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Glycosylation and Oligomerization of the Spike Protein of Marburg Virus

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The oligosaccharide side chains of the glycoprotein of Marburg virus (MW 170,000) have been analyzed by determining their sensitivity to enzymatic degradation and their reactivity with lectins. It was found that they consist of *N*- and *O*-glycans. Studies employing chemical cross-linking showed that the glycoprotein is present as a homotrimer in the viral envelope. © 1991 Academic Press, Inc.

Marburg virus and the morphologically related Ebola virus are negative-stranded RNA viruses of the Filoviridae family (*1*). Both viruses cause a severe hemorrhagic fever with a mortality as high as 90% for Ebola virus (*2*) and about 35% for Marburg virus (*3*). Marburg virus was first isolated in 1967 from laboratory workers, when it caused three outbreaks in Europe at Marburg, Frankfurt, and Belgrade. The workers were infected after contact with tissues of monkeys imported from Uganda (*3*). Since that time, four more episodes of Marburg disease in Africa have been reported (*4*).

Marburg virus is an enveloped, filamentous virus containing a negative-stranded RNA genome with a molecular weight of approximately 4.2×10^6 (*5*). The virion RNA does not bind to oligo-dT–cellulose and is not infectious. SDS–PAGE profiles demonstrate the presence of seven virion proteins. These are the L protein (180K), the glycoprotein GP, the nucleoprotein NP (96K), and proteins VP40 (38K), VP 35 (32K), VP30 (28K), and VP24 (24K). In analogy to Ebola virus, VP35 may be a transcriptase component and VP30 a second nucleoprotein. The functions of VP40 and VP24 are still unknown (*4, 6*). The virion proteins are synthesized from monocistronic mRNA species analogous to those of Ebola virus (*7*). By *in vitro* translation, five different mRNA species could be assigned to proteins NP, VP40, VP35, VP30, and VP24. mRNAs coding for L and GP could not be detected yet (*4*).

GP is the only membrane glycoprotein of Marburg virus and forms the surface projections on the viral envelope. The molecular weight of GP has previously been estimated to be 140,000 but reevaluation with appropriate protein markers indicates that 170,000 may be more accurate (Fig. 1A). To examine the type of glycan–protein linkages, we treated virion proteins with different glycohydrolases. These treatments were performed overnight at 37° after denaturing of the proteins. As shown in Fig. 1B, the electrophoretic mobility of GP was slightly enhanced after incubation with endoglycosidase H (lane 4), and a distinct further increase was obtained by treatment with endoglycosidase F (lane 3), indicating that GP contains *N*-glycans of the oligomannosidic, but mainly of the complex type. However, carbohydrate not linked by *N*-glycosidic bonds was also present. Sensitivity of GP to endo-α-*N*-acetylgalactosaminidase (Fig. 1B, lane 2) indicated that *O*-glycans containing galactose-β(1–3)-*N*-acetylgalactosamine disaccharide units were present. The existence of *O*-glycans was also suggested by *N*-acetylgalactosamine which was detected, in addition to *N*-acetylglucosamine, when purified GP was subjected to amino acid analysis (data not shown).

To further assess the nature of the carbohydrate moiety, the affinity of various lectins to GP was analyzed. The lectins used were conjugated to the steroid hapten digoxigenin (Boehringer, Mannheim, Germany), and their binding was assayed by immunoblotting. The results obtained with peanut agglutinin (PNA) and *Datura stramonium* agglutinin (DSA) are shown in Fig. 2. As indicated by control experiments with fetuin that contains N- and O-linked oligosaccharides and with asialofetuin containing the same glycans devoid of sialic acid, PNA reacts specifically with the galactose-β(1–3)-*N*-acetylgalactosamine cores of *O*-glycans that must not be substituted with terminal *N*-acetylneuraminic acid. The observation that Marburg virus GP is recognized by PNA after incubation with endoglycosidase F or *N*-glycopeptidase F provides further support for the presence of *O*-glycans. It was unexpected, however, that PNA bound also to GP after incubation with endo-α-*N*-acetylgalactosaminidase. This suggests that galactose-β(1–3)-*N*-acetylgalactosamine cores substituted with nonsialic acid residues

