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Delta-Peptide Is the Carboxy-Terminal Cleavage Fragment of the Nonstructural Small Glycoprotein sGP of Ebola Virus[1]

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In the present study we have investigated processing and maturation of the nonstructural small glycoprotein (sGP) of Ebola virus. When sGP expressed from vaccinia virus vectors was analyzed by pulse-chase experiments using SDS–PAGE under reducing conditions, the mature form and two different precursors have been identified. First, the endoplasmic reticulum form sGP_{er}, full-length sGP with oligomannosidic N-glycans, was detected, sGP_{er} was then replaced by the Golgi-specific precursor pre-sGP, full-length sGP containing complex N-glycans. This precursor was finally converted by proteolysis into mature sGP and a smaller cleavage fragment, Δ-peptide. Studies employing site-directed mutagenesis revealed that sGP was cleaved at a multibasic amino acid motif at positions 321 to 324 of the open reading frame. Cleavage was blocked by RVKR-chloromethyl ketone. Uncleaved pre-sGP forms a disulfide-linked homodimer and is secreted into the culture medium in the presence of the inhibitor as efficiently as proteolytically processed sGP. *In vitro* treatment of pre-sGP by purified recombinant furin resulted in efficient cleavage, confirming the importance of this proprotein convertase for the processing and maturation of sGP. Δ-peptide is also secreted into the culture medium and therefore represents a novel nonstructural expression product of the GP gene of Ebola virus. Both cleavage fragments contain sialic acid, but only Δ-peptide is highly O-glycosylated.

Key Words: Ebola virus; glycoprotein; proprotein convertase; proteolytic processing; O-glycosylation.

INTRODUCTION

Ebola virus (EBOV) and Marburg virus, the two species within the family Filoviridae, are among the most pathogenic human viruses causing fulminant hemorrhagic fever in humans and nonhuman primates (Feldmann and Klenk, 1996). The negative-strand RNA genome of these viruses encodes seven structural proteins. Genomic RNA, together with the viral structural proteins VP30, VP35, nucleoprotein NP, and polymerase protein L, form the helical ribonucleoprotein complex. Three other proteins [envelope glycoprotein (GP), VP40, and VP24] are membrane associated (Feldmann and Kiley, 1999). In general, filoviral genes are transcribed into monocistronic subgenomic RNA species (mRNA) (Feldmann *et al.,* 1992; Sanchez *et al.,* 1993). In contrast to all other filoviral genes, including the GP gene of Marburg virus (Will *et al.,* 1993), the organization and transcription of the GP gene of EBOV is unusual and involves transcriptional RNA editing (Volchkov *et al.,* 1995; Sanchez *et al.,* 1996). GP is synthesized as a large precursor, 676 amino acids in length. GP is posttranslationally cleaved by 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.,* 1998a). The amino-terminal fragment GP_1 is not only present in spikes, but is also shed after release of the disulfide linkage (Volchkov *et al.,* 1998b). A nonstructural small glycoprotein (sGP) is synthesized from the unedited GP mRNA and extensively secreted from infected cells as a homodimer in antiparallel orientation (Sanchez *et al.,* 1998; Volchkova *et al.,* 1998). sGP shares its 295 amino-terminal amino acids with GP, but differs from GP in its 69 carboxy-terminal amino acids as a result of transcriptional editing (Volchkov *et al.,* 1995; Sanchez *et al.,* 1996). It is conceivable that sGP, which is secreted in high amounts, might serve as a decoy, binding to protective antibodies and interfering with the specific immune response to transmembrane GP exposed on viral or cellular surfaces. It has also been postulated that recombinant sGP could bind to neutrophils and may inhibit their activation (Yang *et al.,* 1998). Both proposed functions of sGP, inhibition of the inflammatory response and interference with the immune response, could be important pathomechanisms in Ebola hemorrhagic fever.

