale as 18-mers with an offset of six amino acids between the start of each peptide. Peptides 1 to 111 covered the full-length of both GP1 and GP2 with the last 13 peptides corresponding to sGP. Each lyophilized peptide was solubilized in dimethyl sulfoxide and adjusted to a final concentration of 1 to 3 mg/ml with PBS. Pools of four sequential peptides were prepared and used as the antigens in an ELISA. Briefly, flat-bottom plates were coated with 5 µg of streptavidin (Sigma)/ml diluted in water and allowed to evaporate to dryness by uncovered incubation at 37°C overnight. The following day, plates were washed four times with PBS containing 0.1% (vol/vol) Tween 20 (PBS-T). To block nonspecific absorption, PBS containing both 2% (vol/vol) fetal bovine serum and 3% (vol/vol) goat serum (Sigma) was added to each well, followed by incubation at 37°C for 2 h. The plates were then washed again four times with PBS-T. The peptide pools were diluted just before use to a working strength of 1/1,000 in PBS-fetal bovine serum-goat serum (1 to 3 µg of each peptide/ml). To react the streptavidin-coated, blocked plates with the biotinylated peptides, diluted peptide pools were transferred into the appropriate well position of the plate and placed on a shaker for 1 h at 20°C. After the plates were washed as described above, the diluted serum (1/1,000 dilution) to be tested was added to each of the wells of the plate containing captured peptides. The plates were then placed on a shaker and incubated for 1 h at 20°C. Plates were washed as described above; bound antibody was detected after reaction for 1 h at 20°C with horseradish peroxidase-conjugated secondary antibody, goat anti-mouse IgG, goat anti-guinea pig IgG (KPL), and goat anti-human IgG (Sigma) at 1/2,000. The plate was washed again four times with PBS-T. Horseradish peroxidase was detected by reaction for up to 20 min at 20°C with a freshly prepared enzyme substrate solution. The reaction was stopped by the addition of an ABTS stop solution (KPL). The OD was read by using a microtiter plate reader at 405 nm. Background subtracted values were used for graphing.

Generation of glycosylation mutant plasmid DNA. ZEBOV GP N-linked glycosylation mutants were produced by two methods. The original panel of N-linked mutants (A, B, C, D, AB, CD, and ABCD) was generated by using splicing by overlap extension PCR (9). In the first round of PCR, two products were from 5'- or 3'-end-specific primers in addition to forward or reverse internal primers that were designed to facilitate the replacement of serine (S) or threonine (T) from the glycosylation site sequon (N-X-S/T) with alanine (N-X-A). The second round of PCR included the products from the first round with the end-specific primers to generate the final product. These primers contained the NotI and BglII sites which were used for cloning into the NotI and BglII sites of pWRG7077 (28). In addition, flanking primers were designed with the NotI and BglII restriction sites incorporated into the sequence. Subsequent N-linked mutants (ABD, E, F, G and H₂) were generated by PCR-based mutagenesis by using the QuikChange II kit with the high-fidelity PfuUltra polymerase according to the manufacturer's directions (Stratagene). All mutant EBOV plasmids were sequenced on an ABI 3100 genetic analyzer to confirm that the desired mutations were obtained. In conducting this part of the study, the investigator(s) adhered to the *Guidelines for Research Involving Recombinant DNA Molecules* (2a). Sequences for the primer pairs can be obtained upon request.

Immunofluorescent antibody assays (IFA). COS cells seeded on 18-mm-diameter glass coverslips in 12-well cell culture plates were transfected with 2 µg of either empty vector (pWRG 7077) or vector containing either wild-type ZEBOV GP or N-linked mutant DNA by using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. At 48 h posttransfection, coverslips were washed three times with PBS (pH 7.4)

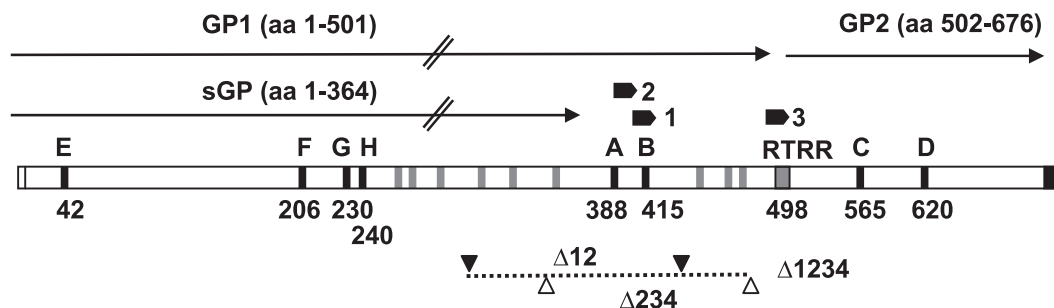


FIG. 1. Schematic of the EBOV GP gene and location of mutations. The positions of the N-linked glycosylation mutations, A to H, are indicated by solid black bars on the GP gene. The position of the amino acid that was changed is indicated under the bar. Other potential N-linked sites that were not mutated are indicated with solid gray bars on the GP gene. The cleavage site of GP1 and GP2 (RTRR) is represented by a gray box, and the transmembrane domain of GP2 is shown as a black box on the GP gene. The coding regions for GP1, sGP, and GP2 are indicated by arrows above the GP gene. The start of the unique regions of GP1 and sGP (amino acid 296) are indicated by double slashes on the arrows. Epitopes recognized by competition group 1, 2, or 3 MAbs (38) are identified as 1, 2, and 3, respectively, above the GP gene. The mucin region deleted in construct Δ1234 is indicated by a dotted line beneath the GP gene, and the start at stop positions of mutant Δ12 is indicated with solid arrowheads above the line, and those of Δ234 are indicated by open arrowheads below the line.

and fixed with either 1:1 methanol-acetone (vol/vol) or PBS containing 2% paraformaldehyde (wt/vol) for 15 min at room temperature. Coverslips were rinsed three times in PBS and blocked for 10 min in PBS containing 5% goat serum (Sigma). Methanol-acetone-fixed coverslips were incubated with a hyper-immune polyclonal guinea pig anti-EBOV serum for 1 h at 37°C. Cells transfected with either N- or O-linked mutant DNA and treated with 2% paraformaldehyde were probed with monoclonal antibody (MAb) 6E3 or MAb 12B5, respectively; these antibodies recognize linear epitopes within GP1 corresponding to amino acids 401 to 417 (6E3) or amino acids 477 to 493 (12B5), as previously described (39). Coverslips were rinsed three times with PBS and incubated for 1 h at 37°C with fluorescein-labeled goat anti-guinea pig IgG (Sigma) or Alexa Fluor 488-goat anti-mouse IgG (Invitrogen). Coverslips were rinsed three times with PBS and once with deionized water and placed on a drop of fluorescence mounting medium containing the counterstain DAPI (4',6'-diamidino-2-phenylindole; Invitrogen) on glass slides. The cells were observed with a Nikon E600 fluorescence microscope.

Preparation of gene gun cartridges, vaccination, and challenge of mice. Cartridges for the gene gun were prepared as described previously (28). Briefly, ~4 µg of plasmid DNA was precipitated onto 0.5 mg of ~2-µm-diameter gold beads. The DNA-coated gold particles were dried on the inside walls of Tefzel tubing and then cut into 0.5-inch sections for the gene gun device. Each cartridge contained ca. 0.25 to 0.5 µg of DNA. Before vaccination, the abdominal fur was removed from mice by shaving with clippers to facilitate the administration of vaccine to two discreet, nonoverlapping sites on the abdominal epidermis. Pathogen-free female BALB/c mice (ca. 6 to 8 weeks old, obtained from the National Cancer Institute, Frederick, MD) were vaccinated by using a hand-held, helium-powered gene gun (Powderject XR-1 delivery device; Powderject, Inc.) at 400 lb/in². Groups of 10 mice per experimental condition were vaccinated three times at 3-week intervals and challenged 21 days after the final vaccination. Anesthetized mice were bled from the retro-orbital sinus at day zero, before each vaccination, and again before viral challenge. One month after the final booster dose, the animals were transferred to a biosafety level -4 facility for EBOV challenge. Mice were injected intraperitoneally with approximately 1,000 PFU of mouse-adapted ZEBOV (1976, isolate Mayinga). The mice were weighed and observed daily for 21 days for signs of illness and mortality. Terminal bleeds were obtained from survivors 3 weeks postchallenge. Research was conducted in compliance with the Animal Welfare Act and used facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care. The investigators adhered to the *Guide for the Care and Use of Laboratory Animals* prepared by the National Research Council (National Institutes of Health Publication no. 86-23, revised 1996).

Immunoprecipitation of EBOV GP. COS cells grown in T-25 cell culture flasks were transfected with 5 µg of plasmid DNA by using Fugene6 (Roche Diagnostics, Basel, Switzerland). After 24 h, the expression products were radiolabeled with Promix (300 µCi of [³⁵S]methionine and 150 µCi of [³⁵S]cysteine; Amersham Pharmacia Biotech, Piscataway, NJ) and immunoprecipitated as described previously (28, 32).

