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Release of Viral Glycoproteins during Ebola Virus Infection

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Maturation and release of the Ebola virus glycoprotein GP were studied in cells infected with either Ebola or recombinant vaccinia viruses. Significant amounts of GP were found in the culture medium in nonvirion forms. The major form represented the large subunit GP_1 that was shed after release of its disulfide linkage to the smaller transmembrane subunit GP_2. The minor form were intact $GP_{1,2}$ complexes incorporated into virosomes. Vector-expressed GP formed spikes morphologically indistinguishable from spikes on virus particles, indicating that spike assembly is independent of other viral proteins. Analysis of a truncation mutant revealed an early and almost complete release of $GP_{1,2}$ molecules, showing that membrane anchoring is mediated by the carboxy-terminal hydrophobic domain of GP_2. We have also compared wild-type virus which requires transcriptional editing for synthesis of full-length GP with a variant that does not depend on editing. Both viruses released comparable amounts of GP_1, but the variant expressed only minute amounts of the small, soluble GP which is the expression product of nonedited mRNA species of the GP gene. The abundant shedding of soluble GP_1 may play an important role in the immunopathology of Ebola hemorrhagic fever in experimentally and naturally infected hosts.

INTRODUCTION

Ebola viruses (EBOV) are among the most severe human pathogens causing hemorrhagic fever, with mortality rates up to 88%. The recent reemergence of Ebola in Zaire and Gabon (1994–1997), as well as the introduction of EBOV-Reston into the United States and Italy by imported macaques from the Philippines, once again showed that these viruses have to be considered pathogens with public health impact even in nonendemic, developed countries.

EBOV subtype Zaire is the prototype of the Ebola species that together with the Marburg species constitute the family Filoviridae (Murphy *et al.,* 1995). Filovirus particles have a characteristic filamentous form. They show a uniform diameter of approximately 80 nm, but vary greatly in length (Geisbert and Jahrling, 1995). Negatively contrasted particles, regardless of serotype or host cell, contain an electron-dense central axis (19–25 nm in diameter) surrounded by an outer helical layer (45–50 nm in diameter) with cross-striations at 5-nm intervals. This central core is formed by the ribonucleoprotein complex that consists of the nonsegmented negative-stranded RNA genome and the virion structural proteins (VP) 30, VP35, nucleoprotein (NP), and polymerase (L) protein. Three other structural proteins (VP40, glycoprotein (GP), and VP24) are membrane-associated. Spikes of approximately 7 nm in diameter are formed by GP oligomers and spaced at about 5- to 10-nm intervals on the surface (Feldmann and Klenk, 1996; Peters *et al.,* 1996).

In general, filovirus proteins are encoded in single reading frames (Feldmann *et al.,* 1992; Sanchez *et al.,* 1993). The EBOV surface GP, however, is encoded in two frames (ORF I and II), and expression of GP occurs through transcriptional editing (Volchkov *et al.,* 1993, 1995; Sanchez *et al.,* 1996). During the intracellular pathway EBOV GP is extensively N- and O-glycosylated, with carbohydrates accounting for >50% of the molecular weight (Feldmann *et al.,* 1994; Volchkov *et al.,* 1995). Furthermore, maturation of GP involves processing of an endoplasmatic precursor ($preGP_{er}$) into an endo H-resistant Golgi-specific precursor (preGP). preGP is subsequently cleaved by a subtilisin-like protease, most likely furin, into the subunits GP_1 (140 kDa) and GP_2 (26 kDa) that are linked by a disulfide bond in the mature complex $GP_{1,2}$ (Volchkov *et al.,* 1998). Sequence analysis suggests that EBOV GP is a type I transmembrane glycoprotein anchored via a hydrophobic domain close to the carboxy-terminal end of GP_2 (Volchkov *et al.,* 1992, 1993). The removal of a signal peptide at the amino-terminal end and oligomerization have to be predicted for EBOV GP processing, as has been shown earlier for Marburg virus (Feldmann *et al.,* 1991; Will *et al.,* 1993). ORF I (amino-terminal) of the GP gene encodes for a small glycoprotein (sGP) that is expressed from unedited transcripts. sGP is a nonstructural protein of EBOV that is effectively secreted into culture medium (Volchkov *et al.,*

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1995; Sanchez *et al.,* 1996). Recently sGP was detected in blood of acutely infected patients (Sanchez *et al.,* 1996).

The molecular mechanisms involved in surface accumulation of $GP_{1,2}$ and in its interaction with other viral proteins in virus assembly and budding are poorly understood. The data presented here demonstrate that EBOV GP is not only incorporated into virions, but that a larger fraction is present as nonvirion molecules. This nonvirion fraction mainly consists of soluble GP_1, but small quantities of $GP_{1,2}$ complexes associated with membrane vesicles are also found. We further show that vector-expressed $GP_{1,2}$ forms spikes on virosomes morphologically indistinguishable from those on filovirus particles, indicating that spike assembly is independent of the expression of any other viral protein. Similar virosomes are also released from EBOV-infected cells.

RESULTS

As shown elsewhere, EBOV GP appears first as an endoplasmic precursor of 110 kDa ($preGP_{er}$), and then as a 160-kDa Golgi-specific precursor (preGP) which subsequently is cleaved into GP_1 and GP_2. Mature GP ($GP_{1,2}$) consists of disulfide-linked GP_1 and GP_2 (Volchkov *et al.,* 1998). In this paper we investigated the release of EBOV GP into culture medium by using a recombinant vaccinia virus (vSCGP8) or transient expression from the plasmid pGEM-mGP8 (vTF7-3 system) that both predominantly express GP without RNA editing (Volchkov *et al.,* 1995). To also study this process in EBOV-infected cells, two subtype Zaire viruses were examined, which differed in the strategy of GP expression. Strain Eckron (EBOV-7U) was used as wild-type virus expressing GP from approximately 20% of the GP-specific mRNAs that are generated by transcriptional editing (Volchkov *et al.,* 1995, 1997; Sanchez *et al.,* 1996). EBOV-8U is a plaque-purified variant of strain Mayinga carrying an insertion of a single uridine residue at the editing site (Sanchez *et al.,* 1993). This virus therefore resembles vSCGP8 in the mechanism of GP expression. To investigate the influence of the transmembrane anchor domain and/or the cytoplasmic tail on transport, maturation and release of EBOV GP we constructed a plasmid expressing an anchor-minus GP (GPΔTm) that was used for transient expression by the vTF7-3 system.