In the present study, synthesis, transport, and processing of sGP have been investigated. We have demonstrated that EBOV sGP is proteolytically cleaved at a site showing the recognition motif for subtilisin-like proteases. Analysis of intracellular processing and transport of EBOV sGP showed that it is first detected as an endoplasmic precursor (sGP_{er}), which is converted into

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an endo H–resistant precursor (pre-sGP). Subsequently, this precursor is cleaved into sGP and carboxy-terminal Δ-peptide, which are both secreted into the culture medium. EBOV Δ-peptide is a glycopeptide, which is O-glycosylated and sialylated.

RESULTS AND DISCUSSION

EBOV sGP undergoes posttranslational proteolytic cleavage

In order to better understand the sequence of sGP maturation steps, pulse-chase labeling experiments were performed. Cells infected with the recombinant vaccinia virus vSCGP7 expressing sGP (Volchkov *et al.,* 1995) were pulse-labeled for 20 min with [^{35}S]cysteine, chased for different time intervals prior to lysis, and proteins from lysed cells and culture supernatants were immunoprecipitated using goat anti-EBOV immunoglobulins. Analysis of sGP expression by SDS–PAGE under reducing conditions showed that immediately after pulse-labeling sGP appeared in a 50-kDa form (sGP_{er}) (Fig. 1) that has previously been identified as the endo H–sensitive endoplasmic reticulum precursor protein (Volchkov *et al.,* 1995). Formation of disulfide-linked dimers in the ER is a limiting step during maturation and transport of sGP (Volchkova *et al.,* 1998). As demonstrated by analysis under nonreducing conditions, a substantial part of the pulse-labeled sGP_{er} remained monomeric during the chase (Fig. 1A). sGP_{er} often appeared as a double band, which was more clearly visible under nonreducing conditions. Since this doublet was converted to a single band after digestion with endo H, it most likely reflects differences in glycosylation (Fig. 1C). Dimers of sGP_{er} first appeared after chase periods of about 20 min. After 40 min, part of dimeric sGP_{er} was converted into the Golgi-specific precursor (pre-sGP). When analyzed under reducing conditions, pre-sGP appeared as a diffuse band of approximately 60 kDa (Fig. 1B) and showed resistance to endo H treatment (Fig. 1C). After digestion with endo H, the Golgi-precursor pre-sGP could be discriminated from the slightly faster migrating sGP band that overlapped with sGP_{er} before endo H treatment. sGP is the mature form of this glycoprotein that also was found in culture medium (Figs. 1A, 1B, and 1C). The reduction in size observed after the final maturation step suggested proteolytic cleavage of pre-sGP, resulting in the removal of a short fragment which, however, was not detected with the anti-EBOV antibodies used in these experiments.

EBOV sGP is cleaved at position R-V-R-R^{324}

The deduced amino acid sequence of EBOV sGP contains the multibasic motif R-V-R-R^{324}, which is a potential cleavage site of furin or related subtilisin-like cellular endoproteases (Fig. 2). It should be noted that the furin cleavage sites of sGP and of the full-length envelope glycoprotein GP are not identical, since they are encoded by different open reading frames (Volchkov *et al.,* 1995). In order to verify that cleavage occurred at position 324, we changed the sequence R-V-R-R^{324} to R-V-R-S^{324} by site-directed mutagenesis using plasmid pGEM-mGP7 with the genomic copy of the EBOV GP gene (Volchkov *et al.,* 1995). HeLa cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) and then transfected with the plasmids coding for wild-type (wt) sGP and mutant $R_{324}S$. Culture medium was collected 18 h posttransfection, separated on a 10% SDS–polyacrylamide gel under reducing conditions, and analyzed for the presence of sGP by immunoblotting using anti-EBOV antibodies. After substitution of R^{324}, pre-sGP was no longer converted into sGP. The substitution had no effect on secretion of this molecule into the medium (Fig. 3A). These data demonstrate that sGP is cleaved at the sequence R-V-R-R^{324}. The cleavage site of sGP shows the typical features of the consensus sequence recognized by furin or the related proprotein convertase PC5/6 (Nakayama, 1997; Steiner, 1998) and is highly conserved in all subtypes of EBOV (Fig. 2). In order to obtain further information on the protease involved in sGP maturation, we carried out expression experiments in the presence of the decanoylated peptidyl chloromethyl ketone decRVKR-cmk, a potent inhibitor of the subtilisin-like endoprotease furin (Garten *et al.,* 1994). As shown in Figs. 1A and 1B, proteolytic processing was abolished under these conditions, and noncleaved sGP was secreted into the medium as efficiently as cleaved sGP.

