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RAPID COMMUNICATION

Proteolytic Processing of Marburg Virus Glycoprotein

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Processing of the transmembrane glycoprotein (GP) of Marburg virus involved the conversion of an endo H-sensitive, ER-specific form into an endo H-resistant, Golgi-specific precursor that was cleaved into GP_1 and GP_2. Cleavage was mediated by furin or another subtilisin-like endoprotease with similar substrate specificity as indicated by mutational analysis of the cleavage site and inhibition using peptidyl chloromethylketones. Mature GP consisted of disulfide-linked GP_1 and GP_2 subunits. © 2000 Academic Press

Key Words: Marburg virus; glycoprotein; proprotein convertase; proteolytic processing; furin.

Introduction. Marburg virus (MBGV) and Ebola virus (EBOV), the two species within the family Filoviridae, are among the most pathogenic agents causing fulminant hemorrhagic fever in humans and nonhuman primates (*10, 32*). The genomes of both filoviruses display linear arranged genes on a single negative-stranded RNA molecule that encodes the seven structural proteins in the order nucleoprotein, virion structural protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and RNA-dependent RNA polymerase (L) (*9*). In contrast to EBOV that requires transcriptional editing to express the transmembrane GP (*34, 37, 38*), the GP gene of MBGV is organized in a way that transcription results in a single subgenomic RNA species used for the synthesis of the full-length envelope glycoprotein (*43*). Thus, MBGV does not express the nonstructural small glycoprotein sGP that is synthesized from the nonedited mRNA species during EBOV infection and secreted into the culture medium (*34, 38, 40*).

MBGV and EBOV GP are trimeric type I transmembrane proteins (*5, 12, 35, 39*). They are heavily N- and O-glycosylated primarily in the variable, extremely hydrophilic middle region of the molecules (*11, 12, 17, 43*). Recently, first experimental data have been published regarding GP functions. The transmembrane GP of EBOV mediates targeting to susceptible cells (*36, 44, 46*), and the asialoglycoprotein receptor has been postulated as a receptor candidate for MBGV on hepatocytes (*2*). GP further carries a putative fusion domain as indicated by computer-assisted sequence analysis (*15*). Recently Ito *et al.* (*22*), using a vesicular stomatitis virus expression system, have demonstrated that mutations in this domain modify the ability of EBOV GP to support infectivity.

Many viral surface glycoproteins undergo posttranslational proteolytic processing. Most of these glycoproteins are cleaved by subtilisin-like eukaryotic endoproteases (proprotein convertases) at multibasic cleavage sites (*16, 26, 27*). In previous reports on MBGV GP such a cleavage process was not described (*1, 14, 43*). However, this type of processing has recently been demonstrated with EBOV GP that is cleaved at the sequence R-T-R-R_{501} into the disulfide-linked fragments GP_1 and GP_2 (*35, 39*). The present study has therefore, been focused on proteolytic processing in order to either identify such a process during maturation of MBGV GP as well or verify another distinction between the two species within the family Filoviridae.

Results and Discussion. Analysis of the amino acid sequence of MBGV GP showed the presence of two multibasic motifs, R-R-K-R_{435} and R-L-R-R_{561}, which are potential cleavage sites of furin or related proprotein convertases (Fig. 1A). In order to investigate if GP undergoes proteolytic cleavage during maturation, synthesis of GP was analyzed by pulse–chase labeling experiments using the vaccinia virus-T7 polymerase expression system (Fig. 2A). HeLa cells (3×10^6) were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3), and subsequently transfected with