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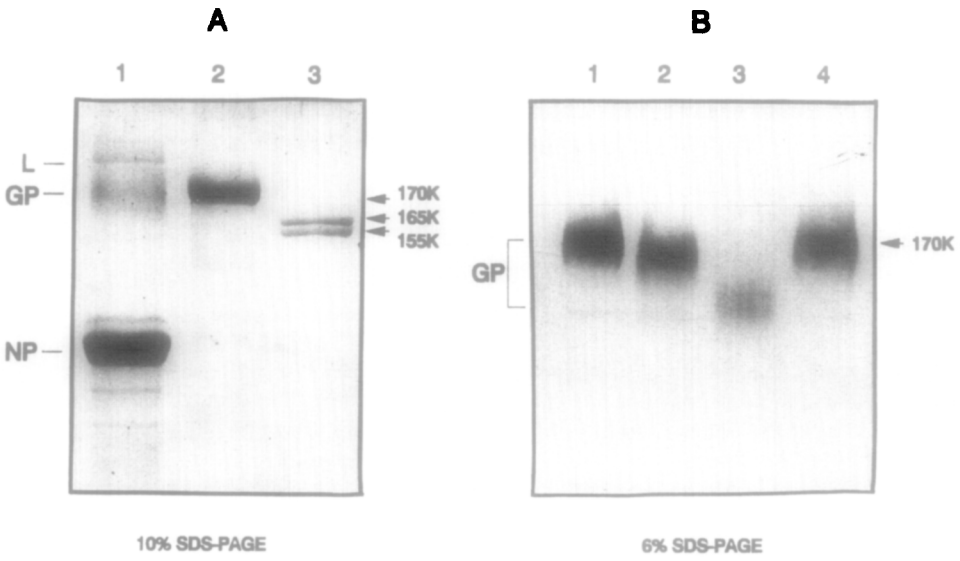

FIG. 1. Electrophoretic mobility of GP before and after glycohydrolase treatment. A. Electrophoretic mobility of GP relative to other viral and to marker proteins. (1) Purified Marburg virus proteins, (2) α_2-macroglobulin (170K), (3) *Escherichia coli* RNA polymerase subunits β and β' (155K and 165K). 10% polyacrylamide gel. The proteins were stained with Coomassie brilliant blue. B. Effect of glycohydrolase treatment of GP labeled with [^{3}H]glucosamine. (1) Untreated virus, (2) treatment with endo-α-*N*-acetylgalactosaminidase (0.01 U/20 μl), (3) treatment with endoglycosidase F/*N*-glycosidase F (0.05 U/μl), (4) treatment with endoglycosidase H (0.05 U/μl). 6% polyacrylamide gel. E6 cells (ATCC Vero clone CRL 1586) (*17*) were infected with Marburg virus, strain Musoke, at a m.o.i. of 10^{-2} PFU/cell. Infected cells were incubated in Dulbecco's medium containing 2% fetal calf serum for 6 to 8 days (c.p.e. approximately 85%). When viral proteins were to be labeled, D[6– ^{3}H]glucosamine or [^{35}S]methionine was added 5 days p.i. at final concentrations of 20 μCi/ml, and labeled virus was harvested 24 hr thereafter. Culture fluid was clarified by centrifugation at 5,000 rpm for 15 min at 4°, and virus was pelleted at 25,000 rpm through a 20% sucrose cushion in TNE (0.01 *M* Tris–HCl, pH 7.4; 0.15 *M* NaCl; 2 m*M* EDTA) for 2 hr at 4°. The pellet was resuspended in TNE and further purified by gradient centrifugation in a SW28 rotor through 0 to 40% (w/v) potassium tartrate, 30 to 0% glycerol in TNE at 27,000 rpm for 16 hr, followed by 20 to 70% sucrose in TNE (containing 1 *M* NaCl) at 27,000 rpm for 4 hr at 4°. The virus band was isolated, diluted in TNE, and pelleted by centrifugation at 35,000 rpm for 30 min at 4°. Glycohydrolase treatments were performed at 37° overnight after denaturing of the proteins by boiling in buffer containing 0.1% SDS, 0.5% octylglucoside, 0.5% β-mercaptoethanol, 50 m*M* sodium acetate, pH 7.0, and 5 m*M* EDTA. The proteins were separated by electrophoresis on polyacrylamide slab gels and detected by fluorography.