Similar results were obtained when Vero-E6 cells infected with EBOV strain Eckron were treated with the inhibitor. Whereas no differences in virus production were noticed with or without decRVKR-cmk, the inhibitor effectively blocked cleavage of sGP (Fig. 3B). Since GP also undergoes proteolytic processing by a subtilisin-like cellular endoprotease (Volchkov *et al.,* 1998a; Volchkov, 1999) treatment with inhibitor resulted in formation of virions containing uncleaved GP.

Δ-Peptide, the carboxy-terminal cleavage fragment, is secreted into the culture medium

The data described so far indicate that proteolytic processing is the last step in sGP maturation. Anti-EBOV antibodies that were used for detection of either secreted sGP or of surface GP were not able to recognize Δ-peptide in immunoprecipitation or immunoblot experiments. To identify the putative carboxy-terminal fragment pre-sGP expressed in the presence of inhibitor was digested with furin *in vitro* (Fig. 3C). Furin treatment resulted in efficient cleavage of pre-sGP into sGP and a second cleavage fragment migrating as a diffuse band that has been designated Δ*-peptide.* Its molecular mass

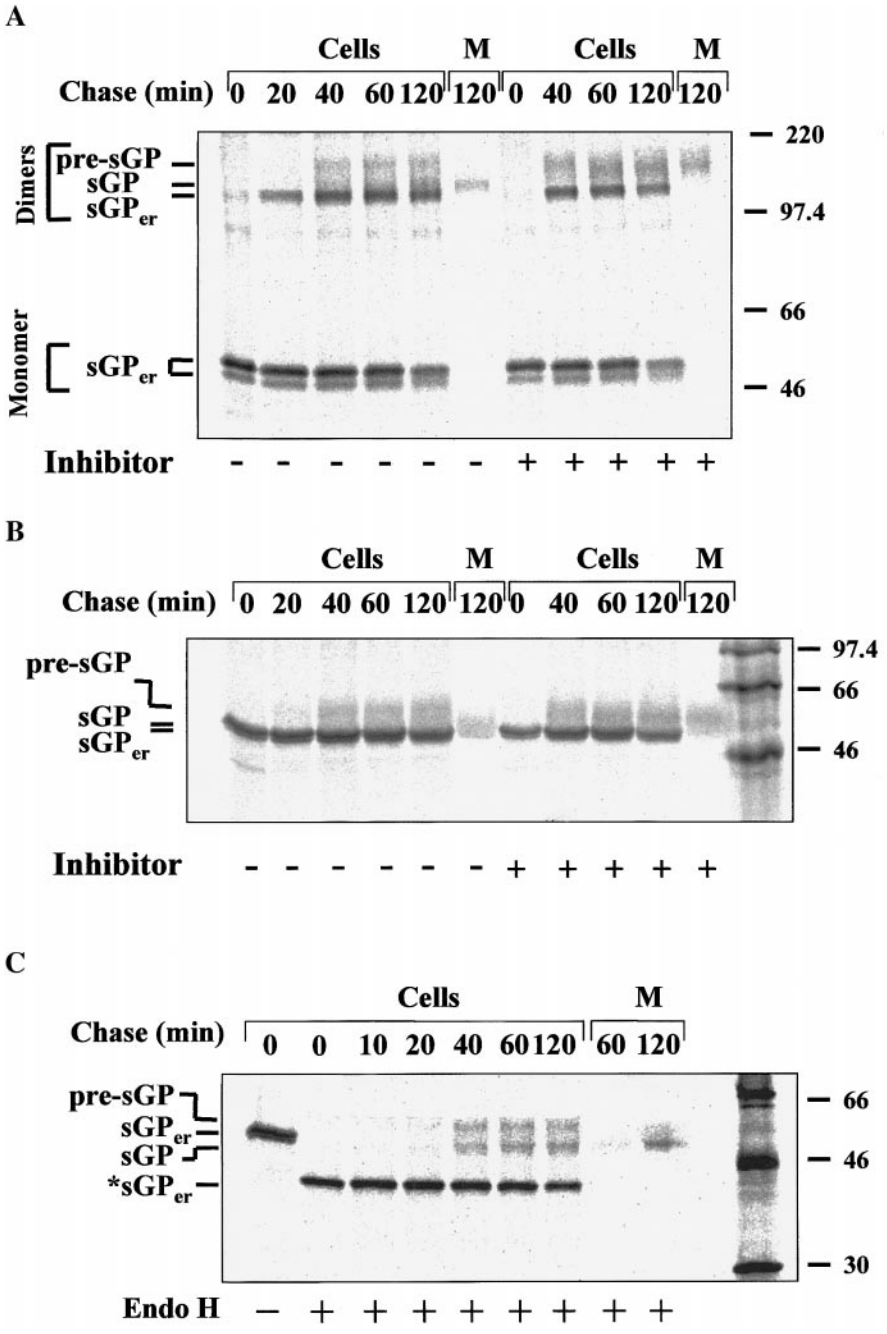

FIG. 1. Synthesis, processing, and transport of EBOV sGP. HeLa cells were infected with vSCGP7. At 4 h postinfection cells were washed, starved in methionine-cysteine-free medium for 1 h, and pulse-labeled for 20 min with 100 