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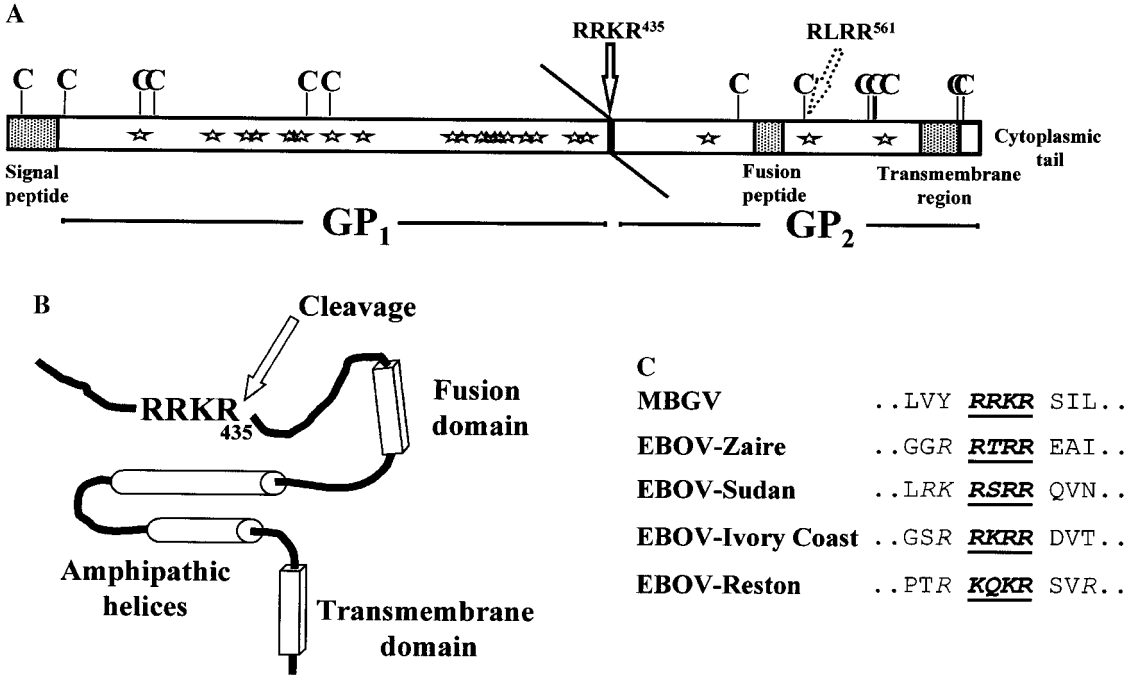

FIG. 1. Schematic illustration of Marburg virus GP. (A) Primary structure showing the co- and posttranslational modifications of $GP_{1,2}$. Potential N-linked carbohydrate side chains are indicated by asterisks (*) and cysteine residues by "C." (B) Proposed structure of GP_2. The ectodomain of GP_2 contains the fusion peptide (526–540), followed by an amino-terminal helix (553–594), a peptide loop (602–609), and a carboxy-terminal helix (613–632). Helices were proposed by the GARNIER program of PC/GENE (IntelliGenetics Inc.). The putative fusion peptide and transmembrane anchor are indicated as boxes, and two amphipathic helices as cylinders. (C) Comparison of the conserved furin-like motifs in the envelope glycoproteins of all known filoviruses.

the plasmid coding for MBGV GP, strain Musoke. SDS–PAGE analysis under reducing conditions showed that GP was processed into two GP-specific polypeptide bands, GP_1 (~160 kDa) and GP_2 (~38 kDa). GP appeared first as a common precursor of about 140 kDa ($preGP_{er}$) that had previously been identified as the endoplasmic reticulum precursor due to its endo H sensitivity (*1*). Later in chase, $preGP_{er}$ was converted into a higher molecular mass precursor preGP, which then was effectively processed into the subunits GP_1 and GP_2. It was difficult to distinguish preGP and GP_1 in pulse-chase experiments due to extensive glycosylation of both proteins and the short life period of the Golgi precursor preGP. However, increasing amounts of GP_2 observed during the chase periods clearly demonstrated the proteolytic processing. The cleavage of GP was abolished when GP was expressed in the presence of the decanoylated peptidyl chloromethylketone decRVKR-cmk (Fig. 2A), a potent inhibitor of the subtilisin-like endoprotease furin (*16*). Under these conditions predominantly noncleaved preGP was synthesized which clearly showed a higher molecular mass than GP_1. These data support the concept of proteolytic processing of MBGV GP mediated by furin or another endoprotease of similar substrate specificity. SDS–PAGE analysis under nonreducing conditions showed that GP_1 and GP_2 are complexed, most likely by a cysteine–cysteine bond, as indicated by the presence of a single band designated as $GP_{1,2}$ (Fig. 2B). As has been observed with EBOV (*40*), MBGV GP was also found in culture medium (Fig. 2C, left). Analysis under nonreducing conditions showed that the released GP represented the soluble GP_1 subunit (Fig. 2C, right). Shedding of GP_1 is most likely due to the release of the disulfide linkage to membrane-anchored GP_2 during protein processing. A similar event has also been observed with the HA_1 subunit of the influenza hemagglutinin (*33*).