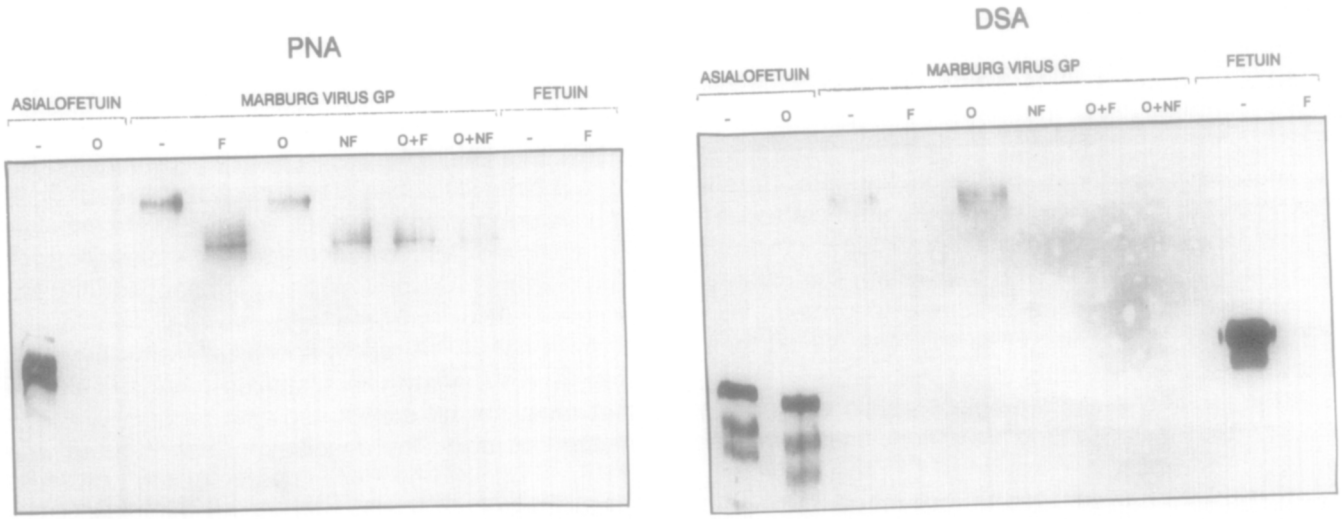

FIG. 2. Reactivity of GP with lectins before and after glycohydrolase treatment. (–) Untreated sample, (F) treatment with endoglycosidase F/*N*-glycosidase F (0.05 U/20 μl), (NF) treatment with N-glycopeptidase F (0.1 U/20 μl), (O) treatment with endo-α-*N*-acetylgalactosaminidase (0.01 U/20μl). Growth and purification of virus and glycohydrolase treatment were carried out as described in the legend of Fig. 1. When two different glycohydrolases were used, the second one was added 3 hr after the first one. Proteins were analyzed on a 10% polyacrylamide slab gel, blotted onto nitrocellulose, and incubated with the lectins DSA and PNA. The digoxigenin conjugated lectins were detected using an anti-digoxigenin antibody (Boehringer Mannheim, Germany).

