Intracellular Transport and Processing of the Marburg Virus

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The surface protein (GP) of Marburg virus (MBG) is synthesized as a 90-kDa precursor protein which is cotranslationally modified by the addition of high-mannose sugars (140 kDa). This step is followed by the conversion of the N-linked sugars to endoglycosidase H (endo H)-resistant species and the addition of O-linked oligosaccharides leading to a mature protein of 170–200 kDa approximately 30 min after pulse labeling. The mature form of GP is efficiently transported to the plasma membrane. GP synthesized using the T7 polymerase-driven vaccinia virus expression system was transported with essentially the same kinetics as the authentic GP. However, the protein that is shown to appear 30 min after pulse labeling at the plasma membrane was slightly smaller (160 kDa) than GP incorporated into the virions (170 kDa). Using a recombinant baculovirus, GP was expressed at high levels in insect cells. Three different species could be identified: a 90-kDa unglycosylated GP localized in the cytoplasm and two 140-kDa glycosylated proteins. Characterization of the glycosylated GPs revealed that processing of the oligosaccharides of GP was less efficient in insect cells than in mammalian cells. The majority of GP remained endo H sensitive containing high-mannose type N-linked glycans, whereas only a small fraction became endo H resistant carrying processed N-glycans and O-glycans. Tunicamycin treatment of the GP-expressing cells demonstrated that N-glycosylation is essential for the transport of the MBG surface protein. © 1996 Academic Press, Inc.

INTRODUCTION

Marburg virus (MBG) and the closely related Ebola virus (EBO) make up the family of Filoviridae, which together with the Paramyxoviridae and Rhabdoviridae constitutes the order Mononegavirales. Filoviruses are highly pathogenic for humans and nonhuman primates causing a severe hemorrhagic fever with fatality rates of 30% for MBG and up to 90% in the case of EBO (for reviews see Martini and Siegert, 1971; Pattyn, 1978). The natural reservoir of filoviruses is still unidentified. The prototype of the filoviruses, MBG, was isolated in 1967 when several laboratory workers were infected after contact with imported monkeys (Martini and Siegert, 1971). MBG virions are bacillus-shaped particles (average length 680 nm, 80 nm diameter) containing seven structural proteins. Four of these are the protein components of the nucleocapsid: the nucleoprotein (NP, Sanchez *et al.,* 1992), the L protein (Mühlberger *et al.,* 1992), and the viral proteins VP35 and VP30. In analogy to the closely related EBO, VP40 and VP24 may be matrix proteins (Elliott *et al.,* 1985). The envelope of the virus is decorated by the only surface protein, GP (M_r 170 kDa), which is inserted into the viral membrane as a homotrimer (Feldmann *et al.,* 1991). The GP gene is 2844 nucleotides in length and encodes a protein of 681 amino acids (Will *et al.,* 1993). In contrast to EBO, where the virion-associated

surface protein is only expressed after mRNA editing, MBG GP is encoded by a single ORF (Volchkov *et al.,* 1996; Sanchez *et al.,* 1996). Two hydrophobic regions have been identified in GP, one at the N-terminus, the other in the C-terminal region. Amino acid sequencing revealed that the N-terminal hydrophobic region is not present in the mature protein, indicating that this region serves as signal peptide, which is cleaved in the ER (Will *et al.,* 1993). The C-terminal hydrophobic domain is probably used as membrane anchor, and the last 8 amino acids constitute the cytoplasmic tail. Two fatty acid attachment sites were identified at the boundary between membrane anchor and cytoplasmic domain (Funke *et al.,* 1995). The sequence of GP contains 19 potential N-linked glycosylation sites and several clusters of hydroxyamino acids which can serve as O-linked glycosylation sites (Will *et al.,* 1993). Oligosaccharide analysis revealed that the sugar side chains account for approximately 50% of the molecular weight. Most of the N-linked sugars belong to the complex type oligosaccharides. Furthermore, GP contains a substantial number of O-linked oligosaccharides, which make up the main part (55 mol%) of the sugar moiety (Geyer *et al.,* 1992). O-glycosylation is reported only from one other viral glycoprotein of the order Mononegavirales, the respiratory syncytial virus G protein (Gruber and Levine, 1985). Another interesting feature of GP is the unusual structure of its oligosaccharides, which completely lacked terminal sialic acids when MBG was grown in E6 cells (Geyer *et al.,* 1992). Since GP is the only membrane protein of MBG, it is

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assumed to be responsible for virus entry into host cells (Becker *et al.,* 1995) and to be the major target for the immune response of the infected organism. Will *et al.* (1993) and Bukreyev *et al.* (1993) reported that the C-terminal part of GP contains a region that shows a high degree of homology to a presumptive immunosuppressive domain observed in some retroviruses. Whether the failure to detect neutralizing antibodies against MBG can be explained by the presence of this domain remains to be seen.

So far, analysis of GP has mainly been done on virion-associated protein. In this study we have analyzed intracellular transport and processing of GP in MBG-infected E6 cells and by using vaccinia virus and baculovirus expression systems.

MATERIALS AND METHODS

Viruses and cell lines

The Musoke strain of MBG isolated 1980 in Kenya (Smith *et al.,* 1982) was grown in E6 cells, a cloned cell line of Vero cells (ATCC CRL 1586). vTF7-3 (Fuerst *et al.,* 1986) and the strain WR of vaccinia virus were propagated in HeLa cells. *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and vVL-GP (see below) were grown in a *Spodoptera frugiperda* cell line (Sf9).

Propagation of viruses and protein labeling

MBG, the recombinant vaccinia viruses, and the recombinant baculovirus were propagated as described previously (Funke *et al.,* 1995; Becker *et al.,* 1994). Labeling of MBG virions was done as follows: Cells were infected at a multiplicity of infection (m.o.i.) of 10^{-2} PFU per cell (Mühlberger *et al.,* 1992). Five days post infection (p.i.) medium was removed and cells were incubated for 16 hr with 20 μCi [^{35}S]methionine (Amersham Buchler) per milliliter of medium. Harvesting of radiolabeled virions was performed as described by Funke *et al.* (1995).

Molecular cloning and construction of recombinant viruses

The vaccinia virus-specific plasmid pTM1 (Fuerst *et al.,* 1986) was kindly provided by B. Moss. All methods for cloning, transformation of bacteria (XL1Blue, Stratagene, and TG1) were carried out according to Sambrook *et al.* (1989). For cloning of the GP gene in different vectors a recombinant pGEM3Zf(+) plasmid containing the ORF of the gene was used (Will *et al.,* 1993). The recombinant plasmid was cut with *Sal*I for cloning the gene into the *Sal*I site of the vector pTM1. The sequence of the cloned insert was verified by DNA sequencing over the entire length. The recombinant pTM1 plasmid (pTM-GP) was used for expression of GP with the vaccinia virus T7 system (Fuerst *et al.,* 1986). Cloning of the GP gene into the baculovirus-specific vector pVL1392 and construction

of the recombinant baculovirus (designated vVL-GP) were described by Funke *et al.* (1995).