Since MBGV GP is processed into two subunits, only one of two putative cleavage sites is recognized. Cleavage at position 561 seemed to be very unlikely because the molecular mass of GP_2 (~38 kDa) estimated by SDS–PAGE was significantly higher than expected for a protein consisting of 120 amino acids with only two potential N-linked glycosylation sites. In order to further support the notion that cleavage occurs at position 435 (R^{-4}-R^{-3}-K^{-2}-R^{-1}) we exchanged amino acids at position −1 (R_{435}L; R-R-K-L) and −2 (K_{434}M; R-R-M-R) using site-directed mutagenesis. Wild-type GP and mutants R_{435}L and K_{434}M were transiently expressed in HeLa cells. After pulse labeling for 20 min with [^{35}S]cysteine and chase incubation for 4 h, GP-specific proteins were immunoprecipitated with a monoclonal antibody. Substitution of Arg_{435} (R_{435}L) resulted in complete loss of cleavage, indicating that GP indeed is cleaved at R-R-K-R_{435} (Fig. 3). Mutation at position −2 (K_{434}M) showed a reduction in

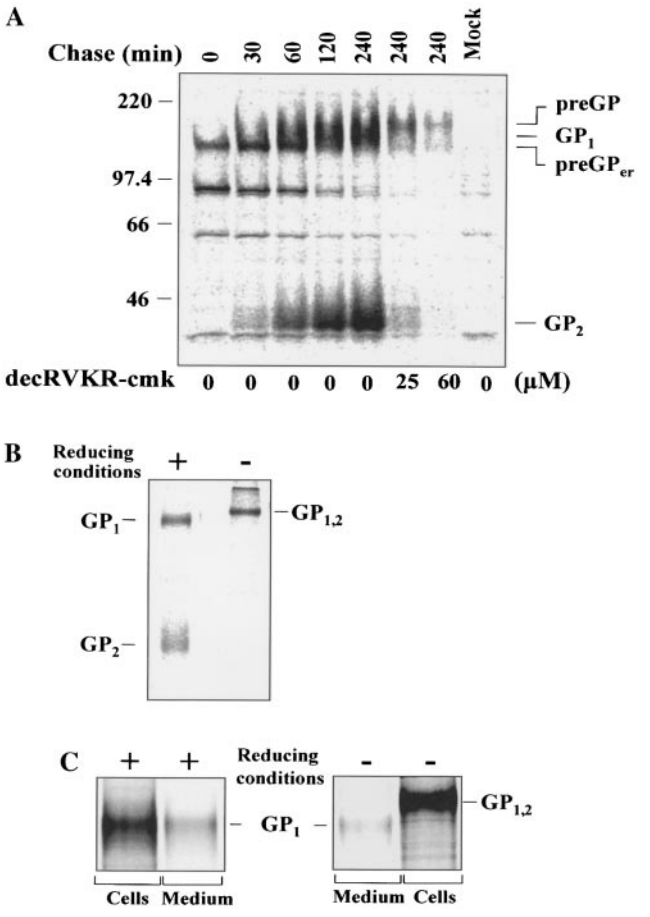

FIG. 2. Transport and proteolytic processing of Marburg virus glycoprotein GP. HeLa cells were infected with vTF7-3, and transfected with a plasmid carrying the entire coding region of MBGV GP (strain Musoke). Proteins were pulse labeled for 20 min with 100 μCi/ml [^{35}S]cysteine and chased for the indicated time intervals. GP-specific proteins were immunoprecipitated from cell lysates and culture medium with guinea pig anti-MBGV immunoglobulins and analyzed on 10% SDS–PAGE under reducing (A, B, and C) and nonreducing conditions (B and C). (B and C) HeLa cells were infected with vTF7-3, transfected with plasmid DNA, metabolically pulse labeled, and chased for 240 min. The presence of the decanoylated peptidyl chloromethylketone decRVKR (25 and 60 μM) during labeling and chase abolished cleavage of preGP.

cleavage efficiency demonstrated by the presence of uncleaved GP and both cleavage products. Thus, the cleavage site of MBGV GP shows the typical features of the consensus sequence recognized by furin or the related proprotein convertase PC5/6, which contains Arg at positions −1 and −4 as a minimal requirement and Arg/Lys at position −2 for cleavage optimization (*31*). Involvement of furin or other furin-like cellular endoproteases in the process of