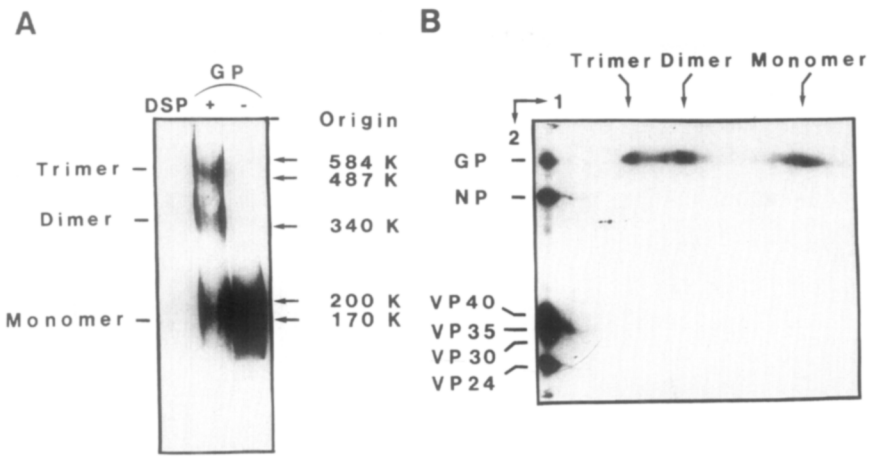

FIG. 3. Electrophoretic analysis of cross-linked GP. A. Unidimensional analysis of [^{3}H]glucosamine-labeled GP cross-linked with DSP (dithiobis(succinimidylpropionate)). Virus was grown in E6 cells, labeled, and purified as described in the legend of Fig. 1. Virus resuspended in Triton-lysis buffer was incubated with DSP in DMSO at a final concentration of 0.8 m*M* for 20 min at 15°. The excess of DSP was adsorped by adding sodium hydrogen carbonate at a final concentration of 20 m*M*. Virus was inactivated by boiling in buffer containing SDS at a final concentration of 3%, but without β-mercaptoethanol. The cross-linked samples were analyzed on a 3.5% polyacrylamide slab gel and detected by fluorography. B. Two-dimensional SDS–PAGE analysis of [^{35}S]methionine-labeled, cross-linked GP. Growth of virus, labeling, and cross-linking were performed as described in the legend of Fig. 1 and as in A above. Proteins were first electrophoresed in a slab gel containing 5% acrylamide and 0.13% bisacrylamide (horizontal direction). The gel lane was cut out, soaked in a reducing solution of 5% β-mercaptoethanol, 10 m*M* DTT, and 0.5 *M* Tris–HCl, pH 6.8, for 30 min. It was placed on top of a slab gel of 10% acrylamide and 0.26% bisacrylamide, using 1% agarose made in reducing solution to embed the gel lane, and then electrophoresed in the vertical direction (*18*).

are present, that are not susceptible to the enzyme, yet still recognized by the lectin. DSA binds specifically to *N*-glycans as demonstrated by the control experiments employing asialofetuin and fetuin. After incubation with endo-α-*N*-acetylgalactosaminidase, Marburg virus GP still binds this lectin, but is no longer able to do so after exposure to endoglycosidase F or *N*-glycopeptidase F. GP bound also weakly to *Galantus nivalis* agglutinin (GNA) which recognizes terminal mannose linked to mannose in *N*-glycans. Treatment with endoglycosidase H abolished reactivity with GNA (data not shown) confirming the presence of high mannose type *N*-glycans on GP.

The subunit organization of GP in the viral membrane was analyzed in cross-linking studies using the homobifunctional reagent dithiobis(succinimidylpropionate) (DSP) which is susceptible to cleavage by reducing agents such as β-mercaptoethanol. Purified virus was incubated in Triton lysis buffer (1 × MNT (10 m*M* [*N*-morpholino]ethanesulfonic acid), 1 m*M* phenylmethylsulfonyl fluoride, 10 m*M* idodoacetamide, 1% Triton X-100, 1:20 Trasylole, 10 m*M* EDTA) with DSP at a final concentration of 0.8 m*M* for 20 min at 15° and analyzed by electrophoresis on 3.5% polyacrylamide slot gels. Figure 3A shows an autoradiogram of [^{3}H]glucosamine-labeled virus analyzed under nonreducing conditions. Whereas in the absence of DSP, only a single GP band was present, two additional bands of lower electrophoretic mobilities were detected, when cross-linking took place. By comparison with cross-linked phosphorylase b (Sigma) and α_2-macroglobulin from horse plasma (Boehringer, Mannheim) as markers, the three bands have been estimated to be 170, 330, and 550 kDa in size, suggesting that they represent GP monomers, dimers, and trimers, respectively. In addition, the DSP-cross-linked, [^{35}S]methionine-labeled complexes of high molecular weight were analyzed by two-dimensional SDS-PAGE. Separation in the first dimension was performed under nonreduced conditions and in the second one under reduced conditions as described in the legend to Fig. 3B. Fig. 3B demonstrates that monomeric, dimeric, and trimeric forms of GP were free of other proteins. Thus GP complexed only with itself, but not with other cellular or viral gene products.