Vaccinia virus transfection system

HeLa cells (2.5×10^6) were infected with vTF7-3 at an m.o.i. of 5 PFU per cell. One hour p.i. cells were transfected with 5 μg pTM-GP and/or pGem-NP (Sanchez *et al.,* 1992) using the lipofectin (Gibco BRL) precipitation technique (Felgner *et al.,* 1987). At 4–6 hr after transfection cells were used for metabolic labeling or immunofluorescence analyses.

Pulse–chase experiments

Experiments were done with 2.5×10^6 E6 cells infected with MBG or 2.5×10^6 HeLa cells infected with vTF7-3 and transfected with pTM-GP. Five-day-p.i. MBG-infected cells or 5- to 6-hr-p.i. vaccinia virus-infected cells were starved for 1 hr with methionine- and cystein-deficient Dulbecco's medium and thereafter labeled with 100 μCi [^{35}S]Promix (Amersham Buchler) for 20 min. Postlabeling chases were in the presence of methionine- and cystein-containing Dulbecco's medium. After different incubation periods medium was removed, and cells were washed with ice-cold PBS and lysed with RIPA buffer (20 mM 2-(N-morpholino)-ethanesulfonic acid (MES), 55 mM Tris, 200 mM NaCl, 10 mM EDTA, 1% (v/v) SDS (pH 7.8), 1% (v/v) Triton X-100, 5% (v/v) Trasylol (Bayer), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM iodoacetamide). Cell lysates were subsequently sonicated and incubated for 1 hr at 4° with a rabbit preimmune serum followed by 1 hr incubation with protein A–Sepharose (Sigma). Immune complexes were precipitated at 14,000 rpm at 4° for 15 min in a tabletop centrifuge, and supernatants were further incubated overnight at 4° with a 1:100 dilution (final concentration) of an anti-GP rabbit serum in RIPA buffer (kindly provided by H. Feldmann). Complexes of GP and antibody were incubated for 1 hr with 30 μl protein A–Sepharose and centrifuged at 14,000 rpm for 15 min. Pellets were washed three times with RIPA buffer, resuspended in sample buffer (20% glycerin, 6% SDS, 375 mM Tris–HCl, pH 6.8, 10% mercaptoethanol, 2.5% saturated bromphenol blue solution), heated for 5 min to 95°, and subjected to SDS–PAGE and fluorography.

Surface immunoprecipitation

Experiments were carried out essentially as described under pulse–chase experiments. The radioactive pulse was for 30 min. After different incubation periods the medium was removed, and cells were rinsed three times with ice-cold PBS supplemented with 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$ (PBS^{2+}) and thereafter incubated with 1 ml of a 1:66 dilution of the anti-GP rabbit serum in PBS^{2+} for 1 hr at 0°. Unbound antibody was removed by washing

three times with ice-cold PBS, and cells were lysed there-after with 1 ml RIPA buffer. Then lysates were incubated for 2 hr with 50 μl protein A–Sepharose, immune complexes were spun down, and pellets were stored at −20°. To precipitate intracellular GP the supernatant was incubated for 1 hr at 4° with 15 μl of the anti-GP antibody for precipitating, followed by a 2-hr incubation with protein A–Sepharose. Immune complexes were further treated as described above and finally loaded onto SDS–PAGE and detected by fluorography.

Fluorography

Radiolabeled proteins were separated on 10% SDS slab gels as described by Laemmli (1970). Thereafter, the gels were fixed in 10% acetic acid, 30% ethanol for 15 min, incubated in Enlightning (Dupont) for another 20 min, dried, and subjected to fluorography.

Immunoblot analysis

At 36 hr p.i. 1×10^6 baculovirus-infected Sf9 cells (m.o.i. of 10 PFU per cell) were washed twice with PBS, resuspended in 500 μl triton lysis buffer (20 mM MES, 55 mM Tris–HCl (pH 7.8), 200 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100, 5% (v/v) Trasylol, 1 mM PMSF, 10 mM iodoacetamide), and proteins were separated by 10% SDS–PAGE (10 μl per lane). After electrophoresis proteins were blotted onto PVDF membranes (Millipore, P-15552) by semidry blot technique. The membranes were blocked with 10% milk powder in PBS at 4° overnight, incubated for 1 hr with the first antibody [either a monoclonal antibody raised against GP, diluted 1:30 in PBS/0.1% Tween 20 (PBS/T), supplemented with 1% milk powder or a guinea pig anti-MBG antiserum, diluted 1:500], washed three times with PBS/T, and subsequently incubated for 1 hr with either an anti-mouse IgG antiserum or an anti-guinea pig IgG antiserum coupled to horseradish peroxidase (Dako) diluted 1:5000 (for ECL) or 1:500 (colorimetrically) in PBS/T supplemented with 1% milk powder. After the second incubation, blots were washed two times with PBS/T and twice with PBS, and thereafter bound antibodies were detected either colori-metrically or with ECL (Amersham Buchler).

Lectin analysis

Lectin blot. Baculovirus-infected Sf9 cells were lysed as described above, and cell lysates were separated by 10% SDS–PAGE. Thereafter, proteins were blotted onto a PVDF membrane that was blocked with a blocking reagent supplied with the Glycan Differentiation kit, (Boehringer Mannheim). Further procedures followed the supplier's prescription. The following lectins were used: *Arachis hypogaea* agglutinin (PNA), which specifically binds unsubstituted galactose β(1-3) N-acetylgalactos-amine cores of O-glycans; *Galanthus nivalis* agglutinin (GNA), which recognizes terminal mannosyl residues linked α(1-3), α(1-6), or α(1-2) to mannose; and *Datura stramonium* agglutinin (DSA), which binds galactose β(1-4) glucosamine in glycans of the complex or hybrid type.

Lectin precipitation. Baculovirus-infected Sf9 cells (1 $\times 10^6$) were washed twice with PBS, resuspended in 750 μl RIPA buffer, and sonicated. After removal of cell debris (13,000 rpm, 10 min, 4°) supernatants were incubated overnight at 4° with 50 μl of agarose-coupled GNA (Sigma). Lectin-bound proteins were precipitated at 13,000 rpm for 15 min at 4° and washed 3× with RIPA. The final pellets were resuspended in 20 μl denaturing buffer (see glycosidase treatment) and boiled for 10 min at 95°. Samples were split into 2 fractions; one was digested with endoglycosidase H (endo H) as described, and the other was left untreated. Samples were separated by SDS–PAGE, blotted onto PVDF membranes, and probed with a monoclonal antibody against GP.