Enzyme-linked immunospot (ELISPOT) assays. Splenocytes harvested and pooled from three BALB/c mice per experimental group were suspended in

RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum. Under aseptic conditions, spleens were minced and sieved through a 70-µm-pore-size nylon cell strainer to remove fibrous tissue. Splenocytes were centrifuged for 10 min at 1,400 rpm to pellet them. Red blood cells were removed with Gey's lysis buffer (4.15 g of NH₄Cl, 0.5 g of KHCO₃, 0.5 ml of 0.5% phenol red, and double-distilled H₂O added to 500 ml). Cells were washed twice in complete medium, counted by using a hemocytometer, and kept on ice until use. Prewet 96-well plates (Millipore Multiscreen_{HTS} polyvinylidene difluoride filter plates) were coated overnight at 4°C with 10 µg of the anti-IFN-γ monoclonal antibody AN18 (Mabtech, Inc., Cincinnati, OH)/ml. The antibody-coated plates were washed five times aseptically with sterile PBS and blocked with complete medium for 30 min at room temperature. Splenocytes were seeded in duplicate at a concentration of 2 × 10⁵ cells/well. Pools of eight sequential peptides (Mimotopes, Raleigh, NC) each were added to the appropriate wells for a final concentration of ~1 µg of each peptide. In separate wells, phorbol myristate acetate and ionomycin (Sigma) were added at a final concentrations of 0.075 and 0.375 µg, respectively, as a mitogen control. Plates were incubated at 37°C in a humidified incubator for 42 to 48 h, washed five times with PBS, and incubated for 2 h with filtered detection antibody R4-6A2 (Mabtech, Inc.) diluted to 1 µg in PBS-0.5% fetal bovine serum. Plates were washed five times with PBS, and streptavidin-bound alkaline phosphatase was added to the wells for 1 h at room temperature. The plates were washed as described above and developed by adding a filtered BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium substrate solution.

Upon visualization of the spots, the reaction was stopped by removing the plate from the holder and rinsing both sides extensively in tap water. Membranes were allowed to dry overnight in the dark and then spots were counted both visually and with a KS ELISPOT Axioskop 2 MAT incident light microscope system (Carl Zeiss, Hallbergmoos, Germany). The results were expressed as the number of spot-forming cells/10⁶ splenocytes.

RESULTS

Rationale for selecting and constructing glycosylation mutant GP genes. EBOV GP has 15 potential N-linked glycosylation sites (N-X-S/T) in GP1 and two sites in GP2 (Fig. 1). We constructed and tested two sets of GP mutants in which individual or combinations of selected glycosylation sites were mutated such that the S or T residues were replaced with A residues (Fig. 1). Each mutant gene was cloned into the expression plasmid, pWRG7077 (28) downstream of a cytomegalovirus promoter. For the first set of mutants, we selected GP1 sites A and B because they flank a previously defined (39) linear, non-neutralizing but protective antibody epitope (Fig. 1, arrow 2). We hypothesized that removing the surrounding

carbohydrate residues might enhance antibody access to this linear epitope.

Sites C and D were selected because they are the only potential N-linked glycosylation sites in GP2 and because they flank the cysteine residue that is thought to be involved in disulfide bridging to GP1 (C609) (10, 27). We theorized that removing one or both of these sites might alter the conformation of GP1 and prevent GP1 and GP2 bridging, resulting either in GP1 secretion from cells or in its accumulation inside cells due to an inability to traffic to the cell surface. Either of these could positively or negatively impact the immunogenicity of GP. In addition, GP2 was shown to contribute to protective immunity to EBOV and MARV challenge (A. Schmaljohn, unpublished information), thus removing these glycosylation sites might enhance immunogenicity by uncovering additional protective epitopes or might reduce immunogenicity by altering the conformation of GP2.

The second set of constructs had mutations in each of the first four of six potential N-linked sites shared by GP1 and sGP (Fig. 1). Sites, F, G, and H were very closely spaced; thus, we theorized that this region was normally shielded from antibodies, so it was possible that removing these sites could uncover additional protective epitopes. Construct E had a mutation in the only potential N-linked site within the first 200 amino acids of GP1/sGP, so its removal results in a long nonglycosylated region of these proteins, which might cause conformational changes. Also, this glycosylation site is near the conserved cysteine residue (C53) that was identified as being involved in the disulfide bridging to GP2 (10); consequently, removing this site might have an impact on the ability of GP1 to associate with GP2.

In addition to the N-linked mutation series, we also generated three expression constructs with in-frame deletions in the mucin domain of GP1 (Fig. 1). The deletion constructs $\Delta 12$, $\Delta 234$, and $\Delta 1234$ were kindly provided by Graham Simmons, University of Pennsylvania, and their generation by overlapping extension PCR was described previously (29). The $\Delta 12$ construct also was missing the N-linked site A, and the $\Delta 234$ and $\Delta 1234$ constructs had deletions of both the A and B N-linked sites (Fig. 1). All three of these constructs were missing the competition group 1 and 2 MAb recognition epitopes. We subcloned each of these genes into the expression plasmid pWRG7077 for additional studies.

In an earlier study, deleting the mucin region of EBOV GP1 improved the expression and processing, as well as the incorporation, of GP1 into pseudotyped retrovirus particles. It was suggested that deleting this region might permit more rapid transit through the Golgi and higher levels of processing to GP1 and GP2 and of cell surface expression (10); thus, we were interested in determining how this might influence immunogenicity.

Expression of the N-linked mutant GP genes. To assay expression, we transfected COS cells with each construct and then radiolabeled the expression products and immunoprecipitated them with antibodies to EBOV. Each of the N-linked mutants generated a GP1 protein similar in size to that of wild-type (wt) GP1 (Fig. 2A and B). Constructs F, G, H, and ABD appeared to express more poorly than the others, although overall ratios of GP1 and GP2 appeared to be similar to those of wt GP (Fig. 2B). Constructs C, D, CD, ABD, and

ABCD all contained deletions of one or both glycosylation sites in GP2. Much less GP2 was precipitated from the C mutant constructs than for the others (Fig. 2A and B) and could only be readily detected by a long exposure of the autoradiograms (results not shown). As expected, constructs with the C and D mutations produced noticeably faster-migrating GP2 compared to wt GP2 (Fig. 2A and B and data not shown).

We further investigated the expression of the constructs with the A, B, C, and D mutations by immunoprecipitation with a panel of previously described MAbs that recognize five different epitopes on GP1. MAbs contained in competition groups 1, 2, and 3 recognized linear epitopes corresponding to amino acids 401 to 417, 389 to 405, and 477 to 493, respectively (Fig. 1) (39). MAbs in competition groups 4 and 5 did not recognize linear epitopes and were not mapped; however, both were found to immunoprecipitate sGP as well as GP1 and thus were surmised to bind within the shared 5'-terminal 295 amino acids of those proteins (39). We found that representative MAbs from each of the five groups were able to immunoprecipitate both GP1 and GP2 from cells transfected with wt GP (Fig. 2D) or with mutant A, B, D, or AB (not shown). However, for cells transfected with constructs containing the C mutation (C, CD, and ABCD), none of the MAbs precipitated GP2 (Fig. 2E and data not shown). In addition, the group 5 MAbs precipitated no detectable GP1 (Fig. 2E and data not shown). These results suggest that the group 5 MAbs recognize a conformational epitope on GP1 and that this epitope is disrupted when the C mutation is introduced. Further, they suggest a role for the N-linked glycosylation of GP2 in maintaining that conformation.

Expression of the O-linked glycosylation mutant GP genes. The O-linked mutants generated GP1 proteins of the expected sizes reflective of the deletion introduced (Fig. 2C). Each construct produced GP2, although less GP2 was precipitated with the polyclonal sera for construct $\Delta 1234$ compared to $\Delta 12$ and $\Delta 234$ (Fig. 2C). MAbs in competition groups 3, 4, and 5 also precipitated both GP1 and GP2 from each construct but, as expected, the MAbs in groups 1 and 2 did not precipitate GP1 or GP2 because both MAb epitopes were deleted in all of these constructs (Fig. 1 and 2F and results not shown). In general, these results suggest that truncating or even removing the mucin region did not greatly influence the antigenicity of GP. This is consistent with earlier findings, which indicated that deleting the mucin region does not negatively impact on viral entry or processing of GP1 (10, 14).