cleavage of EBOV GP has been recently confirmed by Wool-Lewis and Bates (*45*). However, these authors, unlike our observations, did not exclude involvement of other proteases in the processing of EBOV GP. Data obtained now with MBGV GP confirmed our previous observation made on EBOV GP (*39*) and clearly demonstrated a requirement of a furin cleavage site for proteolytic processing of filoviral GPs. This is further supported by the recently determined amino-terminal end of the EBOV transmembrane GP_2 (*35*).

It is interesting to note, that basic di- or tripeptides (R-R, R-K, K-R, R-K-R, or R-R-K) are either part of the furin-like cleavage site or located in the close vicinity in all filoviral GPs (Fig. 1C). The role of these basic paired residues in cleavage of filoviral GP is not clear yet. Theoretically, they could also be recognized by other cellular proprotein endoproteases and, therefore, be involved in cleavage of GP. Data on other viral glycoproteins, such as influenza HA and the F protein of paramyxoviruses, indicate that cleavage can occur at single basic residues (R or K) present in the cleavage sites (*24–27*). In these particular cases, proteins are only cleaved after arrival of viruses at the cell surface (*18, 19*) or even during endocytosis by yet unidentified endosomal endoproteases (*3*). The role of basic paired residues in cleavage has been well documented for the HA of avian influenza viruses and the F protein of Newcastle disease virus. Here, the differences in pathogenicity clearly correlate with the cleavability of these proteins (*25*). The GPC proteins of some arenaviruses, such as lymphocytic choriomeningitis virus, Lassa virus, and Pichinde virus, also contain dibasic sequences at the

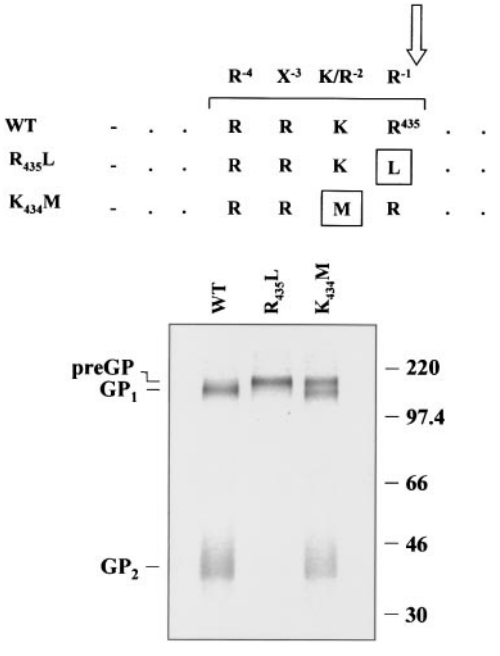

FIG. 3. Site-directed mutagenesis at the cleavage site. The mutants $R_{435}L$ and $K_{434}M$ were generated by PCR mutagenesis using the Quickchange kit (Stratagene). HeLa cells were infected with vTF7-3, transfected with plasmid DNA, metabolically pulse labeled for 30 min with 100 μCi/ml [^{35}S]cysteine, and chased for 240 min. GP-specific proteins were immunoprecipitated from cell lysates with a GP-specific monoclonal antibody (II9G4) and analyzed on 10% SDS–PAGE under reducing conditions. The sequences at the cleavage sites are shown at the top.