μCi/ml [^{35}C]cysteine (Amersham, Germany). During starvation, labeling, and subsequent chase intervals, cells were either treated with 30 μM of decRVKR-cmk or incubated without inhibitor. sGP-specific proteins were immunoprecipitated from cell lysates and culture medium using anti-EBOV immunoglobulins, treated with 2% SDS without (A) or with β-mercaptoethanol (B), analyzed by 10% SDS–PAGE, and subsequently visualized by fluorography. (C) Endoglycosidase digestion of sGP. Immunoprecipitated samples were treated with endoglycosidase endo H and analyzed on 10% polyacrylamide gel under reducing conditions as described above. The positions of sGP, endoplasmic precursor sGP_{er}, and Golgi-precursor pre-sGP are indicated. $*sGP_{er}$, endoplasmic precursor after deglycosylation with endo H.

of approximately 10–14 kDa is significantly higher than the one predicted from the amino acid sequence (~4.7 kDa), suggesting extensive posttranslational modifications present in Δ-peptide. Both cleavage fragments appeared as individual bands when analyzed either under reducing or nonreducing conditions, indicating the absence of disulfide bonds between the molecules (data not shown). These results provide further support to the concept of sGP cleavage by furin or another endoprotease of similar substrate specificity.

In order to better visualize Δ-peptide and also to identify putative posttranslational modifications, a mutant