Immunofluorescent antibody staining. To determine whether GP1 and GP2 proteins were able to interact and traffic to the cell surface, we transfected cells with the DNA constructs and either fixed and permeabilized the cells by treatment with acetone and methanol (to examine intracellular expression) or fixed them with 2% paraformaldehyde but did not permeabilize the cells (to examine surface expression). For detecting N-linked mutant expression products, we immunostained the transfected cells with polyclonal EBOV-immune sera or with a GP1-specific MAb, 6E3. The polyclonal sera detected expression products of GP and of each N-linked mutant both within cells (Fig. 3, top panels, and data not shown) and on the cell surface (not shown). The GP1-specific MAb also detected intracellular expression products from each N-linked mutant in the fixed and permeabilized cells. Surface

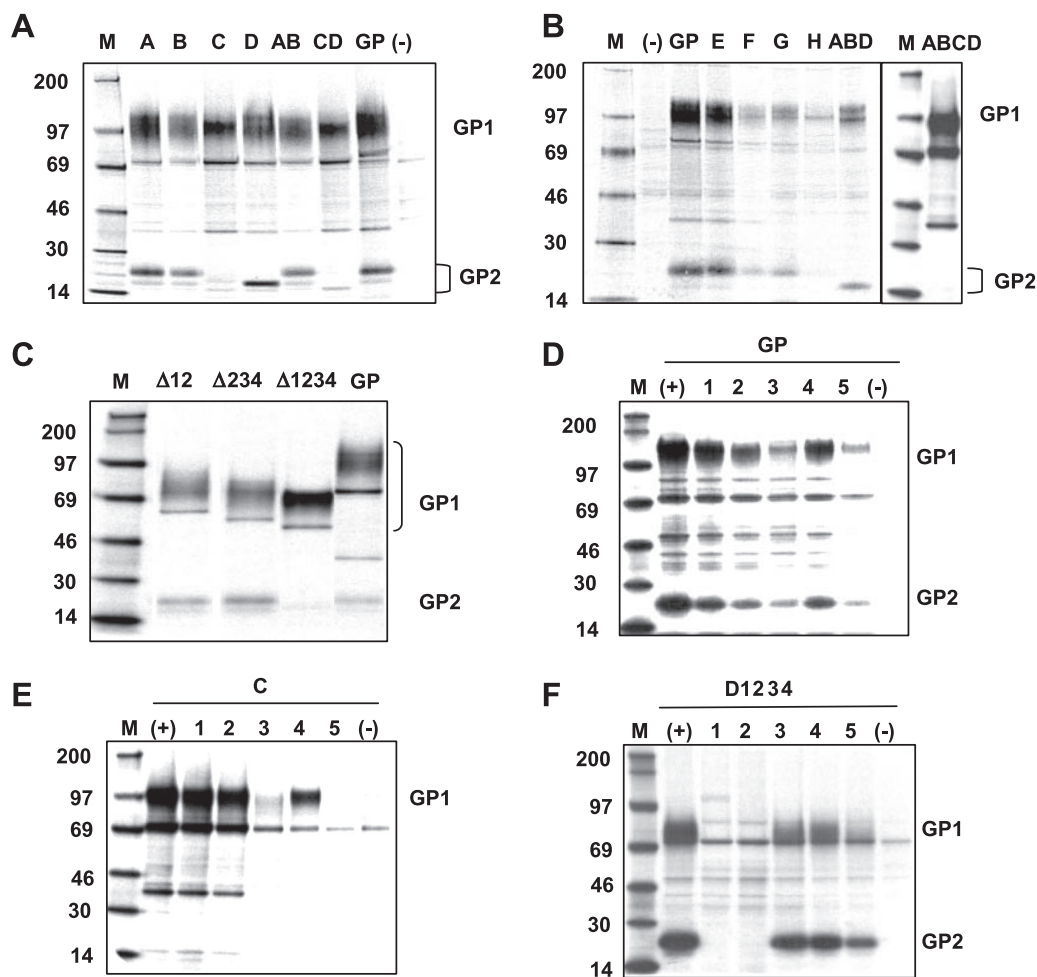


FIG. 2. Expression products of the glycosylation mutant GP genes. Each mutant DNA vaccine construct was transfected into cells, and expression products were radiolabeled and immunoprecipitated using polyclonal guinea pig sera to authentic EBOV (A to C) or GP1-specific MABs from each of five different competition groups (1 to 5 above the autoradiographs) (39). The positions of molecular weight markers (M) are shown on the left, and the positions of GP1 and GP2 are indicated on the right of each panel. (A) Immunoprecipitation of products expressed from N-linked mutants A, B, C, D, AB, CD, or wt GP or a negative control plasmid (-). (B) Immunoprecipitation of products expressed from a negative control plasmid (-), wt GP, or N-linked mutants E, F, G, H, ABD, and ABCD. (C) Expression products of the mucin region deletion constructs compared to those of wt GP. (D) wt GP expression products immunoprecipitated with the MABs or with polyclonal immune sera (+) or nonimmune sera (-). (E) N-linked mutant C expression products immunoprecipitated with the MABs or with polyclonal immune sera (+) or nonimmune sera (-). (F) Mucin deletion mutant $\Delta 1234$ expression products immunoprecipitated with the MABs or with polyclonal immune sera (+) or nonimmune sera (-).

expression was detected with the GP1-specific MAB in cells transfected with all N-linked mutants except those containing the C mutation (Fig. 3 and not shown). Because the 6E3 MAB used for detecting N-linked mutant expression is in competition group 1, and we showed already that this MAB recognized GP1 expressed from the C mutants (Fig. 2), the absence of reactivity with the C-containing mutants in this experiment suggests that GP1 produced by these mutants was not present on the cell surface and that the surface fluorescence observed in cells immunostained with the polyclonal serum was likely due to the recognition of GP2. Further, these data are consistent with the immunoprecipitation results indicating that MABs to GP1 did not precipitate GP2 from constructs with the C mutation [Fig. 2C]. We interpret these findings to reflect poor or no GP1 and GP2 interaction through their normal mechanism of disulfide bonding. Unexpectedly, although im-

muno-precipitation experiments revealed no GP2 production by the ABCD mutant, a small amount of GP1 was detected on the surface of ABCD-infected cells by IFA. The staining was atypical of the fluorescence observed for other cells and was seen in only a few cells (Fig. 3). This result might indicate that the GP1 and GP2 proteins produced by ABCD interacted to some extent and could traffic to the cell surface or, alternatively, GP1 alone was present at the cell membrane.

Similar to the results with the N-linked mutants, the polyclonal serum detected intracellular antigen in methanol-acetone fixed cells that had been transfected with all three of the mucin domain deletion constructs. Each of the three also displayed surface fluorescence when tested with the MAB 12B5 from competition group 3 (Fig. 3 and results not shown). As expected, there was no surface fluorescence when a competition group 1 MAB was used, as this epitope was deleted from

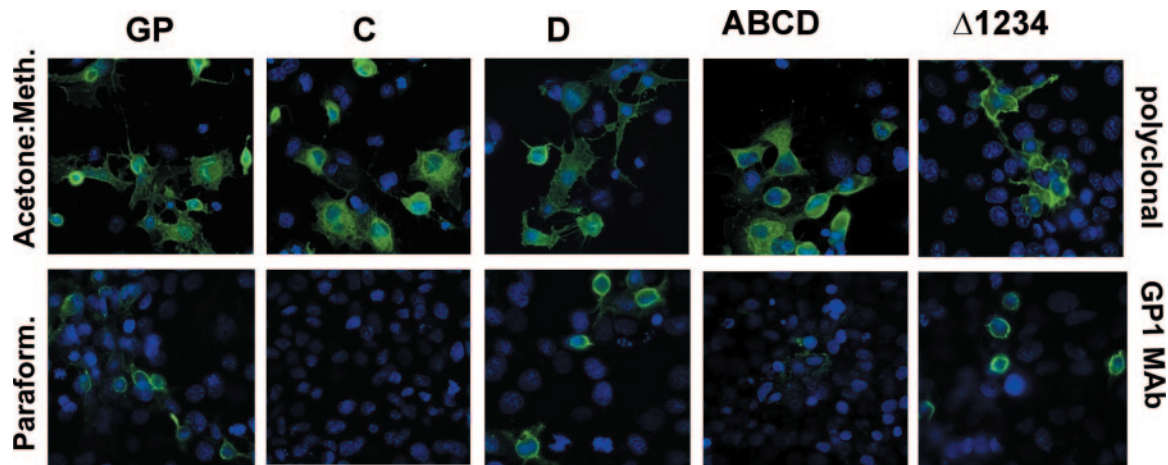


FIG. 3. Detection of expression products by IFA. Cells were transfected with wt GP or N-linked mutants C, D, or ABCD or with the mucin region deletion mutant $\Delta 1234$ and were then either fixed and permeabilized with acetone-methanol or fixed with paraformaldehyde but not permeabilized. Primary antibody was either anti-EBOV polyclonal guinea pig serum or GP1-specific MAbs 6E3, from competition group 1, or 12B5 from competition group 3 (39).

these constructs (not shown). These data indicate that deleting a portion of or even the entire mucin-rich region of GP, including the A and B epitopes, does not significantly alter the antigenicity of GP1 and GP2, nor does it alter the ability of the GP1 and GP2 proteins to interact through disulfide bonding. These data are consistent with an earlier study showing that removing the mucin region does not adversely affect protein folding and conformation (29).

Immunogenicity of glycosylation mutants in mice. To assess the importance of the glycosylation sites and mucin domain in the immunogenicity of EBOV GP, we vaccinated mice with the mutant DNA constructs by gene gun and challenged them with EBOV. In all experiments, control mice vaccinated with an empty plasmid died after challenge with EBOV, whereas most mice (24 of 27) vaccinated with the full-length GP DNA vaccine survived challenge (Table 1). Of the single N-linked mutants, constructs A, B, and D elicited complete protective immunity in mice in three separate experiments. In contrast, vaccination with the construct C DNA protected fewer than half of the mice in two separate experiments. The double mutant AB also elicited complete protective immunity, suggesting that the two glycosylation sites surrounding the known neutralizing and protective epitope defined by MAbs in competition group 2 were not required for immunogenicity. Similarly, the epitope defined by competition group 1 MAbs, which overlaps the B mutation (Fig. 1), was not impacted negatively by removing the B glycosylation site.