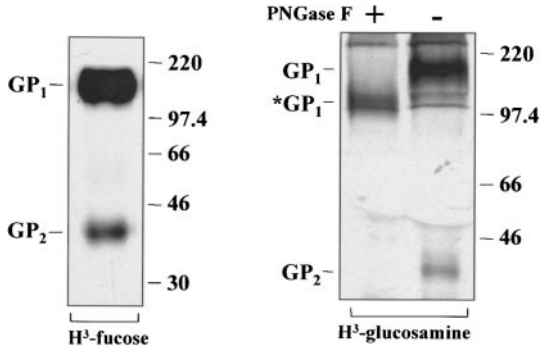

FIG. 4. Identification of the proteolytic subunits of Marburg virus glycoprotein (GP). MBGV, strain Musoke, was grown on Vero cells and labeled for 24 h with different ^{3}H sugars (100 μCi/ml) at day 5 postinfection. Virus particles were harvested and proteins were analyzed on 10% SDS–PAGE (reducing conditions) prior to or after treatment with endoglycosidase PNGase F (Boehringer Mannheim, Germany). *GP_1, GP_1 after removal of N-linked carbohydrates.

proposed proteolytic cleavage site that might be cleaved by plasmin or another serum protease recognizing such cleavage sites (*4, 26, 27*). In the case of MBGV and EBOV GP (*39*), cleavage was almost entirely abolished after mutation of the Arg at position −1 of the cleavage site, although one dibasic motif was still present. Thus, nonfurin-like cellular endoproteases do not seem to play any role in the process of proteolytic maturation of filoviral GP. For EBOV subtype Reston, in which the furin-like cleavage motif is altered at position −4 (Lys instead of Arg), a reduced cleavability of GP has recently been demonstrated (*39*). In contrast to all other EBOV, subtype Reston seems to be low or apathogenic for humans and seems to possess lower pathogenicity for nonhuman primates as well (*13*). Therefore, it is tempting to speculate that cleavability may also determine filoviral pathogenicity.

In order to verify if virion GP of MBGV also consists of two cleavage products, Vero-E6 cells were inoculated with MBGV, strain Musoke, and metabolically labeled for 24 h on day 5 postinfection prior to virus harvest. Labeling with [^{3}H]fucose or [^{3}H]glycosamine revealed, as expected, two glycoproteins, a larger one of approximately 160 kDa and a smaller, weakly labeled one of approximately 38 kDa (Fig. 4). Both proteins were sensitive to treatment with PNGase F (Fig. 4, right), indicating the presence of N-linked carbohydrates on both subunits.

In conclusion, the data presented here demonstrate that MBGV GP is proteolytically processed during its maturation by a subtilisin-like cellular endoprotease. Proteolytic cleavage is an important control mechanism of the biological activity of many viral surface proteins and occurs often next to a hydrophobic domain involved in fusion (*21, 26, 27*). GP_2 of MBGV and EBOV (*15, 28, 41*) have some structural features typical for viral fusion proteins. They include a potential fusion peptide downstream of the cleavage site and two amphipathic helices that are separated by a loop formed by a disulfide bond (Fig. 1B). Such structures have been observed with the transmembrane subunits HA_2 of the influenza virus hemagglutinin (*6, 7*), gp41 of the HIV env protein (*8, 42*), and F1 of the paramyxovirus fusion protein (*23*). Since all of these proteins require cleavage and conformational changes for fusion, it is reasonable to assume that MBGV GP also undergoes proteolytic activation. Taking into consideration a strong structural similarity between filovirus GPs and other viral fusion proteins and a high conservation of the cleavage site among all filoviruses, it is tempting to speculate that proteolytic cleavage is an important factor for the pantropism of filoviruses and their rapid spread through the organism. Recent studies using murine leukemia virions pseudotyped with EBOV GP suggested that proteolytic cleavage was not absolutely necessary for viral infectivity (*45*). However, as already mentioned by the authors, their one-step infection assay may not be sufficient to address the importance of cleavage during multiple cycles of virus replication in an infected host. Furthermore, it cannot be excluded that GP in those pseudotyped particles may have become activated during adsorption or endosomal uptake as discussed above. Further studies are needed to clarify the importance of cleavage in filoviral infectivity and pathogenicity. Recent reports describing the development of minigenome techniques for both filoviruses (*29, 30*) will allow us to establish reverse genetic systems that may shed light into the molecular mechanisms of filovirus pathogenicity in the near future.