Indirect immunofluorescence analysis

Vaccinia virus expression system. Monolayers of 5 $\times 10^5$ HeLa cells grown on glass coverslips (7 cm^2) were infected with vTF7-3 at an m.o.i. of 10 PFU/cell and trans-fected with two plasmids expressing GP (pTM-GP) and NP (pGem-NP; Sanchez *et al.,* 1992) as described above. Six hours after transfection the monolayer was rinsed twice with PBS. For surface staining the cells were shifted to 4° and incubated with a mixture of a mono-clonal antibody against GP (diluted 1:2 in PBS/3% BSA) and a guinea pig anti-NP antiserum (diluted 1:60 in PBS/3% BSA; Becker *et al.,* 1994) for 1 hr at 4°. Then cells were washed twice with ice-cold PBS and fixed with 3% paraformaldehyde in PBS for 15 min at RT. Subsequently cells were incubated in 100 mM glycine for 10 min at RT and then probed for 1 hr with a mixture of a Cy3-conjugated sheep anti-mouse antiserum (Sigma) and a FITC-conjugated rabbit anti-guinea pig antiserum (Dako). Both sera were diluted 1:100 in PBS/3% BSA. Finally, cells were washed and analyzed by immunofluorescence. For intracellular staining cells were permeabilized by incubating with a mixture of acetone:methanol 1:1 at −20° for 5 min. Subsequently cells were washed twice with PBS and the remaining acetone was neutralized by 100 mM glycine for 10 min at RT. Thereafter, cells were washed once with PBS and incubated with a mixture of a monoclonal antibody against GP (diluted 1:2 in PBS/3% BSA) and a guinea pig anti-NP antiserum (diluted 1:60 in PBS/3% BSA) 1 hr at RT. After the cells were washed 3× with PBS, samples were incubated with the second antisera as described above.

MBG. E6 cells (5 $\times 10^5$) were grown on glass coverslips (7 cm^2), infected with MBG, and processed 3 days p.i. as described above for vaccinia virus.

Glycosidase treatment

Vaccinia virus expression system, MBG. The last pellet of the immunoprecipitation analysis was boiled for 5 min

in 20 μl of a denaturing buffer (0.2% SDS, 1% octylgluco-side, 1% mercaptoethanol). The samples were then cen-trifuged for 5 min at 14,000 rpm and supernatants were divided into two fractions. Both were supplied with 10 μl of acetate buffer (100 mM sodium acetate (pH 7.0), 10 mM EDTA), and to one of the fractions 1 μl endo H (1 mU; Boehringer Mannheim) was added. Glycosidase cleavage proceeded overnight at 37°. Thereafter samples were mixed with an equal volume of sample buffer and loaded onto a 10% SDS–PAG. After electrophoresis gels were subjected to fluorography.

Baculovirus expression system. At 36 hr p.i. 1 $\times$ 10^6 vVL-GP-infected Sf9 cells (m.o.i. of 10 PFU per cell) were washed twice with PBS/5 mM PMSF and spun down. After resuspending the cell pellet in 200 μl denaturing buffer (see above), cell lysates were divided into 22.5-μl aliquots and supplied with 22.5 μl of acetate buffer/5 mM PMSF. Proteins were then digested with 4 μl of different glycosidases: Endo H (1 mU/μl), N-glycosidase F (PNGase F, 50 mU/μl), or endo-α-N-acetylgalactosamini-dase (O-glycosidase; 0.3 mU/μl; all enzymes were sup-plied by Boehringer Mannheim). After the incubation pe-riod (37°, overnight) samples were mixed with an equal volume of sample buffer, separated by 10% SDS–PAGE, and thereafter blotted onto PVDF membranes. The blot-ted GP was detected with a guinea pig anti-MBG antise-rum or with the lectin PNA according to the supplier.

Tunicamycin treatment

Sf9 cells (1 $\times$ 10^6) were infected with vVL-GP at an m.o.i. of 10 PFU per cell. At 24 hr p.i. 10 μg tunicamycin/ml TC100 medium without FCS was added. After 10 hr the medium was changed and tunicamycin-containing medium was added again. Three hours later, cells were lysed and proteins were separated by SDS–PAGE, blot-ted onto PVDF membranes, and analyzed by the lectin PNA or a guinea pig antiserum against MBG.

Proteinase protection assay

Sf9 cells (1 $\times$ 10^7) were infected with vVL-GP at an m.o.i of 10 PFU per cell. At 36 hr p.i. cells were washed three times with PBS, incubated for 5 min on ice, scraped into 25 ml PBS, and pelleted at 1500 rpm for 5 min. The pellet was resuspended in 1 vol of hypotonic buffer (10 mM Tris–HCl, pH 7.4, 10 mM KCl, 1.5 mM MgCl$_2$, 10 mM CaCl$_2$) and allowed to stand on ice for 2 hr. Thereafter, the swollen cells were sonicated 30 times and immediately centrifuged at 45,000 rpm at 4° for 30 min. The superna-tant was saved, and the pellet resuspended in 500 μl of the hypotonic buffer. Aliquots of the pellet (50 μl) and the supernatant were incubated for 30 min on ice with 0.5 μg proteinase K (Serva) in the presence or the absence of 1% Triton X-100. Then 16 μl of 50 mM PMSF and 70 μl of 25% trichloric acid were added and samples were incubated at 4° for 30 min before they were centrifuged

at 4° for 30 min. The pellets were washed twice with 750 μl 70% ethanol, dried, and resuspended in 100 μl sample buffer. Samples (10 μl) were loaded onto a 10% SDS–PAGE and blotted onto PVDF membranes. Membranes were analyzed by the immunoblot technique with the described anti-GP monoclonal antibody.

RESULTS

Intracellular transport of the MBG-GP in vertebrate cells

E6 cells were infected with MBG and labeled 5 days p.i. for 20 min with [^{35}S]Promix followed by chase incuba-tions of 10 to 240 min. At the indicated time cells were lysed, and GP was immunoprecipitated with a rabbit anti-GP antiserum. Immediately after the pulse a GP-specific 140-kDa polypeptide band could be detected (Fig. 1A; arrowhead). After 30 min another protein band occurred, with a molecular weight of 170–200 kDa corresponding to the virion-associated protein (arrow). During the chase the 140-kDa species disappeared coincidently with the appearance of the 170-kDa form. After digestion of immu-noprecipitated cell lysates with endo H the 140-kDa form was converted to a 90-kDa protein (Fig. 1B; arrowhead) representing the unglycosylated form of GP (Will *et al.*, 1993), whereas the 170-kDa protein was resistant. These results indicated that the 170-kDa protein is the mature form of GP and the 140-kDa species is a precursor with high-mannose oligosaccharides.