The double mutant CD and the quadruple mutant ABCD protected 16 of 27 mice and 12 of 26 mice (cumulative results from three separate experiments), respectively, again suggesting that deletion C in GP2 had a deleterious effect on the immunogenicity of GP (Table 1). The triple mutant ABD had slightly less protective efficacy than the single and double mutants with these mutations; however, it was only tested in a single experiment, and the results were not markedly different than those from the single mutants or wt GP (Table 1). Likewise, mutants E and F, when tested in a single experiment,

protected 8 of 10 mice, whereas mutants G and H protected 5 of 8 mice and 1 of 10 mice, respectively. Because GP did not completely protect mice in this experiment, it was not possible to assign significance to these results (Table 1); however, comparing protection to the negative control showed that H was not significantly more protective than the control empty plasmid ($P = 0.500$), whereas mutants GP, E, F, and G were more protective ($P = 0.0325, 0.0032, 0.0032$, and 0.0327 , respectively).

Somewhat surprisingly, the three mucin deletion mutants all provided less protective immunity than constructs in which this region was present. These data suggest that although the mu-

TABLE 1. ELISA titers and survival of mice vaccinated with GP glycosylation mutants and challenged with EBOV

Vaccine	No. of repeats	ELISA GMT/expt ^a	No. of surviving mice/total no. of mice	<i>P</i> ^b
Control	4	NA	0/47	
GP	3	3,940, 1,838, 528	24/27	
A	3	6,400, 1,393, 800	27/27	
B	3	7,879, 1,822, 864	26/26	
C	2	800, 418	5/16	0.0028
D	3	6,400, 1,822, 504	26/26	
AB	1	3,901	10/10	
CD	3	566, 1,008, 246	16/27	0.1699
ABD	1	2,263	8/10	
ABCD	3	400, 838, 348	12/26	0.0138
E	1	271	8/10	
F	1	1,056	8/10	
G	1	1,083	5/8	
H	1	835	1/10	
$\Delta 12$	2	2,425, 1,397	10/15	
$\Delta 234$	2	4,032, 1,523	8/17	0.0468
$\Delta 1234$	2	459, 1,181	5/17	0.0017

^a GMT, geometric mean titer. NA, not applicable.

^b Two-tailed Fisher exact tests with stepdown bootstrap corrections were performed to assess differences in the survival rates between the GP and glycosylation mutant groups. The *P* values are shown for comparisons where there was a significant difference compared to survival results in mice vaccinated with wt GP.

TABLE 2. Pre- and postchallenge antibody titers of mice vaccinated with EBOV GP mutant DNA vaccines and challenged with EBOV

Vaccine	GMT ^a	Avg PRNT ₅₀ ^b		S/T ^c	Mean day of death (range)
		Pre	Post		
Control	<100	<1:40	<1:40	0/7	7
GP	3,940	<1:40	1:73	7/7	NA ^d
A	6,400	1:49	1:40	7/7	NA
B	7,879	1:40	1:133	7/7	NA
C	800	<1:40	1:33	3/6	9 (6–12)
D	6,400	1:49	1:107	7/7	NA
CD	566	<1:40	1:27	7/7	NA
ABCD	400	<1:40	1:15	2/6	9 (6–12)
Δ12	2,425	1:52	1:187	6/7	8
Δ234	4,032	<1:40	1:213	4/7	10.5 (9–12)
Δ1234	459	<1:40	1:25	2/7	6

^a GMT, geometric mean antibody titers as determined by IgG ELISA after three vaccinations.

^b The average PRNT₅₀ was determined after vaccinations (Pre) or after challenge with EBOV (Post).

^c S/T, no. of surviving mice/total no. of mice challenged.

^d NA, not applicable.

cin region is thought to be involved in pathogenicity and its deletion does not influence antigenicity, at least in our tests, its deletion negatively influenced immunogenicity.

Neutralizing antibody responses of vaccinated mice. As reported earlier, neutralizing antibodies contribute to protective immunity to EBOV. However, neutralizing responses are generally very low after vaccination or even after challenge with EBOV. With this in mind, we measured the plaque reduction neutralizing antibody responses elicited in mice after vaccination with selected mutants and after challenge with EBOV. We found that although three vaccina-

tions elicited antibody responses readily detected by ELISA, low or no neutralizing antibodies were detected by PRNT (Table 2). The PRNT₅₀ titers from mice that survived challenge rose only slightly.

Antibody epitope mapping. To compare the antibody responses elicited with the GP mutant DNA vaccines, we performed ELISA with sera from vaccinated mice and biotinylated, 18-mer peptides with 12-amino-acid overlaps that represented the entire GP gene. ELISA was performed with pools of four consecutive peptides, with the exception of pools 24 and 32, which each had two peptides (peptides 93 to 94 and peptides 123 to 124, respectively) and pool 28, which had five peptides (peptides 107 to 111). Pools 1 to 11 contained peptides shared between GP and sGP, pools 13 to 28 contained GP-specific peptides, and pools 30 to 32 contained sGP-specific peptides. Pools 12 and 29 each had one peptide that spanned the region where GP and sGP diverge. In pool 12, peptide 48 had 13 shared GP and sGP amino acids and 5 GP-specific amino acids. In pool 29, peptide 112 consisted of 17 shared amino acids and 1 sGP-specific amino acid. The amino acids in common in pools 12 and 29 are boxed in Table 3. Pools 2, 9, 10, 16, 17, 18, 24, 26, and 27 had at least one peptide containing a site that was mutated in GP (Table 3). Peptides 82 and 83 in pool 21 contained the cleavage site for GP1 and GP2 (RRTR, Table 3).

ELISA performed with sera from mice vaccinated with wt GP revealed strong antibody reactivity to peptides in pool 17 and also to pool 8 peptides (Fig. 4). Peptide pool 17 included amino acids 385 to 420 of GP and peptides 65 to 68. Peptide 65 contains the A mutation and the competition group 2 MAb neutralizing epitope. Peptide 66 contains the group 2 MAb epitope but not the A mutation. Peptide 67 contains the group

TABLE 3. Peptides for antibody epitope mapping

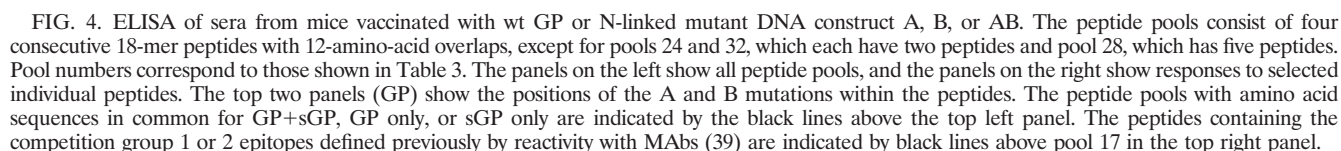
GP group and pool no.	Amino acids	Sequence ^a	Peptides	Glycosylation site	Mutation/peptide no.	MAB group/peptide(s) ^b
GP and sGP						
2	25–60	ILFORTFSIPLGVIHNS TLQVSDVDKL VCRDKLSST	5–8	40–42	E/5,6,7	
9	193–228	FFSSHPLREP VNATEDPSSGY YSTTIRYQATGFGTN	33–36	204–206	F/33,34	
10	217–252	TIRYQATGFGT NETEYLF VDN LT YVQLES RFT PQF	37–40	228–230 238–240	G/37,38H/39,40	
12	265–300	KRSNTTGKLIW KVN PEIDTTIGEWAFWET KK NL TRK	45–48	296–298		
GP specific						
16	361–396	AVSHLTTLGTISTSPQPPTTKPGPD NSTHNT PVYKL	61–64	386–388	A/63,64	
17 ^c	385–420	DNSTHNT PVYKLDISEATQ VEQHRR TD NDST ASDT	65–68	386–388 413–415	A/65 B/68	Grp2/65,66 Grp1/67,68
18	409–444	RRTD NDST ASDTPPATTAAGPLKAENTNTSKGTDLL	69–72	413–415 436–438	B/69	
20 ^d	457–492	ETAGNNNTHHQDTGEESASSGKLGLITNTIAGVAGL	77–80			Grp3/80
21 ^d	481–516	LITNTIAGVAGLITGG RRTR REAI VNAQ PKCN PNLH	81–84			Grp3/81
24	553–576	GLFCGLRQLAN ET TQALQLFLRAT	93–94	563–565	C/93,94	
26	589–624	AIDFLLQRWGGTCHILGPDCCEPHDWT KNIT DKID	99–102	618–620	D/102	
27	613–648	HDWTKNITDKIDQIIHDFVDKTL PDQGD NDN WWT GW	103–106	618–620	D/103	
SGP specific						
29	279–296	PEIDTTIGEWAFWET KK T	112			
	285–308	IGEWAFWET KK TSLEKFAVKSCLF	113–114			

^a Glycosylation sites mutated are in boldface. The amino acid sequence spanning GP1 and sGP divergence is in small caps. The GP1,2 cleavage site is in italics.

^b According to Wilson and Hart (39). Grp2, group 2; Grp3, group 3.