To further analyze the composition of the GP complexes, purified [^{35}S]methionine-labeled virus was subjected to solubilization by nonionic detergent and sedimentation on sucrose density gradients, and the polypeptides present in the fractions obtained from the gradients were assayed by polyacrylamide gel electrophoresis under denaturing and reducing conditions. Figure 4A shows the sedimentation profile of GP monomers (fractions 13–17) that were obtained by treating the virus with β-mercaptoethanol (5%, 10 min, 96°). Most of the other viral proteins were insoluble after this treatment and were removed by lowspeed sedimentation, prior to gradient centrifugation. GP complexes

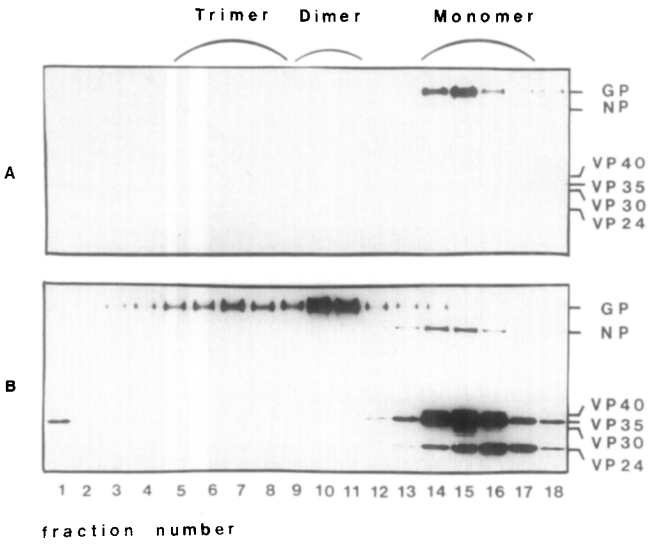

FIG. 4. Sucrose density gradient centrifugation of non-cross-linked and cross-linked GP. Virus was grown, purified, and [^{35}S]methionine labeled as described in the legend of Fig. 1. Cross-linking was performed as described in the legend of Fig. 3. The samples were applied onto a preformed 5 to 30% (w/v) sucrose gradient in MNT and 1% Triton X-100 and centrifuged in a SW 41 rotor at 40,000 rpm for 16 hr at 4°. Fractions were collected from the bottom of the tube, TCA precipitated, and analyzed by electrophoresis on a 10% polyacrylamide slab gel after boiling in buffer containing SDS and β-mercaptoethanol at final concentrations of 3 and 5%, respectively. A. Virus non-cross-linked and treated with β-mercaptoethanol (5%, 10 min, 96°), prior to gradient centrifugation. B. Virus cross-linked and not treated with β-mercaptoethanol.

were obtained under nonreducing conditions, and their sedimentation profile is shown in Fig. 4B. The sedimentation coefficients of monomeric and trimeric GP were approximately 5 and 11 S, respectively, relative to trimeric influenza virus (FPV) hemagglutinin (8.8 S). GP trimers have thus a higher sedimentation coefficient than hemagglutinin trimers, and this is in agreement with the higher molecular weight of GP. Figure 4B shows also that the other viral proteins cosedimented under nonreducing conditions with GP monomers, and that fractions 5–11 containing GP complexes were free of other viral proteins.

The data presented here indicate that GP of Marburg virus is present as a homotrimer in the viral envelope. GP resembles therefore in its quaternary structure the hemagglutinin of influenza virus and the G protein of VSV which form also trimers. Our data show also that GP contains not only *N*-glycans of the complex and the oligomannosidic type, but also carbohydrate side chains in *O*-glycosidic linkages. The latter ones contain the core unit galactose-β(1–3)-*N*-acetylgalactosamine and therefore belong to the *O*-glycans of the mucin type. Whereas *N*-glycans are very common with enveloped viruses, O-linked carbohydrate side chains have been detected so far only in a few viral glycoproteins. These are, on the one hand, the E1 glycoprotein of bovine and murine coronaviruses that contain only *O*-glycans (8–10), and, on the other hand, herpes virus glycoproteins (11–13), vaccinia virus hemagglutinin (14), and the G-protein of respiratory syncytial virus (15, 16) that contain both N- and O-linked side chains, like Marburg virus GP.

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