For further investigations GP was expressed in HeLa cells with the vaccinia virus T7 expression system and pulse-labeled in the same manner as described for MBG-infected E6 cells (Figs. 1C and 1D). As in MBG-infected cells, the high-mannose form of GP (140 kDa, see arrow-head) appeared immediately after the pulse and was subsequently converted into the mature form (see arrow). In addition, a protein was detected migrating at approxi-mately 90 kDa corresponding to the unglycosylated form of GP. After endo H digestion of the high-mannose form of GP the resulting protein comigrated with the unglyco-sylated form (Fig. 1D, arrowhead). The estimated $t_{\frac{1}{2}}$ for gaining endo H resistance was approximately the same as that detected in MBG-infected cells (90 min). The ma-ture form (arrow) of the vaccinia virus-expressed GP was smaller (160 kDa) than the authentic GP (170 kDa). How-ever, the unglycosylated species (90 kDa, arrowheads in Figs. 1B and 1D) of both GPs and the endo H-sensitive form had exactly the same size (140 kDa, arrowheads in Figs. 1A and 1C), indicating that the difference in size is probably caused by an altered glycosylation pattern in the late Golgi apparatus or the trans Golgi network (TGN).

Indirect immunofluorescence analyses were carried out to analyze whether GP is presented at the cell sur-face. MBG-infected E6 cells were reacted with a mono-clonal anti-GP antibody and a guinea pig anti-NP antise-
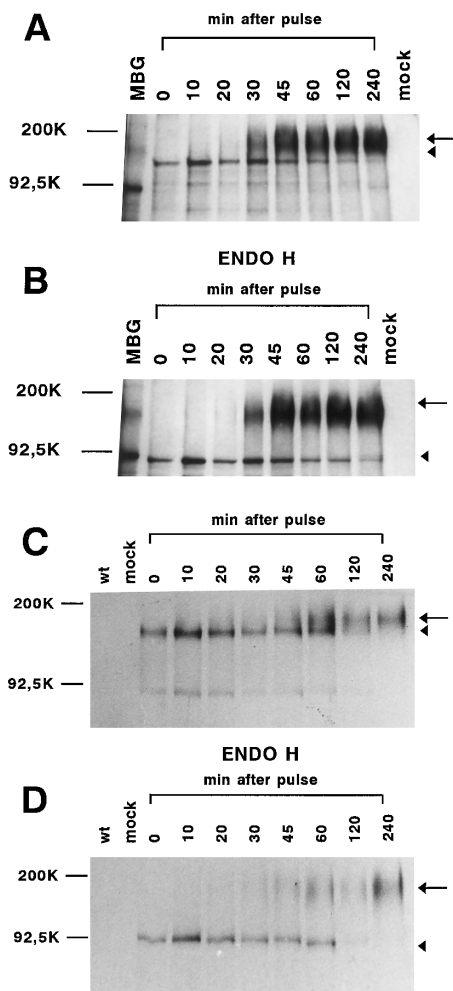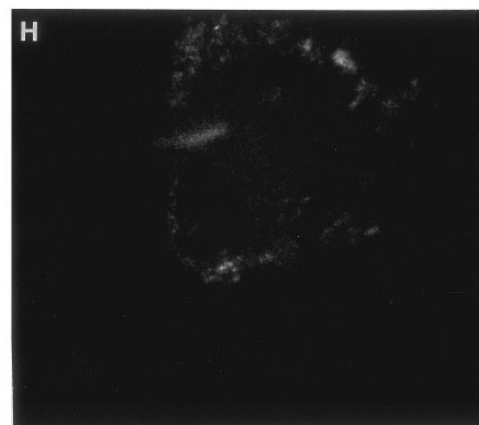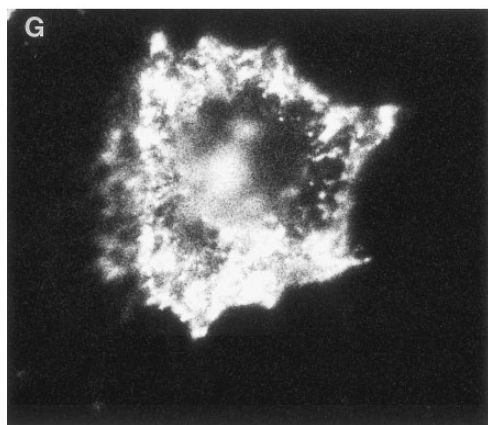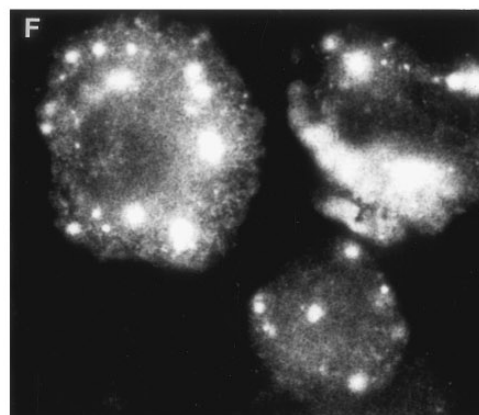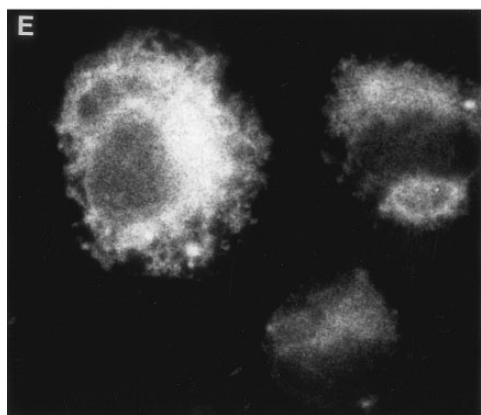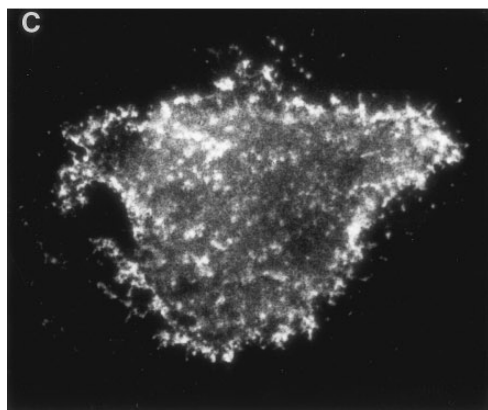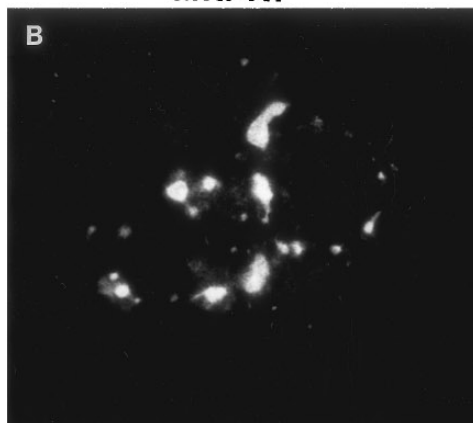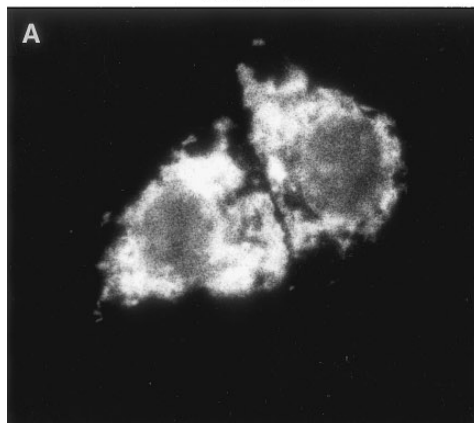
FIG. 1. Pulse–chase analysis of GP. (A and B) MBG-infected E6 cells were labeled with [³⁵S]Promix for 20 min at 5 days p.i. and then chased for various times as indicated. Labeled proteins were immunoprecipitated using a guinea pig anti-GP antiserum. Samples were analyzed by SDS–PAGE followed by fluorography. (A) Untreated proteins. Indicated by an arrow is the mature form of GP; the arrowhead marks the endo H-sensitive form of GP. (B) Proteins digested with endo H. Arrow marks the mature form of GP; arrowhead marks the unglycosylated form of GP. (C and D) vTF7-3-infected HeLa cells were labeled with [³⁵S]Promix for 20 min at 6 hr p.i. and then chased for various times as indicated. Labeled proteins were immunoprecipitated using a guinea pig anti-GP antiserum. Samples were analyzed by SDS–PAGE followed by fluorography. (C) Untreated proteins. Indicated by an arrow is the mature form of GP; the arrowhead marks the endo H-sensitive form of GP. (D) Proteins digested with endo H. The arrow marks the mature form of GP, the arrowhead the unglycosylated form of GP.