^c The group 2 MAB epitope is single underlined, and group 1 MAB epitope is double underlined.

^d The group 3 MAB epitope is underlined.



and was not previously identified as an antigenic epitope in mice, to our knowledge.

Compared to sera from GP-vaccinated mice, sera from mutant A-vaccinated mice, but not from mutant B-vaccinated

mice, showed reduced reactivity with pool 17 peptides. Sera from mutant AB-vaccinated mice showed intermediate activity to peptides in pool 17 (Fig. 4, left panels). ELISA with individual peptides in pools 16, 17, and 18, which encompassed all peptides with A and B mutations, shows that only peptides 66 and 67 were recognized by the GP sera, even though the competition group 2 MAb epitope was represented in both peptides 65 and 66 and the competition group 1 epitope was represented in peptides 67 and 68. This result would be consistent with masking of the epitopes by glycosylation, since peptides 65 and 68 had the A and B glycosylation sites, respectively. This is supported by the results shown for sera from mice vaccinated with the A mutant DNA vaccine, in that increased reactivity with peptide 65 was observed compared to GP. Sera from mice vaccinated with the B mutant DNA vaccine also showed an increase in reactivity to all four peptides relative to GP (Fig. 4).

Sera from mice vaccinated with the AB mutant DNA showed further improved reactivity to peptide 68 (containing the group 1 MAb epitope); however, this was accompanied by a reduction in reactivity to the other three peptides in the pool. These results suggest that glycosylation partially masked the linear epitopes recognized by competition groups 1 and 2 MAbs and that removing one or both glycosylation sites could improve the antigenicity of the protective epitopes recognized by those MAbs.

Sera from mice vaccinated with wt GP showed little reactivity to peptide pools containing the C or D mutations in GP2 (Fig. 5, top left panel). Likewise, little reactivity was observed with the GP sera and the individual peptides in those pools (see Fig. 7, top right panel). Sera from mice vaccinated with the C or D constructs did not show improved reactivity to these peptide pools (Fig. 5, middle two left panels) or individual peptides in those pools (data not shown). Thus, N-linked glycosylation was apparently not masking linear antibody epitopes in GP2.

The C mutant sera had greatly reduced reactivity to pool 17 peptides but not to pool 8 peptides. The D mutant had somewhat reduced reactivity to pool 17 peptides and an apparent increase in reactivity to pool 12 peptides. Pool 12 peptides contained amino acids 265 to 300 of GP and had one N-linked glycosylation site. This pool also contains peptides representing GP and sGP divergence (Table 3). There was no previously reported association of this region with immunogenicity to our knowledge.

The CD mutant showed poor reactivity with all peptide pools and had no reactivity with pool 17 (Fig. 5). ELISA with the C, D, and CD mutant sera and the individual peptides in pool 17 showed that the C and D sera had reduced reactivity with peptides 66 and 67 (Fig. 5, second panel from top on right) compared to GP (Fig. 4, top right panel). Consistent with the pooled peptide results, mutant CD sera had no reactivity with any of the peptides in pool 17. These data are consistent with the poorly protective immune response that C mutants elicited in mice and, along with our data showing that the GP2 of C mutants did not appear to tether GP1 to the cell surface, suggest that there is a need for GP2 and GP1 interaction at the cell surface for immunogenicity.

Comparing ELISA results with sera from mice vaccinated with the triple mutant, ABD or the quadruple mutant ABCD further implicated the C mutation as a negative

factor in both antigenicity and immunogenicity and the A and B mutations as positive factors. Like the AB sera, the ABD sera showed reactivity, albeit reduced compared to GP, to pool 17, whereas the ABCD sera had no reactivity (Fig. 6). ELISA with the ABCD sera and the individual peptides in pool 17 confirmed the absence of reactivity with any of them (not shown). In contrast, the ABD sera showed reactivity to the peptides in pool 17 similar to that of the A and B sera, with stronger reactions to peptides 65, 66, and 67 than to peptide 68, whereas the AB sera had a stronger reactivity to 68 than to the other peptides.

The ABD sera appeared to react with more of the peptide pools than other sera. For example, the ABD sera reacted more strongly with peptides in pools 14 and 29 (Fig. 6). To examine this more closely, we boosted the mice once more with the ABD or the GP DNA vaccines and collected sera 10 days later to repeat the peptide ELISA. We found that the additional vaccination with the ABD construct resulted in increased reactivity to the peptides in pools 12, 14, and 29 and especially to the peptides in pool 17. However, after challenge with EBOV, responses to pool 14 peptides were not boosted, whereas those to 17, 12, and 29 were (Fig. 6, ABD survivors, note the scale change). In addition, the survivor mice showed an increased response to peptide pool 20, which contains the previously described linear nonprotective epitope recognized by competition group 3 MAbs (39) (Fig. 6, ABD survivors).

Sera from mice vaccinated with wt GP that were boosted 10 days before assay (Fig. 6, GP boost) showed a predominant response to pool 17 peptides, and those that survived challenge with EBOV (Fig. 6, GP, survivors) had boosted responses to pool 17, 12, and 29 peptides and also smaller increases in responses to pools 15 and 20 (Fig. 6, GP survivors, note the scale change). As noted above, pool 20 contains the MAb group 3 epitope. No antibody epitopes of importance have been reported for amino acids covered by pool 15; however, this pool included the carboxy-terminal amino acids of sGP, which were also shared with GP (Fig. 1 and Table 3). These data suggest that the AB and ABD mutations allowed continued access to pool 17 epitopes, whereas addition of the C mutation abrogated that response. In addition, the AB mutation appeared to broaden the response to additional epitopes in GP.

Because pools 12 and 29 overlap, with pool 29 containing both GP-specific and sGP-specific peptides, we were interested to determine whether the reactivity observed might be to sGP. ELISA with the ABD sera and individual peptides in pool 29 demonstrated reactivity only with peptides 112 and 113, both of which contained shared GP and sGP sequences, but not to peptide 114, which is sGP specific. Consequently, we concluded that the pool 29 reactivity was exclusive to antigens shared by GP and sGP.

Analysis of sera from mice vaccinated with the E, F, G, or H constructs, which contained shared GP1 and sGP antigens, revealed that deleting the E and H sites reduced reactivity with almost all peptide pools (Fig. 7). Similarly, these sera had low ELISA titers with authentic EBOV antigen and provided incomplete protection to challenge (Table 1). Sera from mice receiving the G construct showed reduced reactivity to all peptide pools except 17. Incomplete protection was elicited with this construct, which might suggest that this antigenic region, which contains the