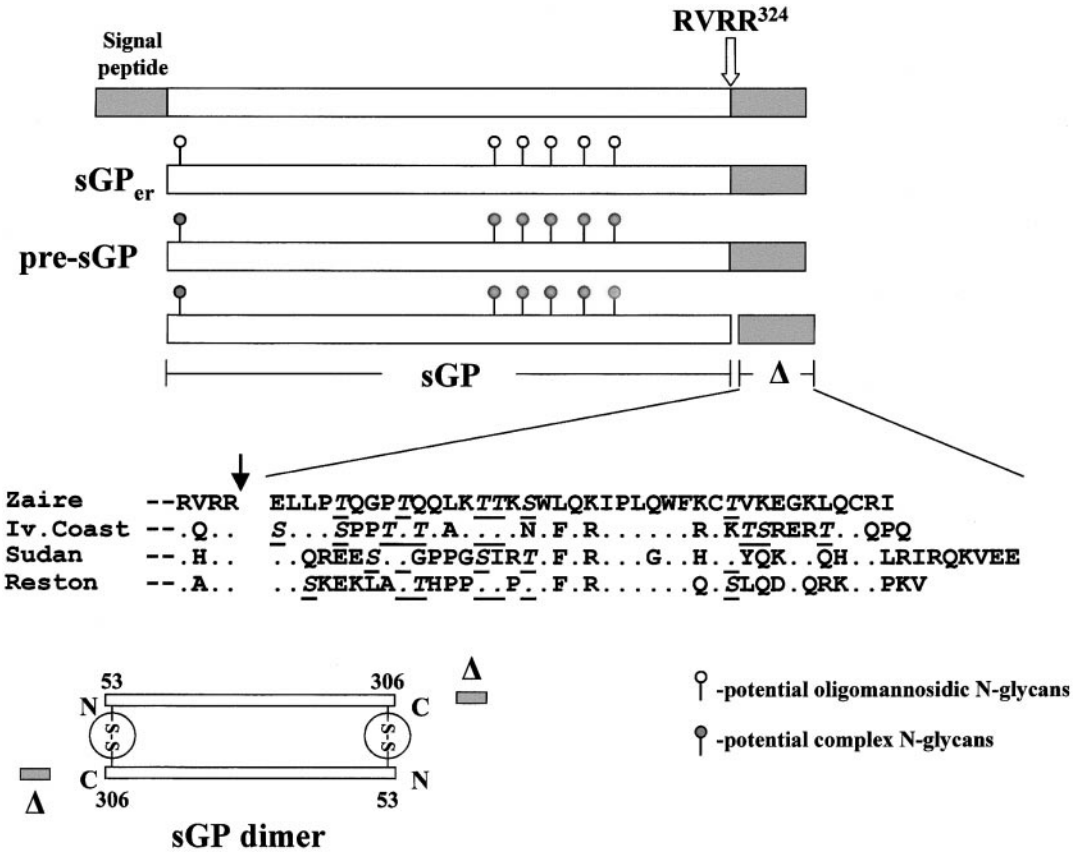

FIG. 2. Schematic representation of EBOV sGP precursors and proteolytically processed products, and amino acid sequence alignment of the Δ-peptides of four subtypes of EBOV. Signal peptide and the carboxyterminal Δ-peptide are indicated by gray boxes. Threonine and serine residues, putative sites for O-glycosylation are underlined. Cysteine residues involved in sGP oligomerization are shown as S-S. Potential N-linked glycosylation sites are indicated. The cleavage site at position 324 is indicated by an arrow.

containing a 6xHis tag at the carboxy-terminal end has been constructed. Immunoblotting and immunoprecipitation assays using monoclonal anti-His antibodies allowed to detect the Δ-peptide cleaved *in vivo* (Figs. 4A, 4B, and 4C). Both noncleaved sGP precursors (sGP_{er} and pre-sGP) as well as the carboxy-terminal Δ-peptide were found in cells (Fig. 4A). In medium, besides minor amounts of pre-sGP, secreted Δ-peptide was clearly detected. Because of the carboxy-terminal location of the 6xHis tag, mature sGP was not recognized by anti-His antibodies, but detected with anti-EBOV immunoglobulins (Fig. 4B). Only noncleaved precursor has been detected with anti-His antibodies with an sGP-His mutant containing an Arg to Ser^{324} substitution was expressed in HeLa cells. This precursor molecule showed a higher molecular mass than normally processed sGP when analyzed with anti-EBOV antibodies. In contrast to mature sGP that is secreted so efficiently that it usually could not be identified in cells using immunoblot analysis, Δ-peptide was retained in cells for longer time intervals. Whereas most of the sGP found in the culture medium is cleaved, experiments with furin inhibitor and the cleavage-site mutant demonstrated that cleavage is not required for secretion. Pulse-chase analysis of the processing of sGP-His using anti-His antibodies revealed that Δ-peptide was detected in the cell lysates shortly after appearance of the Golgi-precursor and almost disappeared at the 120-min time point due to secretion into the culture medium (Fig. 4C). It should be noted that Δ-peptide migrated as a very diffuse band when analyzed by SDS–PAGE after immunoprecipitation, but showed a sharp band in the immunoblotting experiments using anti-His antibodies.