GP of Ebola virus is released into culture medium

The mature $GP_{1,2}$ complex consists of the two disulfide-linked cleavage products GP_1 (140 kDa) and GP_2 (26 kDa) (Volchkov *et al.,* 1998). In order to study the kinetics of GP processing Hela cells infected with vSCGP8 were pulse-labeled for 20 min with [^{35}S]cysteine and chased for different time intervals. Cell lysates and medium were analyzed separately for the presence of GP by quantitative immunoprecipitation using anti-EBOV immunoglobulins (Fig. 1). GP_1 was not only found in cells, but also

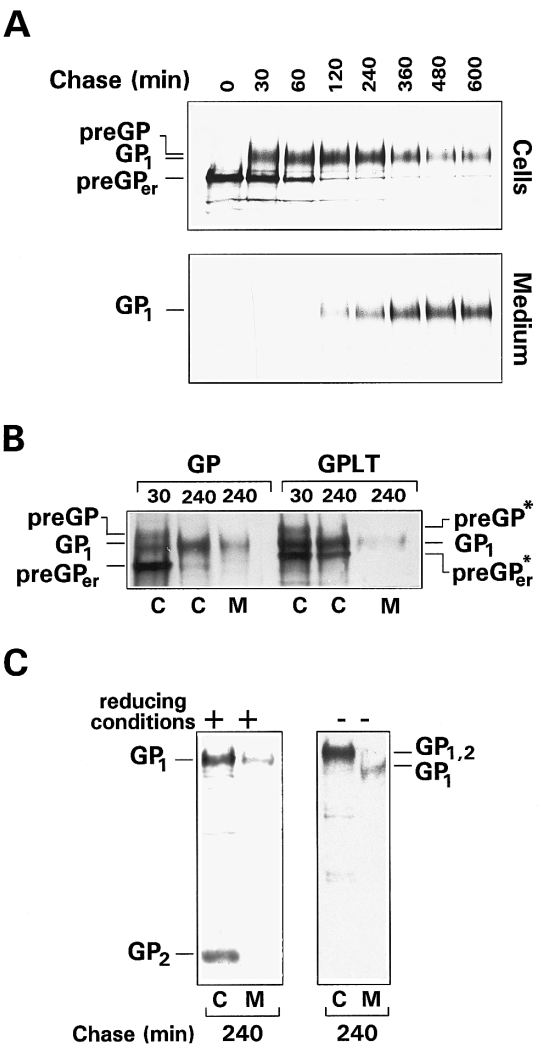

FIG. 1. Expression of recombinant GP. HeLa cells were infected with either vSCGP8 or vTF7-3 and subsequently transfected with either pGEM–mGP8 or pGEM-GPLT. Cells were labeled with [^{35}S]cysteine for 20 min at 4 h p. i. and subsequently chased for various times, as indicated. Labeled proteins from cell lysates and culture medium were immunoprecipitated using goat anti-EBOV immunoglobulins (1:100) and subjected to SDS–PAGE. (A) Pulse–chase analysis (infection with vSCGP8). Samples of cell lysates (top part) and medium (bottom part) were run on 10% SDS–PAGE under reducing conditions. (B and C) Proteolytic processing and release of GP_1 (transient expression). Samples of cell lysates and medium subjected to 8% SDS–PAGE under reducing conditions (B) and 10% SDS–PAGE under reducing and nonreducing conditions (C). Key: C, cell lysate; M, culture medium; (−) nonreducing conditions; GPLT, EBOV GP tail elongation mutant; GP, wild-type EBOV GP; (+) reducing conditions.

detected in culture medium. The kinetics of GP_1 release correlated well with the processing of intracellular GP (Fig. 1A). Following a 20-min pulse GP was first detected as the endoplasmatic precursor ($preGP_{er}$) that was converted into the Golgi-specific precursor (preGP) within 30 min of chase [Figs. 1A and 1B (left part)]. The size difference between preGP and GP_1 is more striking if the tail elongation mutant GPLT is expressed (Fig. 1B, right part). The number of intracellular cleavage products in-

creased within the first 120 min after synthesis (Fig. 1A). Over the next 360 min of chase the amount of intracellular GP_1 decreased, while extracellular GP_1 concomitantly increased. Finally, both remained at constant levels for at least 600 min postsynthesis, and no additional release occurred thereafter. Quantification (Fuji BAS 1000 Bio-Imaging Analyzer) revealed that the proportion of intra- and extracellular GP_1 changed from 86:14 after 240 min to 24:76 after 10 h of chase. Within the last 360 min postsynthesis the total amount of intracellular and released GP did not decrease, indicating that significant degradation or turnover did not take place within this period (Fig. 1A). SDS–PAGE analysis under reducing and nonreducing conditions confirmed that the released protein was predominantly GP_1, which could only be distinguished from $GP_{1,2}$ complexes under nonreducing conditions (Fig. 1C).

An anchor-minus mutant (GPΔTm) was constructed by introducing a stop codon at position 651 to investigate the functional role of the carboxy-terminal hydrophobic domain. Pulse–chase analysis demonstrated that synthesis and processing of GPΔTm was similar to that of wild type (Fig. 2A), but $GP\Delta Tm_{1,2}$ was released immediately after maturation (240 min of chase) in contrast to wild type GP (Figs. 1A and 2A). Immunoblot analysis confirmed that only molecules with mature carbohydrates were secreted (Fig. 2B, lanes 3, 4, 7, 8, 11, and 12), as has been observed for sGP earlier (Volchkov *et al.,* 1995). Released and intracellular GP_1 of both wild-type GP and GPΔTm were resistant to endo H digestion, but shifted to a decreased molecular weight after PNGase F treatment (Fig. 2B), indicating a similar maturation process for both molecules. The finding that intracellular $preGP_{er}$ migrated faster than GP_1 even after PNGase F digestion (Fig. 2B, lane 5) suggested that GP_1 contained additional O-linked oligosaccharides, as described previously (Feldmann *et al.,* 1994). Expression of GPΔTm resulted mainly in the release of $GP_{1,2}$ complexes and only a small number of soluble GP_1 and truncated GP_2 molecules, as demonstrated under nonreducing SDS–PAGE conditions (Fig. 2C, left part). This was in contrast to expression of wild-type GP, where mainly GP_1 was released into culture medium (Fig. 1C) and GP_2 remained membrane-associated (Fig. 2C, right part). These data illustrated that disulfide linkage occurred prior to proteolytic processing and that the hydrophobic domain indeed serves as a transmembrane anchor.