rum under native conditions (before fixing, without permeabilization, at 4°). For control, infected cells were permeabilized by acetone treatment and stained with the same antibodies. Staining of NP, which is located strictly intracellularly, serves to indicate the integrity of the plasma membrane. As can be seen in Fig. 2 both NP and GP gave a signal in permeabilized cells (Figs. 2A and 2B), whereas under native conditions only GP could be detected (Figs. 2C and 2D), reflecting its surface local-

ization. The same immunofluorescence experiments were performed with recombinant GP expressed with the vaccinia virus T7 expression system. In Figs. 2E–2H, HeLa cells were infected with vTF7-3 and then doubly transfected with plasmids encoding the GP and the NP gene under the control of the T7 promoter. Immunofluorescence data revealed that GP and NP were both expressed in the same cells (Figs. 2E and 2F), but only GP could be detected at the surface (Figs. 2G and 2H). vTF7-3-infected cells without transfection and mock-infected cells demonstrated no significant staining (data not shown). These findings suggest that the vaccinia virus-expressed GP, like the authentic GP, is transported to the plasma membrane. The time course of surface presentation of GP is shown in Fig. 3. HeLa cells infected with vTF7-3 and transfected with a recombinant pTM1 plasmid encoding GP (pTM-GP) were subjected to pulse–chase labeling. GP localized at the surface was identified by incubation of intact cells with anti-GP antibody for 1 hr at 0° and by binding of the GP–antibody complexes to protein A–Sepharose following cell lysis. After removal of surface-bound GP, intracellular GP was immunoprecipitated from cell lysates. Whereas the high-mannose form of GP was detected immediately, the intracellular mature (endo H-resistant) form of GP was detected only 30 min after the pulse (Fig. 3A). GP arrived at the cell surface almost simultaneously (Fig. 3B).

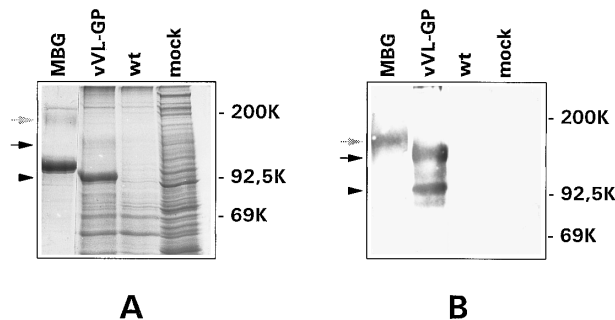## Expression of GP in insect cells using a baculovirus vector

The lepidopteran cell line Sf9 was infected with the recombinant baculovirus vVL-GP containing the ORF of the GP gene of MBG. At 36 hr p.i. cells were harvested, and expression of GP was detected by Coomassie blue staining (Fig. 4A) and by Western blot analysis using a monoclonal antibody against GP (Fig. 4B). GP was expressed in two forms in Sf9 cells: a 140-kDa (black arrow) and a 90-kDa species (arrowhead). Western blot analysis revealed that the two species were present in almost equal amounts, whereas in the Coomassie blue-stained gel the 140-kDa form was underrepresented. Since glycosylated proteins are known to be poorly stained by Coomassie blue, these observations suggest that the 140-kDa GP might represent the glycosylated and the 90-kDa GP the nonglycosylated protein. This view is supported by the findings that the unglycosylated in vitro translation product of GP gene is a 90-kDa protein (Will et al., 1993) and that only the 140-kDa form reacts with lectins (see below). Large quantities of GP were synthesized in insect cells, which, in contrast to the vaccinia virus system, could even be detected by Coomassie blue staining. Serial dilutions of cell lysates analyzed by Western blot revealed that the amount of GP expressed with the baculovirus system exceeds the amount of vaccinia virus-expressed GP by approximately 300 times (not

anti-GP   **anti-NP**
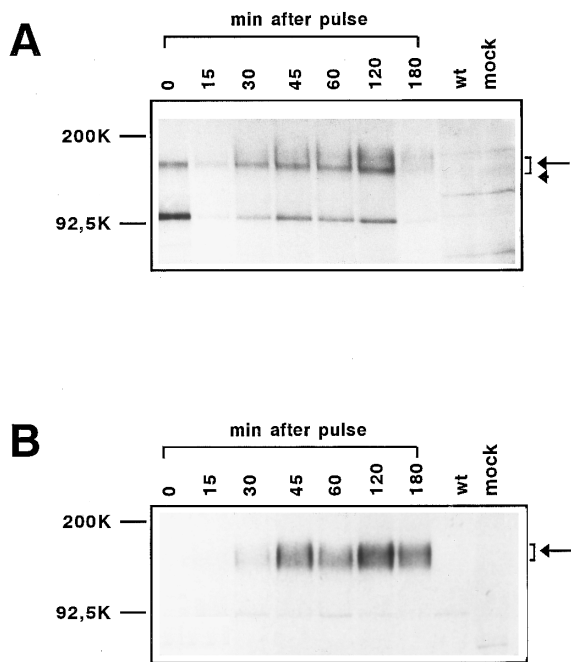
FIG. 3. Surface immunoprecipitation of GP in HeLa cells. HeLa cells were infected with vTF7-3 and subsequently transfected with pTM-GP. Cells were labeled with [^{35}S]Promix for 30 min at 6 hr p.i. and then chased for various times as indicated. Labeled proteins were immunoprecipitated using a guinea pig anti-GP antiserum. Samples were analyzed by SDS–PAGE followed by fluorography. (A) Intracellular GP. Indicated by an arrow is the mature form of GP, the arrowhead marks the endo H-sensitive form of GP. The band migrating at 90 kDa represented a nonglycosylated form of GP. (B) Surface GP. The arrow marks the mature form of GP.