Δ-peptide is O-glycosylated and sialylated

EBOV Δ-peptide expressed in HeLa cells showed a molecular mass that is larger than the one calculated from the amino acid content. Amino acid sequence analysis showed that Δ-peptide of EBOV subtype Zaire does not contain sites for N-linked glycosylation, but that there are five threonine and one serine residues that may serve as attachment sites for O-glycans. Moreover, comparison of Δ-peptide sequences from all known EBOV subtypes revealed that all of them contain in the amino-terminal part at least one dipeptide Pro-Thr, which is known as a favorable motif for O-glycosylation (Fig. 2). The presence of O-linked carbohydrates in the structure

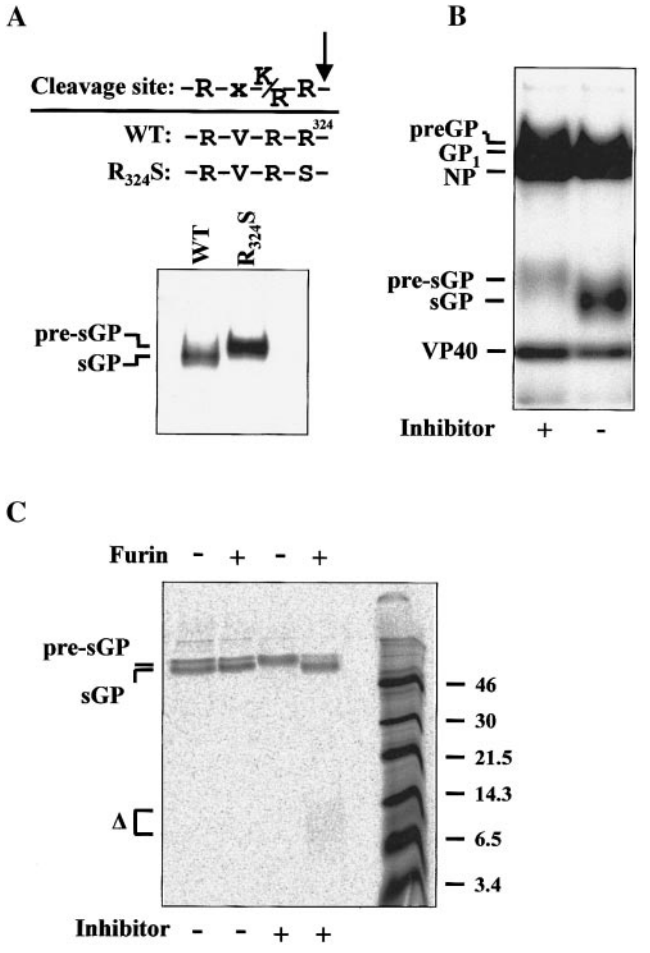

FIG. 3. EBOV sGP is cleaved at site R-V-R-R^{324}. (A) Synthesis and secretion of sGP cleavage site mutant $R_{324}S$ (R-V-R-S). HeLa cells were infected with vTF7-3 and transfected with the plasmids pGEM-mGP7 (WT) or pGEM-mGP7/$R_{324}S$ ($R_{324}S$). At 18 h posttransfection, culture supernatants were separated by 10% SDS–PAGE under reducing conditions and analyzed for the presence of sGP-specific proteins by immunoblotting using goat anti-EBOV immunoglobulins. The consensus sequence of the furin cleavage site as well as the sequences at the cleavage sites of wild-type sGP and mutant $R_{324}S$ are shown on the top. (B) Effect of decanoylated peptidyl chloromethyl ketone (decRVKR-cmk) on cleavage of EBOV sGP. Vero-E6 cells infected with EBOV were washed 3 days postinfection with Dulbecco's medium and incubated with or without decRVKR-cmk at a concentration of 25 μM. At 6 h posttreatment, culture supernatants were separated by 10% SDS–PAGE under reducing conditions and analyzed by immunoblotting using anti-EBOV immunoglobulins. (C) Cleavage of sGP by recombinant furin *in vitro.* HeLa cells were infected with vSCGP7. At 4 h postinfection, cells were washed, starved in methionine-cysteine-free medium for 1 h, and pulse-labeled for 20 min with 100 μCi/ml [^{35}C]cysteine (Amersham, Germany). During starvation, labeling, and a subsequent chase of 240 min, cells were either treated with 60 μM of decRVKR-cmk or incubated without inhibitor. sGP-specific proteins were immunoprecipitated from culture medium using goat anti-EBOV immunoglobulins. Immunoprecipitated samples were incubated for 2 h at 37°C with or without purified recombinant furin. The samples were subsequently treated for 10 min at 37°C with SDS (2% end concentration) and analyzed on 15% polyacrylamide SDS gels under reducing conditions.