Released GP is present in a soluble and a membrane-associated form

To investigate the physical properties of released GP, culture medium of cells expressing recombinant GP was subjected to sucrose equilibrium gradient analysis and detergent solubility studies (Fig. 3). Released GP was detected on top of the 10–40% sucrose density gradient

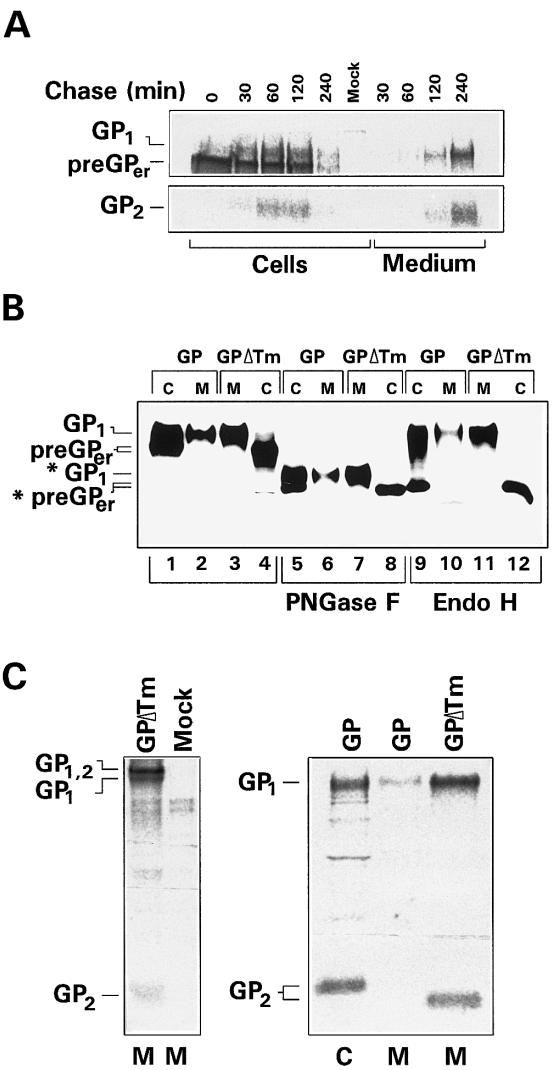

FIG. 2. Expression of anchor-minus GP (GPΔTm). HeLa cell monolayers were infected with the recombinant vaccinia virus vTF7-3 followed by transfection with pGEM-mGP8 or pGEM-GPΔTm. (A) Pulse–chase analysis of GPΔTm. Four hours posttransfection cells were labeled with [^{35}S]cysteine for 20 min and subsequently chased for various times as indicated. Labeled proteins from cell lysates and culture medium were immunoprecipitated using goat anti-EBOV immunoglobulins (1:100) and subjected to 10% SDS–PAGE. (B) Endoglycosidase digestion of GP and GPΔTm. Proteins from cell lysates (C) and culture medium (M) were treated with endo H or PNGase F and subsequently separated on 10% SDS–PAGE and blotted onto PVDF membranes. Two forms of GP (GP_1 and $preGP_{er}$) were detected intracellularly by immunoblotting using goat anti-EBOV immunoglobulins (1:3000). Only GP_1 was released into culture medium. Lanes 1, 5, and 9—lysates of cells transfected with pGEM-mGP8; lanes 2, 6, and 10—medium of cells transfected with pGEM-mGP8; lanes 3, 7, and 11—medium of cells transfected with pGEM-GPΔTm; lanes 4, 8, and 12—lysates of cells transfected with pGEM-GPΔTm. The positions of $preGP_{er}$ and GP_1 before and after* endoglycosidase treatment (PNGase F, endo H) are indicated. (C) Immunoprecipitation of GP and GPΔTm from culture medium. Labeling was performed at 4 h posttransfection with [^{35}S]cysteine for 20 min. Subsequently, cells were chased for 240 min. Culture medium was immunoprecipitated with goat anti-Ebola immunoglobulins and precipitated proteins were subjected to 10% SDS–PAGE under nonreducing (left part) and reducing (right part) conditions.