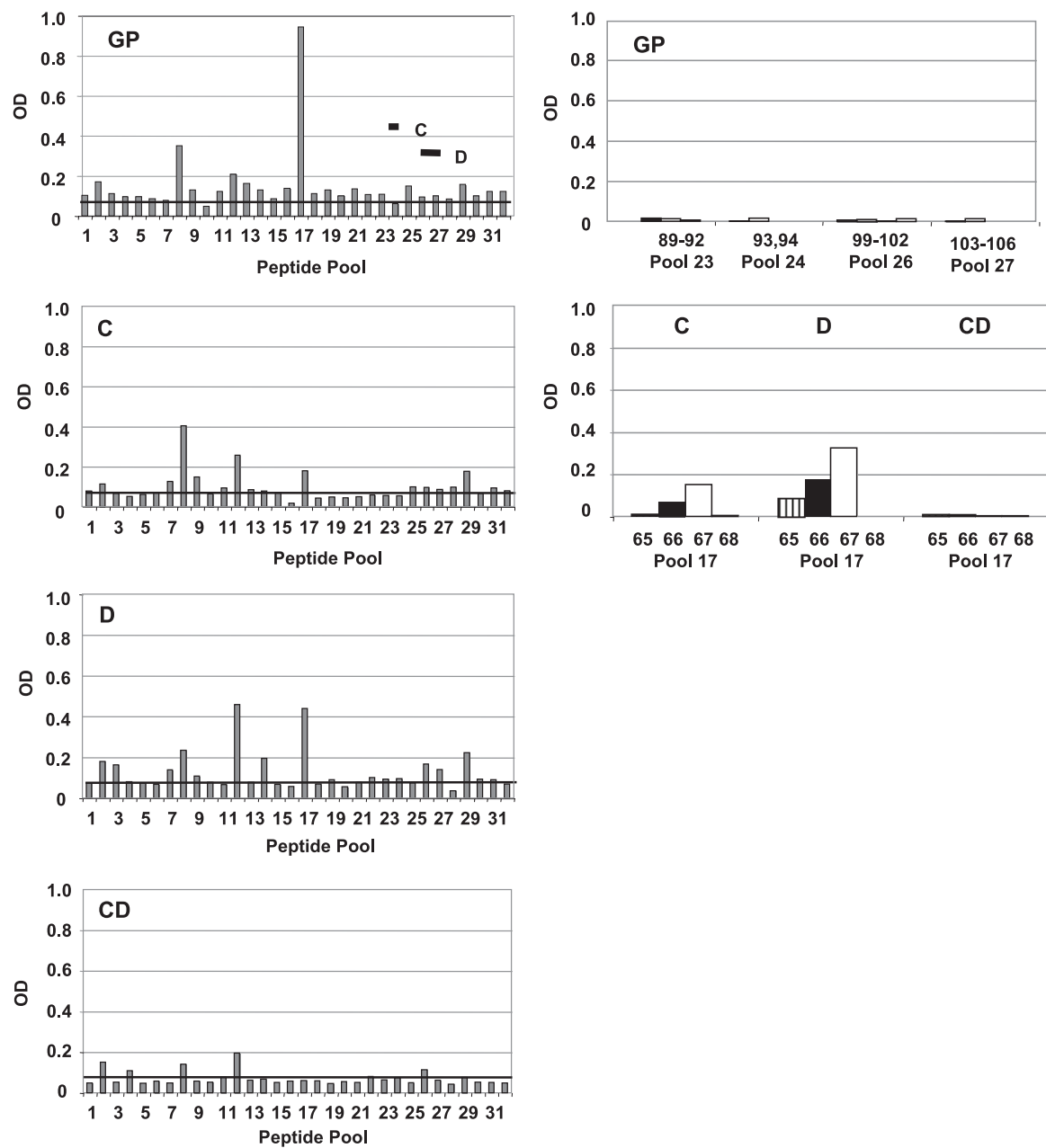


FIG. 5. ELISA of sera from mice vaccinated with wt GP or N-linked mutant DNA construct C, D, or CD. The peptide pool numbers correspond to those shown in Table 3. The panels on the left show all peptide pools, and the panels on the right show responses to selected individual peptides. The top left panel (GP) shows the positions of the C and D mutations within the peptide pools.

group 1 and 2 antibody epitopes (39), was not, in itself, sufficient for protective immunity in mice; however, as indicated earlier, this result was only significant compared to the negative control, because in this experiment, wt GP did not protect all of the mice. Construct F sera maintained reactivity with the pools 17, 12, and 29 but also had increased reactivity with pool 20, which was not prominently recognized by sera from any of the other N-linked constructs but did contain the competition group 3 MAb recognition site (Table 3), which was boosted in mice that survived EBOV challenge (Fig. 6) and which was found to be poorly protective in BALB/c mice

(39). Consistent with this and with the idea that pool 17 responses were insufficient for protective immunity, only partial protective immunity was elicited in mice vaccinated with the F mutant construct. Analysis of the O-linked mutant sera showed, as expected, a complete loss of reactivity with peptide pools containing the deleted regions, which included the A and B sites and the group 1 and 2 MAb recognition sites (Fig. 7). As with the F construct, the $\Delta 12$ construct elicited an increased response to pool 20. Construct $\Delta 234$ elicited an increased response to pool 14 (Fig. 7), which was also seen with the ABD construct

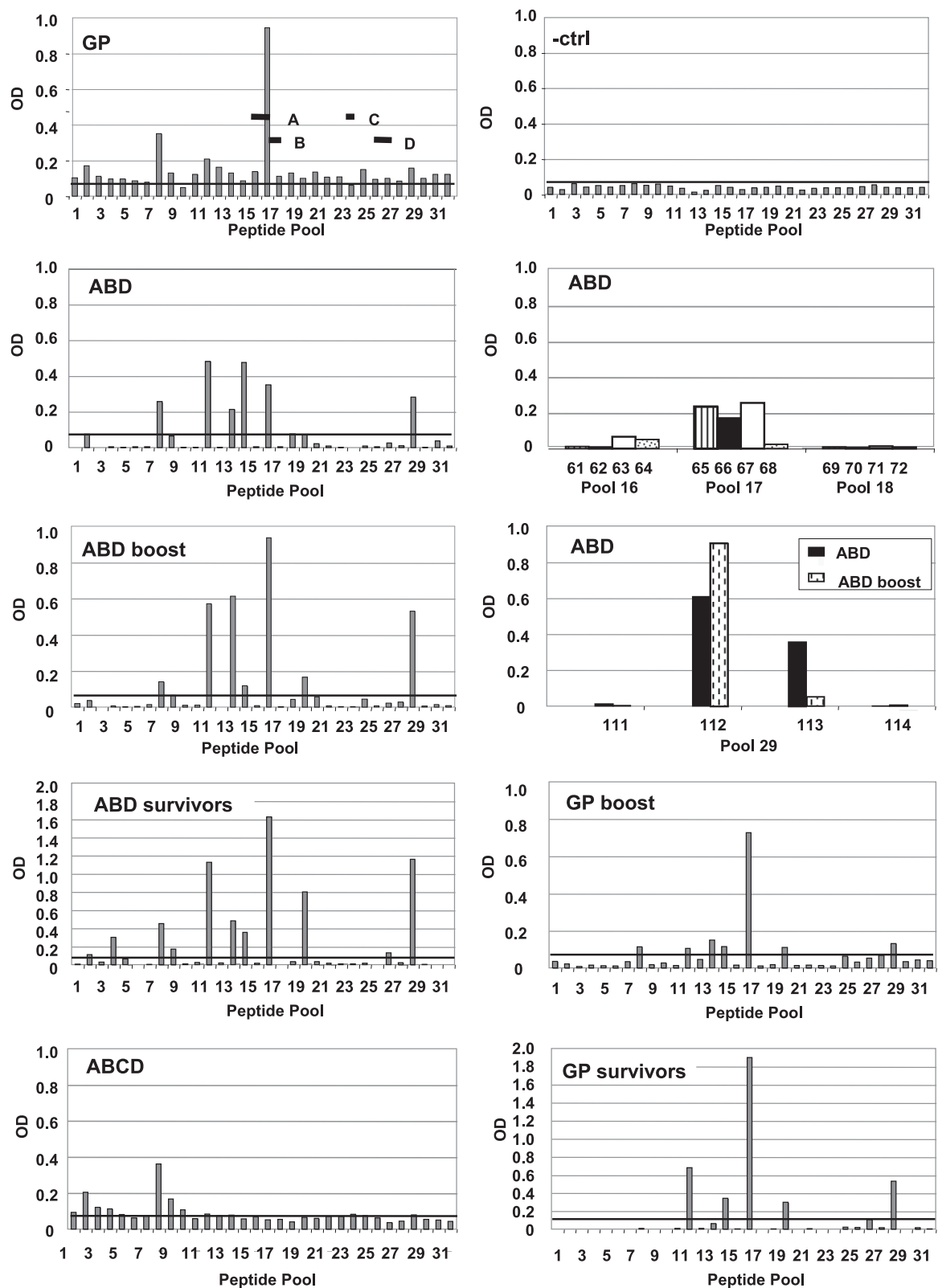


FIG. 6. ELISA of sera from mice vaccinated with wt GP, a negative-control plasmid (-ctrl), or N-linked mutant DNA construct ABD or ABCD. The peptide pool numbers correspond to those shown in Table 3. Sera from mice receiving three vaccinations (ABD or GP) or an additional vaccination 10 days before assay (ABD boost or GP boost) or from those that survived challenge with EBOV (ABD survivors or GP survivors) were compared. The ABD sera were also assayed against selected individual peptides from pool 16, 17, 18, or 29.

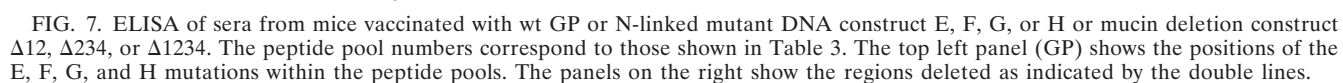


TABLE 4. Peptides used for IFN- γ ELISPOT assay

GP group and pool no.	Amino acids	Sequence ^a	Peptides	Glycosylation site	Mutation/peptide(s)
GP1 specific					
1	1–50	MGVTGILQLPRDRFKRTSFFLWVILFQRTFSIPLGVIHNST LQVSDVDK	1–8	40–42	E/7,8
4	121–170	CLPAAPDGIRGFPRCRYVHKVSGTGPCAGDFAFHKEGAF FLYDRLASTVI	25–32		
5	161–210	LYDRLASTVIYRGTTFAEGVVAFLILPQAKKDFSSHPLRE PVNATEDPS	33–40	204–206	F/40
6	201–250	EPVNATEDPSSGYSTTIRYQATGFGTNETEYLFEVDNLT YVQLESRFTP	41–48	204–206	F/41
10	361–410	AVSHLTTLGTISTSPQPPTTKPGPDNSTHNTVPYKLDISEAT QVEQHRR	73–80	386–388	A/76,77,78
11	401–450	ATQVEQHRRRTDNDSTASDTPPATTAAGPLKAENTNTSK GTDLLDPATT	81–88	413–415	B/81,82,83
GP2 specific					
13	471–520	EESASSGKLGLITNTIAGVAGLITGGRRTRREAIVNAQPKC NPNLHYWTT	95–102		
15	551–600	QDGLFCGLRQLANETQALQLFLRATTELRTFSILNRKAI DFLLQRWGGT	111–118	563–565	C/111,112,113
16	591–640	DFLLQRWGGTCHILGPDCCIEPHDWTKNITDKIDQIIHDF VDKTLPDQGD	119–126	618–620	D/122,123,124

^a Previously defined T-cell epitopes are underlined (20, 21). Glycosylation sites mutated are in boldface. The GP1,2 cleavage site is in italics.

(Fig. 6). Construct Δ 1234 elicited poor reactivity to all peptide pools, although there was some reactivity to pool 20 peptides, which contains the non-neutralizing and nonprotective linear epitope recognized by competition group 3 MAbs (Fig. 7).