FIG. 4. Expression of GP in insect cells. Sf9 cells were infected with vVL-GP or AcMNPV (wt) or not infected (mock). At 36 hr p.i. cells were lysed, and proteins were separated by SDS–PAGE. (A) Coomassie blue staining. (B) Western blot probed with a monoclonal antibody against GP. The position of MBG GP is marked by an dashed arrow, the position of the glycosylated GP expressed in Sf9 cells by an black arrow, and the position of the unglycosylated form of GP expressed in Sf9 cells by an arrowhead. MBG, MBG structural proteins.

shown). The 90-kDa form of GP could only be detected in insect cells, where the expression level was significantly higher than in mammalian cells. Thus, it was of interest to find out whether the occurrence of nonglycosylated GP was due to exhausted glycosylation machinery or to insufficient translocation into the ER. In the first case, the nonglycosylated GP would be translocated into the ER without further processing by glycosyltransferases. In the second case, GP would be localized in the cytosol. To discriminate between these possibilities, a proteinase protection assay in combination with a solubility analysis was performed on lysates of the vVL-GP-infected Sf9 cells. Incubation with proteinase K in the presence or the absence of Triton X-100 was used to discriminate between cytosolic and ER-sequestered proteins (Fig. 5). After centrifugation of cell lysates, only the nonglycosylated form of GP was found in the supernatant. The glycosylated form and a small fraction of the 90-kDa form

remained in the pellet. In the presence of Triton X-100, the 90-kDa protein was digested by proteinase K in the pellet fraction as well as in the supernatant. Glycosylated GP, however, was protected. This experiment shows that, unlike glycosylated GP, the nonglycosylated form is not translocated into the ER.

GP expressed in Sf9 cells is significantly smaller than the authentic GP present in virions (Fig. 4, dashed arrow) reflecting different glycosylation pathways in insect and mammalian cells. To further analyze carbohydrate processing of GP in insect cells, vVL-GP-infected Sf9 cells were lysed 36 hr p.i., and cell lysates were digested with different glycosidases followed by Western blot analysis and detection with a guinea pig anti-MBG antiserum or with different lectins (Fig. 6). The recombinant GP was recognized by PNA (specific for the core unit of O-glycans; Fig. 6B) and GNA (specific for high-mannose type oligosaccharides; Fig. 6C), suggesting that the protein contained O-linked oligosaccharides and high-mannose type N-glycans. DSA, which recognizes oligosaccharides of the complex type and is known to detect the authentic GP (Feldmann et al., 1991), failed to bind to GP expressed in insect cells (data not shown). After treatment with endo H, which cleaves high-mannose type glycans, GP detected by the specific antibody migrated at approximately 90 kDa, whereas treatment with PNGase F led to a 93-kDa product (Fig. 6A; GP is marked by arrowheads). Since PNGase F removes all N-glycans and endo H only high-mannose type N-glycans, it was surprising that the endo H-digested GP was smaller than that treated with

FIG. 2. Surface transport of GP. E6 cells were infected with MBG or HeLa cells were infected with vTF7-3 and subsequently transfected with pTM-GP and pGem-NP. Cells were stained with a monoclonal anti-GP antibody and with a guinea pig anti-NP serum. Bound antibody was detected with a rabbit anti-mouse IgG coupled to Cy3 and a rabbit anti-guinea pig IgG coupled to FITC. A, C, E, and G show the Cy3-emitted fluorescence (GP-specific signal), and B, D, F, and H show the FITC-emitted fluorescence (NP-specific signal). (A and B) MBG-infected E6 cells, permeabilized; (C and D) MBG-infected E6 cells, surface; (E and F) vTF7-3-infected HeLa cells, transfected with pTM-GP and pGem-NP, permeabilized; (G and H) vTF7-3-infected HeLa cells, transfected with pTM-GP and pGem-NP, surface.
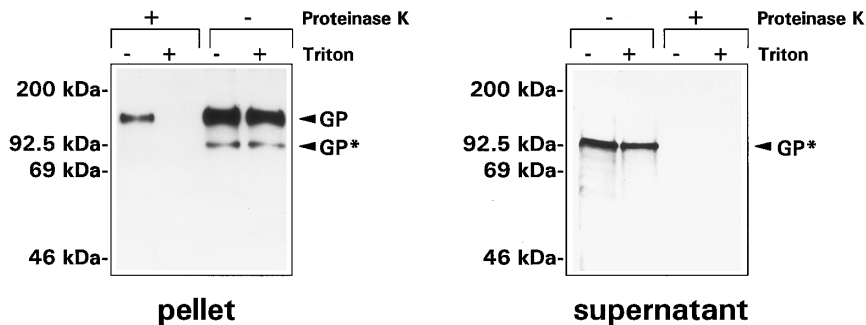
FIG. 5. Proteinase protection assay of GP expressed in Sf9 cells. Sf9 cells were infected with vVL-GP and harvested 36 hr p.i. After incubation in a hypotonic buffer cells were sonicated and centrifuged. Pellet and supernatant were separated and treated with proteinase K in the presence or the absence of Triton X-100. The unglycosylated GP is marked by an asterisk.