Materials and Methods. Viruses and cell cultures. Strain Musoke of MBGV was propagated on Vero-E6 cells (ATCC CRL 1586) and purified from tissue culture medium as described previously (*12*). The recombinant vaccinia viruses vTF7-3 expressing T7 polymerase (provided by B. Moss, NIH, MD) was propagated in CV-1 cells. CV-1, HeLa, and Vero-E6 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO, Germany) containing 10% FCS (GIBCO).

Pulse–Chase Experiments. HeLa cells (3×10^6) were infected with vTF7-3 at a m.o.i. of 10 pfu/cell. The inoculum was replaced after 1 h by transfection medium containing plasmid DNA [9 μg; 15 μl lipofectin (BRL, Germany)]. Cells were washed 6 h postinfection, starved in cysteine-free medium for 1 h, and labeled with 100 μCi/ml [^{35}S]cysteine (Amersham, Germany). After a 20-min pulse, the inoculum was replaced with medium for a chase. Labeled cells were lysed immediately in Co-IP buffer [1% Nonidet P-40 (NP-40), 0.4% sodium deoxycholate, 0.5% BSA, 5 mM EDTA, 100 mM NaCl, 20 mM Tris–HCl, pH 7.6, 25 mM iodoacetamide, 1 mM PMSF] at 4°C. Immunoprecipitation was performed using guinea pig anti-MBGV immunoglobulins (kindly provided by A. A. Chepurnov, Institute of Molecular Biology, Koltsovo, Russia) or with a MBGV GP monoclonal antibody [II9G4,

kindly provided by M. Hevey and A. Schmaljohn, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD (*20*)]. Precipitated proteins were visualized by fluorography or quantified with the Fuji BAS 1000 bio-imaging analyzer (Raytest, Germany).

Metabolic Labeling. Vero-E6 cells (75 cm^2 flasks) were inoculated with MBGV, strain Musoke, at an m.o.i. of 1 pfu/cell for 1 h. Cells were washed 5 days postinfection with Dulbecco's medium, starved in glucose-free medium for 1 h, and metabolically labeled with 500 μCi/ml [^{3}H]glucosamine or [^{3}H]fucose (Amersham). The virus was harvested 24 h postlabeling and partially purified from culture medium by centrifugation through a 20% sucrose cushion (120 min at 100,000*g*). The pelleted virus was resuspended in PBS and lysed with 2% SDS, 1% NP-40, and 0.4% deoxycholate. Viral proteins were subjected to 10% SDS–PAGE under reducing conditions and visualized by fluorography.

Endoproteolytic Cleavage Inhibition Assay. For cleavage inhibition studies, HeLa cells infected with vTF7-3, and subsequently transfected with a plasmid carrying the entire coding region of MBGV GP (strain Musoke), were incubated during starvation, pulse, and chase periods either without or with the decanoylated peptidyl chloromethylketone (decRVKR-cmk) at concentrations of 25 or 60 μM. Immunoprecipitation analyses were carried out as described previously (*39*).

Site Directed Mutagenesis. Construction of the plasmid which directs synthesis of the wild-type MBGV GP was described earlier (*43*). In order to generate the cleavage site mutants of GP ($R_{435}L$ and $K_{434}M$), PCR mutagenesis was performed using the Quick-change kit (Stratagene, Germany). All clones were verified by sequence determination.

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