Peptide screening of T-cell responses. Both humoral and cell-mediated immunity have been reported to be required for protective immunity to EBOV. We, and others, previously demonstrated that GP DNA vaccination elicits cytotoxic T-cell responses in mice (32, 40). Here, we attempted to answer two questions: (i) does glycosylation influence the ability of the GP DNA vaccines to elicit cell-mediated immunity as measured by IFN- γ ELISPOT assays, and (ii) do the T-cell specific responses of the GP and GP-mutant DNA-vaccinated mice correlate with those reported in earlier studies. To date, two studies have identified three GP-specific T-cell epitopes in BALB/c (*H-2^d* restricted) mice at positions 161 to 169, 231 to 239 (21), and 141 to 155 (20). In the first study, the T-cell epitopes were identified in mice vaccinated with liposome-encapsulated, lethally irradiated EBOV, and in the second study the epitope was identified in mice vaccinated with Venezuelan equine encephalitis virus replicons expressing EBOV GP.

To gauge the influences of glycosylation on the ability of the GP DNA vaccines to elicit cell-mediated immunity, we performed IFN- γ ELISPOT assays with spleen cells from mice that were boosted with each DNA vaccine 10 days before harvest. For stimulating the cells in vitro, we used pools of 15-mer peptides with 10-amino-acid overlaps (Table 4). Each pool contained eight peptides except for pool 12, which contained six peptides. Pool 13 contained peptides with the cleavage signal for GP1 and GP2.

Vaccination with the wt GP DNA vaccine elicited responses predominantly to peptides in pools 4, 5, and 10 (Fig. 8). Pool 4 contains two of the previously described BALB/c (*H-2^d*)-specific T-cell epitopes (20, 21). Pool 5 also contains one of

these same epitopes (21). Mutant A-vaccinated mice did not exhibit responses to the T-cell epitopes in pools 4, 5, or 10 and yet were all protected from challenge with EBOV in three separate experiments (Table 1). Conversely, the AB and ABD mutants did elicit responses to peptides in pools 4 and 5, and yet these mice were incompletely protected from challenge (Table 1). These results indicated that the T-cell epitopes contained in pools 4 and 5 were neither required nor sufficient for complete protective immunity.

Although removing the A glycosylation site did not enhance reactivity to pool 10 peptides, which contains the A mutation, removing both the A and the B sites did result in enhanced reactivity with peptides in pool 10 (AB and ABD, Fig. 8). In contrast to T-cell responses in wt GP-vaccinated mice, reactivity with group 8 peptides was pronounced in ELISPOT assays performed with AB-vaccinated mouse splenocytes. T-cell responses to peptides in this pool were not described previously, and this response suggests that one or more novel T-cell epitopes may be unmasked in the mutant AB GP.

As described above, the C mutation DNA vaccines elicited poor protective immunity in BALB/c mice, evidently due to the inability of GP1 and GP2 to interact efficiently. ELISPOT assays performed with cells from the C mutant-vaccinated mice exhibited a slight response to peptides in pool 15, which contains the C mutation, strong responses to pool 5 peptides, and intermediate responses to the peptides in pools 8 and 10. These data are consistent with our observation with the ABD mutant and indicate that a T-cell response to pool 5 peptides, containing an epitope previously shown to offer protective immunity to BALB/c mice (peptide 1) (21), was not sufficient for protection. Likewise, strong reactivity to peptide pools 4 and 5 was observed for the O-linked glycosylation mutant-vaccinated mice (Δ 234 and Δ 1234) (Fig. 8), although both elicited significantly less protective immunity than wt GP-vaccinated mice (Table 1).

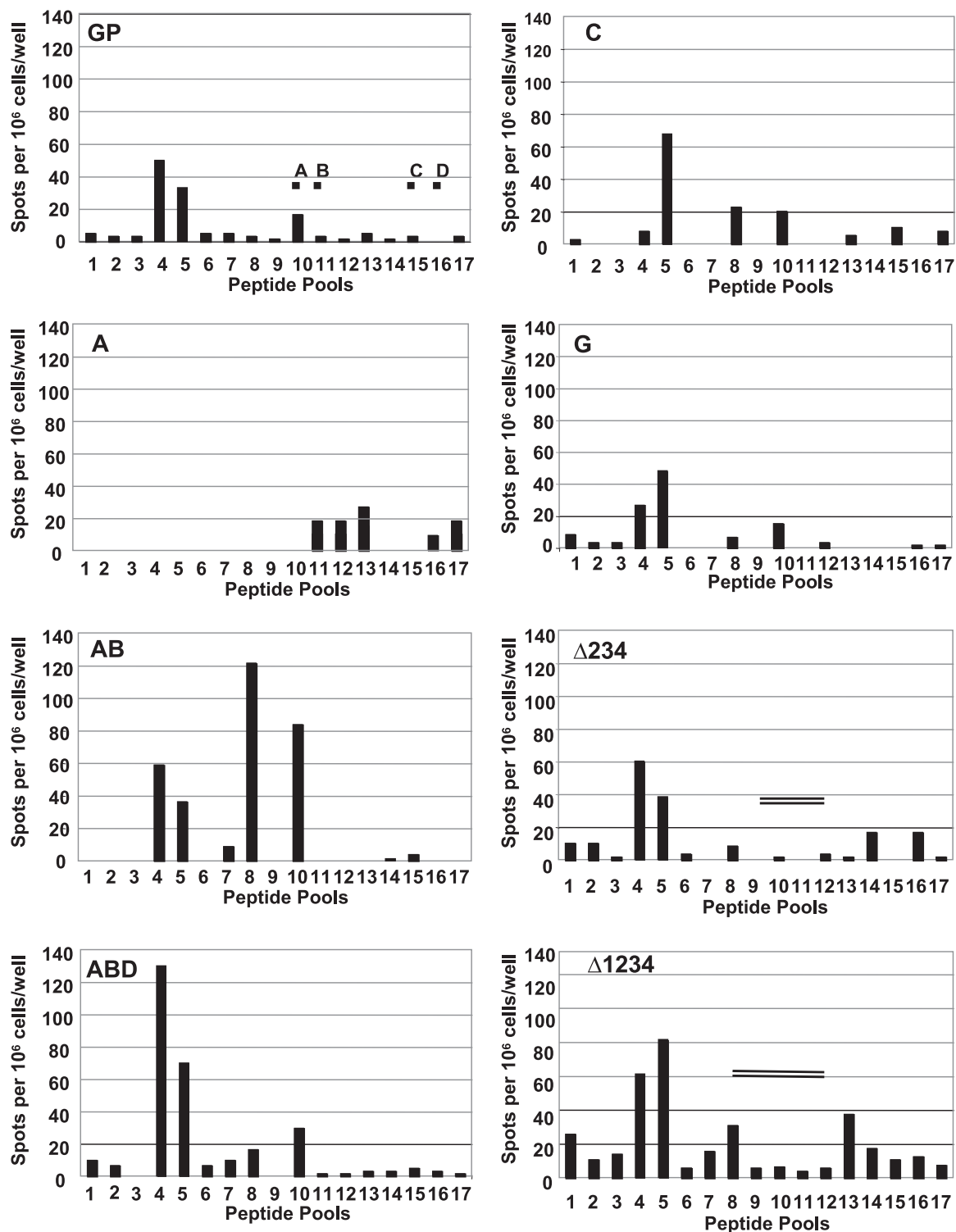


FIG. 8. IFN- γ ELISPOT assay using splenocytes from mice vaccinated with the wt GP DNA vaccine, with the N-linked mutants A, AB, ABD, C, or G or the mucin deletion constructs Δ 234 or Δ 1234. The peptide pool numbers correspond to those in Table 4. The top left panel (GP) shows the locations of the A, B, C, or D mutation within the peptide pools. The double lines show the portion of the GP gene deleted in the mucin deletion constructs.

Another epitope previously reported to elicit cytotoxic T cells in mice (peptide 2) (21) was not detected in our ELISPOT assays with any of the mutants (Fig. 8 and results not shown). This epitope, EYLFEVDNL, was contained in pool 6 in

peptides 46 and 47. Peptide 46 also contains the G and H mutations, and peptide 47 contains the H mutation (Table 4). Since removal of the G (not shown) or H (Fig. 8) glycosylation sites did not increase reactivity to peptides in pool

6, the absence of reactivity with this epitope was likely not due to masking by either of these glycosylation sites and may have been due to the differing vaccination platforms (irradiated virus, versus DNA).

DISCUSSION

We studied the antigenicity and immunogenicity of EBOV GP with mutations in N-linked glycosylation sites or deletions in the mucin region. We used gene-gun delivery of the DNA vaccines for the *in vivo* analysis of the mutant DNAs because we have found this approach to be an efficient means of eliciting both humoral and cell-mediated immune responses to a variety of pathogens, including filoviruses (15, 24, 28, 30, 32).

One earlier study addressed some of the same issues that we did, in that individual N-linked glycosylation sites were mutated and the mucin region was deleted (10). That study differed, however, in that (i) asparagine residues rather than serine/threonine residues were changed in the consensus N-linked glycosylation sequence (N-X-S/T), (ii) multiple mutations were not studied, (iii) a vaccinia virus (VACV)-T7 system was used for transient expression, (iv) pseudotyped retroviruses were used for testing transduction of cells with mutated GP genes, and (v) immunogenicity was not assessed.