PNGase F. One possible explanation for this phenomenon is that PNGase F is more sensitive to sterical hindrance than endo H (Long *et al.,* 1991). The O-glycosidase-cleaved GP had approximately the same size as
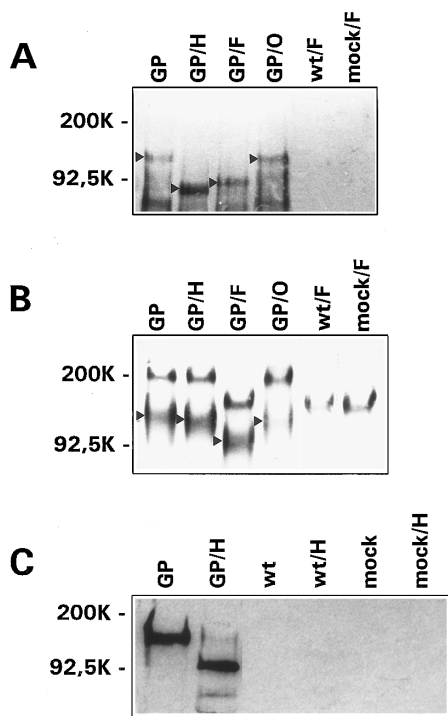

FIG. 6. Oligosaccharide analysis of GP expressed in Sf9 cells. (A and B) Sf9 cells were infected with vVL-GP (GP) or AcMNPV (wt) or not infected (mock). At 36 hr p.i. cells were lysed and subjected to glycosidase treatment. Digested samples were separated by SDS–PAGE and blotted onto PVDF membranes. (A) Staining of proteins with a guinea pig anti-MBG antiserum. The prominent GP band is marked by arrowheads. (B) Staining of proteins with the lectin PNA. GP recognized by PNA is marked by arrowheads. (C) Sf9 cells were infected with vVL-GP (GP) or AcMNPV (wt) or not infected (mock). At 36 hr p.i. cells were lyzed and precipitated using the agarose-coupled lectin GNA. Thereafter, samples were subjected to endo H treatment. Digested samples were separated by SDS–PAGE, blotted onto PVDF membranes, and probed with a monoclonal antibody against GP. H, digestion with endo H; F, digestion with PNGase F; O, digestion with O-glycosidase.

the untreated protein (Fig. 6A). The smaller protein bands (<90 kDa) stained by the antiserum were GP-specific and might represent degradation products due to intracellular proteases. When the endo H-digested cell lysates were probed with PNA, the lectin recognized a protein, which was only slightly smaller than the undigested GP (GP is marked by arrowheads, Fig. 6B). PNGase F treatment resulted in a pronounced shift of the PNA-detected GP to 93 kDa. Cleavage of O-linked oligosaccharides with *O*-glycosidase did not change the size of the PNA-detected protein, but diminished the signal strength (Fig. 6B). Interestingly, PNA did not stain the above-mentioned degradation products, suggesting that the fragments recognized by the GP-specific antibody were due to degradation of unglycosylated or high-mannose type glycosylated GP. A cellular glycoprotein, which was also recognized by PNA migrates at 200 kDa and is shifted by PNGase F treatment to approximately 150 kDa (Fig. 6B). These experiments suggested the existence of two differently processed forms of GP: an abundant endo H-sensitive fraction and a minor endo H-resistant fraction, which could not be detected by the specific antiserum, probably for quantitative reasons. This form, however, was recognized by the more sensitive lectin PNA. To determine the ratio between the endo H-sensitive and -resistant species, GP was precipitated using agarose-coupled GNA. Since it is known that the virion-associated authentic GP still carries a small amount of high-mannose type oligosaccharides (Geyer *et al.,* 1992), it was expected that both species of glycosylated GP could be recognized by the lectin. GNA precipitates were digested with endo H, showing that the overwhelming part of glycosylated GP was endo H sensitive (Fig. 6C). However, the endo H-resistant form was also detectable. The results of the glycosidase digestion can be summarized as follows: (i) PNGase F digestion of GP resulted in a 93-kDa protein recognized by the GP-specific antibody and by PNA. (ii) After treatment with endo H, the antibody and the lectin PNA recognized two different protein bands: the antibody reacted with a 90-kDa protein, and PNA bound to a 140-kDa form. These results
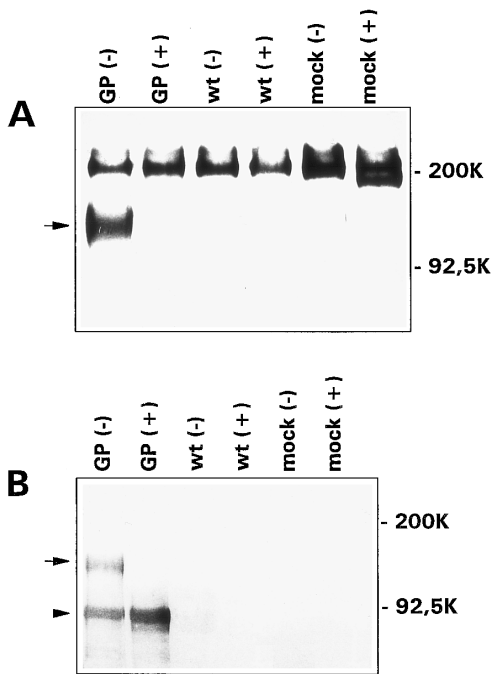
FIG. 7. Tunicamycin treatment of GP expressed in Sf9 cells. Sf9 cells were infected with vVL-GP (GP) or AcMNPV (wt) or not infected (mock). At 24 hr p.i. tunicamycin was added and cells were harvested 13 hr later. Cell lysates were subjected to SDS–PAGE and blotted onto PVDF membranes. (A) Lectin analysis with PNA. (B) Immunoblot with a guinea pig anti-MBG antiserum. The glycosylated form of GP is marked by an arrow, the unglycosylated form by an arrowhead; (+) with tunicamycin treatment; (−) without tunicamycin treatment. A cellular glycoprotein, also stained by PNA, migrates at 200 kDa.

provide evidence that the glycosylated GP exists in vVL-GP-infected insect cells in at least two different forms of similar size. One of these species, recognized by PNA and resistant to endo H, represents processed GP. The other species, which is sensitive to endo H and does not bind to PNA, is the immature GP localized either in the ER or between the ER and the medial Golgi apparatus.

To analyze the effect of N-glycosylation on the transport of GP, vVL-GP-infected Sf9 cells were treated with tunicamycin to prevent the addition of N-linked glycans (Takatsuki et al., 1975). Western blot analysis of vVL-GP-infected cells revealed that GP synthesized in the presence of tunicamycin was not detected by PNA (Fig. 7A). This observation indicates that transport of GP to the Golgi apparatus, where O-glycosylation occurs, depends on N-glycosylation. Using a GP-specific antiserum it could be demonstrated that no glycosylated form of GP is present, whereas the 90-kDa unglycosylated form of GP seemed to be accumulated in the presence of tunicamycin (Fig. 7B).