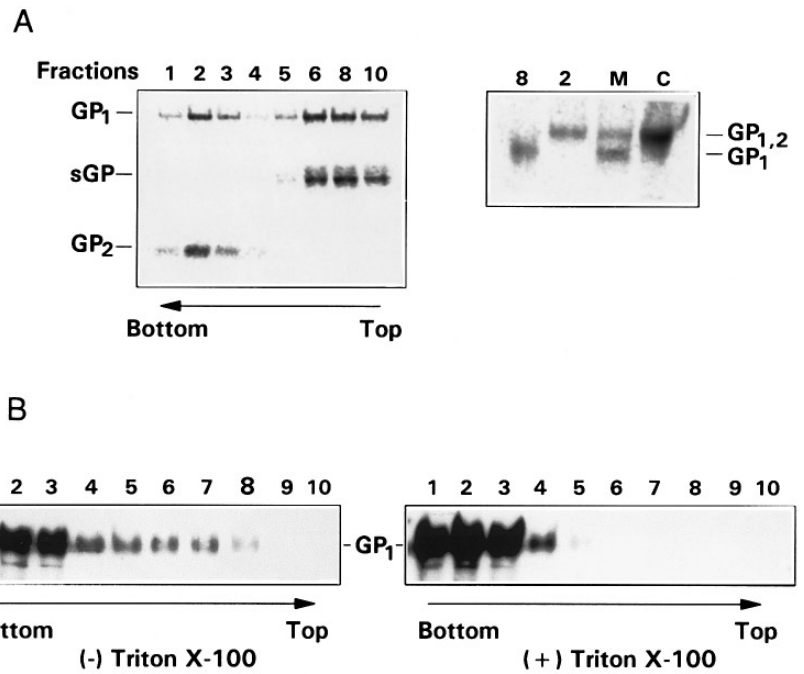

FIG. 3. Centrifugation analysis of medium obtained following recombinant expression of GP. HeLa cells were infected with vTF7-3 at an m.o.i. of 10 and transfected with pGEM-mGP8. Culture media of [^{35}S]cysteine-labeled or unlabeled cultures were clarified by low-speed centrifugation. (A) Sucrose equilibrium gradient analysis of labeled material. An aliquot of the medium was loaded onto a linear sucrose gradient (10 to 40%, w/w) and 12 fractions (1 ml) were collected from the bottom to the top following centrifugation at 36,000 rpm and 4°C for 20 h in a SW 41 rotor (Beckman). Samples were immunoprecipitated by goat anti-EBOV immunoglobulins prior to subjection to 10% SDS–PAGE under reducing (left part) or 8% SDS–PAGE under nonreducing conditions (right part). The numbers indicate the gradient fractions which are loaded on the gel from the bottom to the top. (B) Flotation analysis of unlabeled material. Aliquots of the medium were mixed with sucrose (final concentration of 50%) in the absence (left part) or the presence (right part) of Triton X-100 (1 mg/ml). Subsequently, samples were laid under a linear sucrose gradient (10–40%, w/w) with or without Triton X-100 (1 mg/ml) and subjected to centrifugation at 40,000 rpm and 4°C for 20 h in a SW60 rotor (Beckman). A total of 11 fractions (0.4 ml) were collected and detection of EBOV proteins was performed by immunoblotting using goat anti-EBOV immunoglobulins (1:3,000 dilution). The numbers indicate the gradient fractions which are loaded on the gel from the bottom to the top. Note, membrane-associated GP ($GP_{1,2}$) appears as GP_1 under reducing conditions (see designation).

(fractions 6–10) as well as in gradient fractions 1 to 3 at sucrose concentrations of approximately 30–35% (Fig. 3A). Quantitative analysis with the Fuji BAS 1000 Bio-Imaging Analyzer (Raytest, Germany) showed that the ratio between moderately and quickly sedimenting GP was approximately 10:1 (Fig. 3). Slowly sedimenting GP (fractions 6–10) represented soluble GP_1, as determined by comparison under reducing and nonreducing SDS–PAGE conditions, whereas fractions 1–3 contained $GP_{1,2}$ complexes (Fig. 3A). These complexes could be detected several hours postsynthesis and not at early stages, as shown in Fig. 1. Due to editing properties of T7 RNA polymerase (Volchkov *et al.,* 1995) sGP was detected in fractions containing slowly sedimenting GP. Flotation analysis on a 10–40% sucrose gradient revealed that the main part of the released GP stayed on the bottom of the gradient (GP_1), whereas a minor fraction floated into the gradient (Fig. 3B, left part). When culture medium was subjected to flotation analysis on a sucrose gradient containing 0.1% Triton X-100, released GP totally remained on the bottom of the gradient (Fig. 3B, right part). All these results indicate that GP is released into culture medium in two forms: as soluble GP_1 (major fraction) and as membrane-bound nonvirion $GP_{1,2}$ complexes.

GP and sGP are released during EBOV infection

In order to also investigate the release of EBOV GP during virus replication Vero-E6 cells were infected with two subtype Zaire viruses (EBOV-7U and EBOV-8U) that differed in their strategy of GP expression (see above). Culture medium was subjected to sucrose equilibrium centrifugation and analyzed for the presence of viral glycoproteins by SDS–PAGE and immunoblot analysis using goat anti-EBOV immunoglobulins (Fig. 4A). With both viruses synthesis and release of GP was comparable. Similar to recombinant GP, significant amounts (approximately 50%) of GP were released into the culture medium in a soluble form (GP_1) (Fig. 4A, fractions 8 to 10). Other virion structural proteins were found mainly in the gradient fractions containing intact virus particles (Fig. 4, fractions 1 to 3) or prior to ultracentrifugation of culture medium (Fig. 4B), indicating a specific release mechanism rather than cytolysis for the appearance of extracellular GP_1 during EBOV infection. As shown in Fig.