In our study, eight N-linked glycosylation sites were mutated and tested. Only one, located in GP2 at amino acid 565, had a deleterious influence on the antigenicity and immunogenicity of EBOV GP. The most likely reason for the observed decrease in antigenicity is that this mutation prevented GP1 and GP2 from disulfide bonding. The data supporting this conclusion come from both our immunoprecipitation and immunostaining studies. Although we do not have any GP2-specific antibodies, MAbs to GP1 will generally immunoprecipitate GP2 due to its interaction with GP1 through disulfide bonding. Immunoprecipitates of expression products from cells transfected with the C mutants contained little or no GP2, providing indirect evidence that GP1 and GP2 could not disulfide bond. Further, we could detect GP1 inside cells transfected with any of the constructs, and we also could detect GP1 on the surfaces of cells transfected with any of the constructs except those with the C mutation. Unfortunately, we do not have reagents available to determine whether the C mutation results in GP2 that cannot be inserted into the cell membrane or whether it is inserted but has a conformation that precludes binding to GP1.

Not only was GP2 poorly detected in immunoprecipitates from C constructs with MAbs to GP1, there also was no GP1 detected with a group 5 MAb. These results are consistent with earlier findings, which indicated that the group 5 MAbs recognize a conformational epitope in GP1 (39). Our results indicate that this epitope was disrupted when the C mutation was introduced and further suggest a role for GP1,2 dimerization and the N-linked glycosylation of GP2 in maintaining the antigenic conformation for GP1 as well as GP2.

Our results with the C mutant differ from those reported earlier (10), in that the C-like mutant in those studies produced little to no detectable GP1 or GP2 in the transient VACV-T7 expression assays, whereas in our studies with the DNA constructs GP1 was detected at levels similar to those obtained with wt GP when precipitated with polyclonal antibodies to EBOV. Also, in the earlier study, the C-type GP2 was detected

in pseudotyped retroviruses, a result seemingly in conflict with these authors' findings with the VACV-T7 expression.

Although one of the two N-linked glycosylation sites near the GP2 cysteine involved in disulfide bonding to GP1 was apparently important, the other was not. The putative GP2 disulfide-binding residue, C609 (10, 27), is found 44 amino acids downstream of the C mutation and 11 amino acids upstream of the D mutation. The D mutation had little or no influence on the antigenicity of the expressed GP, as measured by the immunoprecipitation or immunofluorescent antibody tests, and elicited complete protective immunity in mice.

We also did not find an effect similar to that evoked by the C mutation on GP1-GP2 interaction when the only glycosylation site near the GP1 cysteine (C53) involved in bonding to GP2 was removed. Mutant E, which had a deletion in the N-linked glycosylation site at amino acid 42, produced both GP1 and GP2 detectable by immunoprecipitation and displayed GP1 on the cell surface at levels similar to those observed with wt GP. This glycosylation site, therefore, does not appear to have a large influence on the conformation of this region of GP, even though it is the only glycosylation site in the first 200 amino acids of GP1 and sGP.

The deleterious influence of the C mutation was also reflected in its poorly protective efficacy even after three administrations. Mice vaccinated with any of the C-mutation-containing constructs were significantly less protected from challenge with EBOV compared to that achieved with wt GP, whereas mice vaccinated with the other constructs had similar levels of protection to that of wt GP. In fact, mice vaccinated with constructs containing the A, B, D, or AB mutations were completely protected, whereas in one of our experiments, wt GP failed to protect. Although these results were not statistically significant, there is the possibility that the mutations might have improved immunogenicity by uncovering additional protective epitopes or generating a more favorable conformation of GP. To address this possibility, we used two different overlapping peptide libraries to measure antibody and T-cell responses.

The results that we obtained by antibody peptide ELISA indicate that there are differences in antibody responses to the glycosylation mutant GPs, some of which were reflected in changes in protective immunity and some of which were not. Our results are mostly consistent with those found in an earlier study with MAbs to EBOV (39), in that there are only a small number of linear epitopes recognized by the mouse humoral immune response. The predominant response of wt GP-vaccinated mice was to peptides containing two previously defined linear epitopes. Within this pool, two of the peptides contained the amino acid sequence recognized by group 2 MAbs, which were neutralizing and protective in mice, and two contained the amino acid sequence recognized by group 1 MAbs, which were not neutralizing but could passively protect mice from challenge with EBOV (39). Sera from mice vaccinated with the A and B mutation constructs, which flank both of these epitopes, showed increases in reactivity with the specific peptides containing amino acids for that glycosylation site. Because these peptides have the wt GP amino acid sequence, rather than that of the mutant, the results may have been even more dramatic if peptides with the mutations were used.

Interestingly, deleting both the A and the B sites further

improved the reactivity to the peptide with the B glycosylation site but removed the reactivity to the A site. A mutant containing both of these deleted glycosylation sites, plus the deletion of a site in GP2 (ABD), appeared to elicit a broadened humoral response, with responses to additional peptides not seen with GP. The results with the ABD mutant also suggest that not all newly exposed epitopes were relevant for protection. For example, increased responses to epitopes in peptide pool 14 appeared after ABD vaccination but were not boosted in survivors to EBOV challenge. In contrast, increased responses to epitopes in pool 12 after ABD vaccination were further boosted in mice that survived EBOV challenge. Further studies would be needed to determine whether specific regions of GP that are unmasked in a mutant such as ABD actually are enhancing protective immunity. In general, these results suggest that it is possible to uncover or improve access to certain epitopes by removing glycosylation. However, this was not a consistent finding with our mutants in that none of the others showed increased reactivity with peptides containing the mutated glycosylation sites.

Our results with the mucin deletion mutants differ in some ways from what would be expected. This region was previously found to be associated with cell rounding and detachment in cell culture assays and has been suggested to relate to EBOV pathogenicity (29, 31, 41). In one study, deletion of this region enhanced the ability of pseudotyped retroviruses to transduce cells (10), but in another study deletion of this region did not influence the infectivity of pseudotyped retroviruses (29). In the latter report, the authors concluded that the regions of EBOV GP involved in binding to cellular receptors and mediating membrane fusion are not within the mucin region. Further, they surmised that deletion of the entire mucin region does not negatively influence GP folding. Consistent with this, in our studies there was no apparent difference in the antigenicity of the mucin deletion mutants compared to wt GP in immunoprecipitation and immunofluorescence assays with polyclonal or monoclonal antibodies. In addition, because this region includes the N-linked sites 319, 335, 348, 438, and 454, which we did not mutate, their deletion provides us with indirect evidence that these sites may not greatly impact the antigenicity of GP either.

Despite the apparently normal antigenicity of the mucin deletion mutants, none of them elicited complete protective immunity in mice, and the larger two of the deletion constructs were significantly less protective than GP. In part, a likely explanation for this is that this region includes two known mouse-protective linear epitopes (39). Deletion of these epitopes might result in reduced protective efficacy of the mucin deletion DNA vaccines. Although we know that these epitopes can be involved in protection, clearly they are not absolutely required, in that some mice receiving the mucin deletion vaccines were still protected from challenge, indicating that other gene regions and epitopes also are involved in protection. Likewise, a response to these specific epitopes does not necessarily predict complete protective efficacy. For example, our F and G constructs, which elicited good responses to the linear epitopes in this region, did not completely protect mice from challenge with EBOV. Conversely, our A construct elicited reduced reactivity to these linear epitopes but provided complete protective immunity. These findings indicate that no

single epitope is either required or sufficient for protection against EBOV challenge in mice.

Our findings with wt GP IFN- γ ELISPOT assays and overlapping peptides are consistent with earlier identification of two T-cell epitopes in EBOV GP (20–22). We found that some of the mutant GPs also elicited responses to pools of peptides containing these epitopes, whereas others did not. These results indicate that these particular epitopes were neither required nor sufficient for protective immunity in mice, in that good responses to peptides containing those epitopes did not necessarily correlate with protection, nor did the absence of a response correlate with poor protection. Our results also suggest that additional T-cell epitopes are uncovered by using some of the glycosylation mutants for vaccination but that these sites were not necessarily found where the glycosylation has been removed. For example, although with the AB mutant there was an increase in response to peptides in pool 10, which includes the A mutation, there was no increase in pool 11, which contains the B mutation. In addition, there was an even larger response to peptides in pool 8, which was not seen with any of the other mutants. The significance of these potential T-cell epitopes remains to be determined.

In summary, the present study has produced several novel findings. First we demonstrated for the first time the importance of GP2 glycosylation on the antigenicity and immunogenicity of EBOV GP, and present evidence showing that the most likely reason for this negative impact involves disruption of the GP1-GP2 interaction. Second, we showed that previously identified B-cell and T-cell epitopes in GP were neither required nor sufficient by themselves for protective immunity to EBOV challenge in mice and we identified new regions that might be important. Third, we demonstrated that the mucin region cannot be removed without adversely influencing protective immunity. And finally we provide information to suggest that it might be possible to enhance immunity by specific changes in the glycosylation of GP.

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