DISCUSSION

GP is the only surface protein of MBG (Feldmann et al., 1991) and is suggested to play a key role in recogniz-

ing target cells (Becker et al., 1995) and in mediating fusion of the viral envelope with cellular membranes. GP is the first viral component that comes into contact with the invaded host, and antibodies against GP should have the potential to neutralize the infection. However, neutralizing antibodies against MBG were never detected. Also, all efforts to produce a protecting vaccine against MBG have failed so far. It is therefore of interest that depletory effects of MBG on the lymphatic system of infected hosts have been reported (Ryabchikova et al., 1994) and that a stretch of 26 amino acids has significant homology to a immunosuppressive domain found in some retroviruses (Will et al., 1993; Bukreyev et al., 1993). To better understand its role as initiator of infection and as a target and a modulator of the immune response it is necessary to obtain detailed knowledge of the structure and maturation of GP. This includes elucidation of the complex events involved in GP processing. Because of the high pathogenicity of infectious virus, it is desirable to employ vectorial expression of GP in such studies. The potential of vector-expressed GP as a diagnostic tool and as a vaccine is also of considerable interest. We have therefore compared here expression and post-translational maturation of GP in (i) MBG-infected cells, (ii) HeLa cells infected with vTF7-3 and transfected with pTM-GP, and (iii) Sf9 cells infected with vVL-GP.

In vertebrate cells infected with MBG, GP molecules started to gain endo H resistance (corresponding to the arrival in the medial Golgi compartment) 30 min after synthesis. The $t_\frac{1}{2}$ for Golgi transport was approximately 90 min. These data are in line with results obtained for other viral glycoproteins showing that the $t_\frac{1}{2}$ for Golgi localization ranges between 15 and 120 min, depending on the number of attached oligosaccharides and the size of the protein (for review see Doms et al., 1993). Expression of GP with the T7 polymerase-driven vaccinia virus system resulted in a specific protein that was transported with kinetics similar to authentic GP, as indicated by an endo H resistance obtained after 45 min. GP expressed with the vaccinia virus system showed a different complex glycosylation pattern resulting in an apparent molecular weight of approximately 160 kDa compared to 170 kDa with authentic GP. It is reported for some other viral glycoproteins that expression in the vaccinia virus system leads to altered glycosylation (Rapp et al., 1994; Yaswen et al., 1993). Surface localization of GP could be detected in MBG-infected cells, suggesting that MBG budding occurs at the plasma membrane. This observation confirms the results of Schnittler et al. (1993), who showed by electron microscopic studies that virions were released at the plasma membrane. GP expressed with the vaccinia virus system was also transported to the membrane, indicating that the slightly altered complex glycosylation did not influence the transport competence of recombinant GP. In addition these experiments

show that GP is transported to the plasma membrane without the need of other viral proteins. First GP molecules arrived at the cell surface 30 min after the radioactive pulse. After 180 min, almost all pulse-labeled GP molecules were located at the plasma membrane, indicating that, despite the unusually high glycosylation of GP, its transport is neither very slow nor inefficient. The rate-limiting step of the transport of GP is processing in the ER and/or early Golgi compartments. Compared to ER–Golgi transport, Golgi–plasma membrane transport was very fast and could not be measured under the chosen experimental conditions. Endo H resistance and plasma membrane presentation occurred in our experiments simultaneously. It is well established that processing of glycoproteins in the ER and in the early Golgi (for example folding, oligomerization) are the most time-consuming steps during the transport of viral glycoproteins. Later steps proceed with higher velocity (Doms *et al.*, 1993; Earl *et al.*, 1991).

GP could be efficiently expressed in insect cells using the baculovirus system. It was shown that the recombinant GP contained N- and O-linked oligosaccharides. The expression rate was approximately 300 times higher than that of the vaccinia virus system. Three different GP species were detected in insect cells infected with the recombinant baculovirus: (i) a mature 140-kDa form with processed N-glycans and O-linked sugar side chains, (ii) a 140-kDa protein carrying high-mannose type N-glycans, and (iii) an unglycosylated 90-kDa form. The difference in size between the mature GP in insect cells and the authentic GP is due to different glycosylation in the Golgi apparatus in insect and mammalian cells. The N-glycans in insect cells are trimmed to trimannosyl cores without further elongation, resulting in endo H-resistant short sugar side chains ($Man_3GlcNAc_2$ or $Man_3(Fuc)GlcNAc_2$; Kuroda *et al.*, 1990). In addition, the O-glycosidically linked oligosaccharides are also different compared to those in mammalian cells. They consist predominantly of the monosaccharide GalNAc and in lower amounts of the disaccharide $Gal\beta$1-3GalNAc (Wathen *et al.*, 1991; Thomsen *et al.*, 1990). Lectin analysis of the GP expressed in Sf9 cells in combination with glycosidase digestion revealed that in addition to the mature GP a second premature form of GP occurred with essentially the same size as the mature protein, which was endo H sensitive, containing only high-mannose type N-glycans. This form made up the major part of the three species, indicating that most of GP was not efficiently processed. For other glycoproteins expressed with the baculovirus system it is also reported that N-linked oligosaccharides remained partially [e.g., the human immunodeficiency virus type 1 (HIV-1) gp 160, Wells and Compans, 1990] or fully (e.g., surface antigen polypeptides of hepatitis B virus, Lanford *et al.*, 1989) endo H sensitive. On the other hand some glycoproteins are known to be almost fully processed when expressed via the baculovirus system

(e.g., soluble interferon-γ receptor, Manneberg *et al.*, 1994; influenza A virus hemagglutinin, Kuroda *et al.*, 1990). It seems that insect cells process highly glycosylated proteins like MBG GP or HIV-1 gp 160 less efficiently than glycoproteins with only a few N-glycosylation sites, such as the influenza A virus hemagglutinin. However, the addition of O-glycans to some molecules of GP indicated that the protein was transported to the Golgi apparatus because O-glycosylation presumably occurs in the cis Golgi (Wilson *et al.*, 1993). The unglycosylated form of GP in insect cells was shown to be located in the cytoplasm. Hann and Walter (1991) showed that depletion of yeast cells from signal recognition particles (SRP) gave rise to unglycosylated forms of naturally glycosylated proteins that were not translocated into the ER. These data are in line with our observations that the relation between glycosylated and nonglycosylated GP was lowered when the overall expression level of GP was increased (data not shown). In this case the need of SRP for translocation of the newly made GP into the ER exceeded the available amount, leading to nonglycosylated forms of the protein.

Treatment with tunicamycin not only prevented N-glycosylation of GP but also O-glycosylation (Fig. 7B), underlining the importance of N-glycans for the transport of GP, as has been reported for other proteins expressed with the baculovirus system (Tate and Blakely, 1994; Jarvis *et al.*, 1990).

In this paper we presented data on transport and processing of GP in MBG-infected E6 cells and of recombinant GP expressed in HeLa cells. It was shown that GP expressed with the vaccinia virus system was transported in a similar way as the authentic GP, thus providing a tool for mutational analysis of structural and functional features of GP and for the production of vaccines. Using the baculovirus system GP could be expressed in large amounts, serving as a source for large-scale production of GP. However, glycosylation of the mature protein in insect cells was considerably altered, and for the most part oligosaccharides of GP remained in the high-mannose form.

ACKNOWLEDGMENTS

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