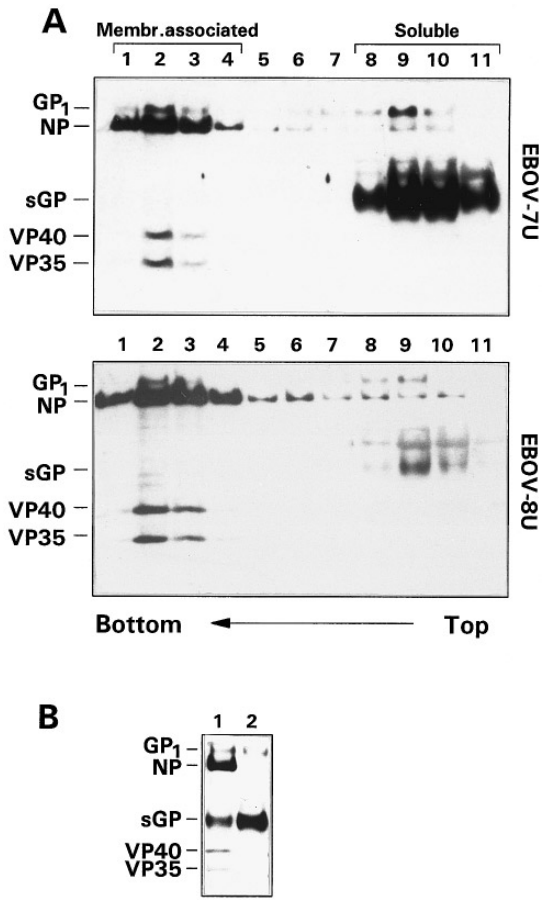

FIG. 4. Release of nonvirion glycoproteins from EBOV-infected cells. Vero-E6 cells were infected with EBOV-7U and EBOV-8U with an m.o.i. of 10^{-2}. Culture medium was harvested 5 days postinfection. (A) Sucrose equilibrium gradient analysis of culture medium obtained following infection with EBOV-7U (top part) and EBOV-8U (bottom part). Aliquots of the culture media were separated on linear sucrose gradients (10 to 40%, w/w) and 12 fractions (1 ml) were collected from the bottom to the top of the gradient following centrifugation at 36,000 rpm and 4°C for 20 h in a SW41 rotor (Beckman). Proteins were analyzed following 10% SDS–PAGE by immunoblotting using goat anti-EBOV immunoglobulins (1:3000). The numbers indicate the gradient fractions which are loaded on the gel from the bottom to the top. (B) Analysis of culture medium prior to or after clearance of viral particles by ultracentrifugation. Ultracentrifugation was performed in a Beckman SW28 rotor at 20,000 rpm for 2 h at 4°C. Proteins were subjected to 10% SDS–PAGE under reducing conditions and blotted onto PVDF membranes, and viral proteins were detected with goat anti-Ebola immunoglobulins (1:3000). Lane, 1, culture medium of EBOV-7U-infected cells; lane 2, culture medium of EBOV-7U-infected cells after ultracentrifugation. *Note.* two times the material was applied in lane 2).

4A, EBOV-7U and EBOV-8U drastically differed in the amount of sGP expression. Whereas infection with EBOV-7U led to a very efficient expression and release of sGP, only trace amounts of sGP were synthesized and secreted during EBOV-8U infection (Fig. 4A).

Vector expressed GP forms spikes

Membrane-associated GP isolated by flotation analysis from culture medium of vSCGP8-infected cells (Fig. 3B) was examined by electron microscopy after negative staining with potassium phosphotungstate. In fractions containing floated GP (Fig. 3B, left part; lanes 7 and 8) virosomes were detected. These particles varied in form and diameter, but showed uniform spike structures on their surfaces (Figs. 5A–5C). The spikes could not be distinguished morphologically from those on characteristic filamentous EBOV particles, as shown in Fig. 5F. Immunogold labeling with goat anti-EBOV immunoglobulins confirmed that the spikes on these particles obtained after vSCGP8 infection were formed by GP (Figs. 5D and 5E). This result clearly demonstrated that vector-expressed GP formed spikes on membrane surfaces and that no other viral proteins were needed in this maturation process. Similar vesicles were also found during EBOV infection (Fig. 5G), when gradient fractions with lower densities (Fig. 4A, fractions 3 and 4) were compared with fractions containing virus particles (Fig. 4, fractions 1 to 3).

DISCUSSION

The release of structural proteins in nonvirion form has been observed before with other viruses, and the following different mechanisms have been described: (i) Release as a result of cell lysis during viral infection. This has been demonstrated for soluble VSV proteins that account for about 3% of total viral proteins in an infected culture (Kang and Prevec, 1971; Little and Huang, 1978). (ii) Release of viral structural glycoproteins as a result of proteolytic cleavage by cellular proteases. This has been demonstrated for the soluble G protein of VSV (Irving and Gosh, 1982; Chen and Huang, 1986) and rabies virus (Dietzschold *et al.,* 1983; Morimoto *et al.,* 1993), soluble HA of measles virus (Malvoisin and Wild, 1994), soluble G protein of RSV (Hendricks *et al.,* 1987), HA_1 subunit of influenza A hemagglutinin (HA) (Roberts *et al.,* 1993), and GP120 of HIV (Veronese *et al.,* 1985). (iii) Budding of virus-like particles with incorporated viral proteins. This has been described for surface antigen of the hepatitis B virus (Moriarty *et al.,* 1981), the E proteins of a number of flaviviruses (Mason *et al.,* 1991; Yamshchikov and Compans, 1993; Allison *et al.,* 1995), envelope proteins of lentiviruses (Kräusslich *et al.,* 1993; Yamshchikov *et al.,* 1995), the small envelope protein (E) of coronavirus (Vennema *et al.,* 1996), and for recombinant VSV G protein expressed using the Semliki Forest virus (SFV) system (Rolls *et al.,* 1994, 1996). In addition, viral glycoproteins are also released after removal of the transmembrane anchor region by genetic engineering. This has been shown for rubella virus glycoprotein (Seto *et al.,* 1995), influenza virus hemagglutinin (Gething and Sambrook, 1982), and VSV glycoprotein G (Rose and Bergmann, 1982).

After infection with EBOV-7U and EBOV-8U, mature $GP_{1,2}$ was incorporated into virus particles, and GP_1 was

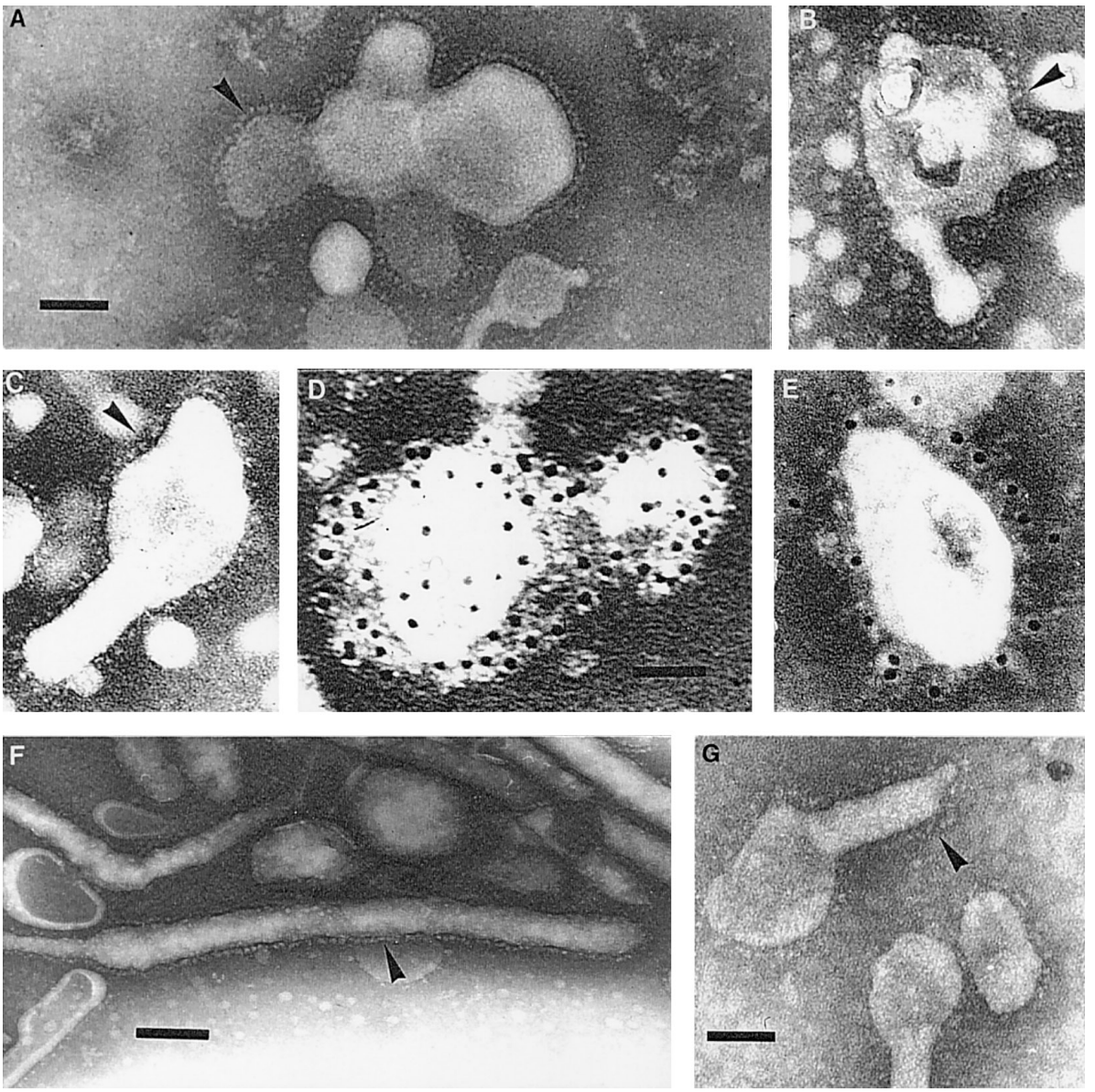

FIG. 5. Electron micrographs of virosomes. Different fractions of the sucrose equilibrium gradient or flotation analyses of EBOV-7U- or vSCGP8-infected cells were analyzed by electron microscopy after potassium phosphotungstate staining. For immunogold staining fractions were treated with goat anti-EBOV immunoglobulins (1:100) followed by a rabbit anti-goat serum conjugated with gold particles (10 nm) prior to potassium phosphotungstate staining. Spike structures on virosomes and virus particles are indicated by black arrowheads. (A to C) Fractions containing virosomes (vSCGP8 infection). Bar in A, 80 nm; B and C are identical in magnification with A. (D and E) Immunogold staining of virosomes (vSCGP8 infection). Bar in D, 50 nm; E is identical in magnification with D. (F and G) Fractions containing EBOV particles and virosomes, respectively (EBOV-7U infection). Bar in F, 170 nm; bar in G, 80 nm.

extensively released in a soluble, nonvirion form (Fig. 4). The observation that other viral proteins were not present in the medium in soluble form indicated that the release of GP_1 was not due to destruction of infected cells or viral particles. The extracellular appearance of GP_1 was also demonstrated in cells expressing recombinant GP (Figs. 1–3). As indicated by the earliest appearance of GP_1 in the culture medium, GP processing and transport took approximately 90–120 min (Fig. 1A). Similar maturation kinetics have been observed with several other viral glycoproteins (Doms *et al.,* 1993).

Expression of an anchor-minus mutant of EBOV GP (Fig. 2) showed a processing pathway similar to that observed with wild-type GP, but an earlier and almost complete release of soluble disulfide-linked $GP_{1,2}$ molecules in contrast to soluble GP_1 for wild type. These data indicate that (1) the proposed carboxy-terminal hydrophobic domain functions as the membrane anchor of GP, although additional hydrophobic domains are present on the molecule (Volchkov *et al.,* 1992), and (2) essential signals for processing and transport are not located in the transmembrane anchor and cytoplasmic domain of GP.

Our data show that GP_1 is released from the complex with GP_2 during processing (Fig. 1). A similar mechanism had been postulated for the shedding of the HA_1 subunit of the influenza HA (Roberts *et al.,* 1993) and of GP120 of HIV (Veronese *et al.,* 1985). Since GP120 and GP41 of HIV are not linked by disulfide bonds, release of GP120 may occur spontaneously. In the case of influenza virus an acid-induced conformational change of the cleaved HA in the trans-Golgi network and/or in transport vesicles destined for the plasma membrane may make the disulfide linkage between HA_1 and HA_2 susceptible to reduction (Roberts *et al.,* 1993). Since $GP_{1,2}$ also contains a disulfide linkage that is formed prior to proteolytic cleavage, as demonstrated by the analysis of the anchor-minus mutant (Fig. 2), such a mechanism is also suggested for the release of GP_1. As an alternative mechanism, disulfide linkage may not be always completed between the GP_1 and GP_2 domains of $preGP_{er}$ during early processing of GP. Thus, proteolytic processing in the Golgi of these molecules would lead to a release of GP_1 molecules.

A minor fraction of nonvirion GP ($GP_{1,2}$) was membrane-associated and released as spikes on virosomes (Fig. 5). The spikes of these vesicles were morphologically indistinguishable from virion spikes and vesicles appeared either after vector expression of GP or after virus infection (Fig. 5). We cannot exclude the possibility that virosomes result from a cytopathic effect caused by EBOV or vaccinia viruses in culture rather than a specific mechanism triggered by GP expression. Morphological studies on EBOV-infected animals, however, demonstrated areas of cellular membrane proliferation. These areas seemed not to be directly involved in virus budding (Riabchikova *et al.,* 1993; Geisbert and Jahrling, 1995), and further studies have to clarify if they could be associated with a specific release of virosomes. The biological function of released nonvirion membrane-associated GP is unknown and may be irrelevant due to limited quantities. However, the release of virosomes from plasma membranes of vSCGP8-infected cells clearly demonstrated that spike formation is mediated by GP expression alone and independent of the expression of other viral proteins.

The abundant release of GP_1 and secretion of sGP suggest biological functions which have not yet been determined. During filovirus infection in humans and animals lymphoid depletion and necrosis of lymphoid tissue including destruction of noninfected macrophages and other antigen-presenting cells (APCs) have been described. Furthermore, a lack of cellular immune response against virus-infected cells has been reported (Ryabchikova *et al.,* 1996; Zaki and Peters, 1997). These observations may implicate that macrophages or other APCs are lysed by $CD4^+$-bearing T-cells after presenting processed GP_1 and sGP in context with MHC class II antigens. The destruction of these cells might be responsible for the insufficient cellular and humoral immune responses observed in the course of EBOV infection. This concept is supported by the observations that uninfected cells exposing processed soluble viral glycoproteins of HIV (Siliciano *et al.,* 1988), VSV (Browning *et al.,* 1990), and influenza virus (Morrison *et al.,* 1986, 1988) are efficiently lysed by virus-specific $CD4^+$ T-lymphocytes (MHC class II-restricted). Class II MHC antigen is mainly expressed on the surface of professional APCs including B lymphocytes and can also be found after activation on other cells, such as endothelial cells. The lysis of GP_1- and sGP-exposed endothelial cells by $CD4^+$-bearing T-cells may in addition to destruction by virus replication (Schnittler *et al.,* 1993) further contribute to the bleeding symptoms observed in patients. Released GP_1 and secreted sGP may also interfere with humoral defense mechanisms. Specific antibodies may bind to these molecules and therefore be no longer available for virus elimination.

If nonvirion glycoproteins are determinants of viral pathogenicity during EBOV infection, this role seems to be more related to shedding of GP_1 than to sGP secretion. This is supported by the following observations: (i) EBOV-7U (wild type) and EBOV-8U (variant) that display high pathogenicity in animal models, such as guinea pigs and monkeys (Chepurnov *et al.,* 1995; Ryabchikova *et al.,* 1996), produce comparable amounts of released GP_1, but EBOV-8U expresses only minute amounts of sGP (Fig. 4). (ii) Subtype Reston EBOV produces high levels of sGP (Sanchez *et al.,* 1996), but is less pathogenic for man and some monkeys than the African EBOV subtypes (Peters *et al.,* 1994; Fisher-Hoch *et al.,* 1992). (iii) Marburg hemorrhagic fever is a comparable disease in human and nonhuman primates, but there is no evidence for sGP expression during infections with Marburg virus (Will *et al.,* 1993; Bukreyev *et al.,* 1995; Feldmann and Klenk, 1996; Becker *et al*., 1996).

Finally, released GP_1 and sGP may interfere with cellular receptors and, therefore, reduce the efficiency of Ebola virus replication. This does not seem to hold true for infections in humans and experimental animals, but may play a role in establishing a persistent infection in the yet unknown natural host.

MATERIALS AND METHODS

History and propagation of viruses

The EBOV strain Eckron, subtype Zaire, was received from the Institute Voor Tropische Geeneskunde, Antwerp, Belgium. This virus strain, as EBOV strain Mayinga, was isolated from a case of the 1976 outbreak of Ebola hemorrhagic fever in Zaire (S. R. Pattyn, personal communication) and represents a wild-type strain (EBOV-7U) with an editing site of seven consecutive uridine residues (GenBank Accession No. U81161; Volchkov *et al.,* 1997). The EBOV strain Mayinga, subtype Zaire, was

received from the Special Pathogens Branch, Centers for Disease Control and Prevention (Atlanta, GA). This virus represents a variant with eight consecutive uridine residues at the editing site (EBOV-8U) (Sanchez *et al.,* 1993, 1996). EBOV were cultured in Vero cells, clone E6 (ATCC CRL 1586), as described earlier (Feldmann *et al.,* 1991). The recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase (vTF7-3) was kindly provided by B. Moss, National Institute of Health (Bethesda, MD) and propagated in Vero-E6 cells. The recombinant vaccinia virus expressing the EBOV GP (vSCGP8) (Volchkov *et al.,* 1995) was propagated on CV-1 cells. Vero-E6, CV-1, and HeLa cells were maintained in Dulbecco's medium containing 10% fetal calf serum (FCS) (GIBCO, Germany).

Expression of recombinant GP

Construction of the recombinant vaccinia virus expressing EBOV GP (vSCGP8) was described previously (Volchkov *et al.,* 1995). This construct contains eight consecutive uridine residues at the editing site (genomic RNA sense) and thus expresses full-length virion GP from a single large ORF. The anchor-minus mutant of GP (GPΔTm) was made by introduction of a point mutation into the native GP sequence of the plasmid pGEM-mGP8 (Volchkov *et al.,* 1995) using a PCR technique and four synthetic oligonucleotides. Briefly, in a first round of PCR two overlapping GP fragments were synthesized using N1/N2 and N3/N4 pairs of oligonucleotides [N1 (5′-GAAGGATCCTGTGGGGCAACAACACAATG; mRNA sense, nucleotides 114 to 142) and N2 (5′-GCCGGTATCTATTGTCTCCATCCTGTC; complementary to nucleotides 2099 to 2073 mRNA sense), N3 (5′-GACAGGATGGAGACAATAGATACCGGC; mRNA sense, nucleotides 2073 to 2099), and N4 (5′-AAAAAGCTTCTTTCCCTTGTCACTAAA, complementary to nucleotides 2492 to 2466 mRNA sense); numbering according to the EBOV GP gene sequence, GenBank Accession No. U31033]. Oligonucleotides N2 and N3 were complementary to each other in their sequences and carried mutations (TGG to TAG, underlined) resulting in a stop codon of the ORF at a position that encoded the amino-terminal end of the proposed transmembrane anchor (position 651). In a second step, full-length mutated GP was synthesized via PCR using oligonucleotides N1 and N4 on a mixture of the previously amplified DNA fragments. First- and second-step PCR were performed using the Expand High-Fidelity System (Boehringer Mannheim, Germany). Oligonucleotides N1 and N4 were synthesized with 5′-terminal *Bam*HI and *Hin*dIII sites, respectively, to facilitate cloning into the plasmid vector pGEM3Zf. The plasmid pGEM-GPLT is a derivative of plasmid pGEM-mGP8 (Volchkov *et al.,* 1995) with an insertion of a single nucleotide at the stop codon leading to a frameshift and thus expression of a GP tail elongation mutant (GPLT) with 36 additional amino acids at the carboxy-terminal end. All mutated clones were verified by sequence analysis using a cycle sequencing technique based on the dideoxy chain-termination method.

For transient expression of GP, GPΔTm, and GPLT, 1×10^6 HeLa cells were infected with vTF7-3 at a m.o.i. of 10 PFU/cell. One hour postinfection (p. i.), the inoculum was replaced by the transfection medium containing 3 μg recombinant plasmids and 5 μl lipofectin (BRL, Germany). At 16–20 h posttransfection, medium and cells were harvested separately and analyzed for the presence of GP. To analyze comparable amounts of intracellular and released GP, medium was concentrated 10 times using Centricon-50/Centriprep-50 according to the instructions of the supplier (Amicon Inc., U.S.A.).

Pulse–chase experiments and immunoprecipitation

HeLa cells (3×10^6) were infected with either vSCGP8 or vTF7-3 (transient expression) at a m.o.i. of 10 PFU/cell. For transient expresssion, the inoculum was replaced after 1 h by transfection medium containing recombinant plasmid DNA (3 μg; 5 μl lipofectin (BRL). Cells were washed 4 h p. i., starved in methionine/cysteine-free medium for 1 h, and labeled with 100 μCi/ml [^{35}S]cysteine (Amersham, Germany). Following a pulse of 20 min, medium was replaced by medium containing an excess of unlabeled cysteine (chase). Cells were treated with lysis buffer (1% Nonidet P-40, 0.4% sodium deoxycholate, 0.5% BSA, 5 mM EDTA, 100 mM NaCl, 20 mM Tris–HCl, pH 7.6, 25 mM iodacetamide, 1 mM PMSF) at 4°C and culture medium was collected separately. To minimize nonspecific precipitations cell lysate and medium samples were cleared by centrifugation at 4°C for 15 min at 14,000 rpm in an Eppendorf centrifuge and subsequently preadsorbed with 50 μl of a 50% (vol/vol) suspension of protein A–Sepharose (Sigma, Germany) in lysis buffer for 1 h at 4°C, followed by centrifugation as indicated above. Following preadsorption on HeLa cells for 1 h at 4°C, goat anti-EBOV immunoglobulins were incubated overnight at 4°C with the resulting supernatants. A rabbit anti-goat secondary antibody coupled to protein A–Sepharose was added to precipitate the immune complexes. The precipitates were washed three times with lysis buffer and TNE (1:1) and subsequently incubated in buffer containing 2% SDS for 10 min at 37°C. Prior to loading onto 8, 10, or 12% polyacrylamide gels (SDS–PAGE) samples were boiled with or without 5% β-mercaptoethanol. Precipitated proteins were visualized by fluorography or quantified with the Fuji BAS 1000 Bio-Imaging Analyzer (Raytest, Germany).

Sucrose equilibrium gradient and flotation analyses of proteins

Vero E6 cells were infected with EBOV at 10^{-2} PFU/cell for 1 h and culture medium was collected 5 days postinfection. HeLa cells were infected with vTF7-3 and subsequently transfected with plasmid DNA as described under

"Pulse–chase experiments and immunoprecipitation," and culture medium was collected 10 h postlabeling. Cell culture medium was clarified by low-speed centrifugation (1500 *g,* 10 min 4°C). A fraction of 6 ml was subjected to centrifugation (Beckmann SW41 rotor, 20 h, 36,000 rpm, 4°C) through a linear 10–40% (w/w) sucrose gradient prepared with PBS buffer. Subsequently, factions of 1 ml were collected from the bottom of the gradient and analyzed for the presence of EBOV proteins using an immunoprecipitation technique. For flotation analysis, HeLa cells were infected with vSCGP8 at a m.o.i. of 10 PFU/cell and culture medium was collected 20 h postinfection. Sucrose was added to cell culture medium at a final concentration of 50% (w/w), and half of the sample was treated with Triton X-100 (1 mg/ml). Aliquots (1 ml) of each portion were laid under a linear 10–40% sucrose gradient prepared in PBS buffer containing or lacking Triton X-100 (1 mg/ml) and subjected to centrifugation (Beckmann SW60 rotor, 20 h, 40,000 rpm, 4°C). Fractions (0.4 ml) were collected and EBOV proteins were detected by immunoblotting.

Electron microscopy and immune electron microscopy

Aliquots of different fractions of the sucrose equilibrium gradient or flotation analyses (vSCGP8-infected HeLa cells; EBOV-infected Vero-E6 cells) were fixed with PBS-buffered glutaraldehyde (1%) and paraformaldehyde (2%) for 30 min. For negative staining, samples were adsorbed to Formvar-coated nickel grids, stained with 4% potassium phosphotungstate and examined with an EM 109 (Zeiss) electron microscope. For immune electron microscopy, samples were adsorbed to Formvar-coated nickel grids and floated onto a solution of PBS containing 1% bovine serum albumin and 0.05% Tween 20 (PBST). Grids were treated with goat anti-EBOV immunoglobulins (1:100 in PBST). Controls were treated with the appropriate preserum at the same dilution. The grids were rinsed six times in PBS and floated onto a 1:50 solution of rabbit anti-goat antibody conjugated with 5-nm gold particles (Sigma, U.S.A.). After washing, samples were fixed for 2 min in 1% glutaraldehyde and negatively stained with 2% potassium phosphotungstate.

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Note added in proof. Recently new data concerning biological functions of Ebola virus glycoproteins have been presented by Takada *et al*., 1997 (*Proc. Natl. Acad. Sci. USA* 94, 14764–14769) and Yang *et al*., 1998 (*Science* 279, 1034–1036).

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