of Δ-peptide has been confirmed by digestion with neuraminidase and/or O-glycosidase (Fig. 5A). A reduction in size of the Δ-peptide has been observed already after neuraminidase treatment alone, indicating the presence of sialic acid. Δ-Peptide was converted to lower molecular mass forms also after treatment with only O-glycosidase. Since O-glycosidase cannot remove O-linked carbohydrates that contain sialic acid, this result indicates that not all sugars of Δ-peptide are sialylated. The molecular mass of Δ-peptide has been reduced to approximately 5 kDa after digestion with both neuraminidase and O-glycosidase and now coincided with the one predicted from the amino acid sequence or with the size of a synthetic peptide (data not shown). sGP digested with neuraminidase also showed a change in electrophoretic mobility, indicating the presence of sialic acid. Since digestion with O-glycosidase did not reveal any effect on electrophoretic mobility of sGP, it appears that only N-linked carbohydrates are present on this molecule (Fig. 5B).

There are many viral transmembrane glycoproteins including EBOV surface glycoprotein GP (Volchkov *et al.,* 1998a; Volchkov, 1999; Wool-Levis and Bates, 1999), that are cleaved by furin or other cellular proprotein convertases during their intracellular transport through the Golgi apparatus. Many of these proteins are directly or indirectly associated with fusion activity (Klenk and Garten, 1994a,b; Stadler *et al.,* 1997; White, 1990). However, the range of proteins that are initially synthesized as inactive precursors and are subsequently cleaved to develop full activity is significantly larger, covering many peptide hormones, cell-adhesion factors, growth factors, and receptors (Molloy *et al.,* 1999). To our knowledge, the cleavage of EBOV sGP is the first example where a secreted glycoprotein of viral origin is proteolytically processed by subtilisin-like endoproteases. High conservation of the sGP cleavage site in all EBOV subtypes suggests that this cleavage plays an important role in infection.

MATERIALS AND METHODS

Viruses and cell cultures

Strain Eckron of the Zaire subtype (provided by the Institut voor Tropische Geneeskunde, Antwerp, Belgium) was propagated on Vero-E6 cells (ATCC CRL 1586) as described previously (Volchkov *et al.,* 1997). The recombinant vaccinia viruses vTF7-3, expressing T7 polymerase (provided by B. Moss, NIH, Bethesda, MD), and vSCGP7 expressing EBOV sGP (Volchkov *et al.,* 1995) were propagated in Vero-E6 and/or CV-1 cells. CV-1, HeLa, and Vero-E6 cells were maintained in Dulbecco's medium containing 10% FCS (GIBCO, Germany).

Pulse-chase experiments and immunoblot analysis

sGP expression in HeLa cells using the vaccinia/T7 polymerase expression system (transient expression) or recombinant vaccinia virus vSCGP7, immunoblot, and

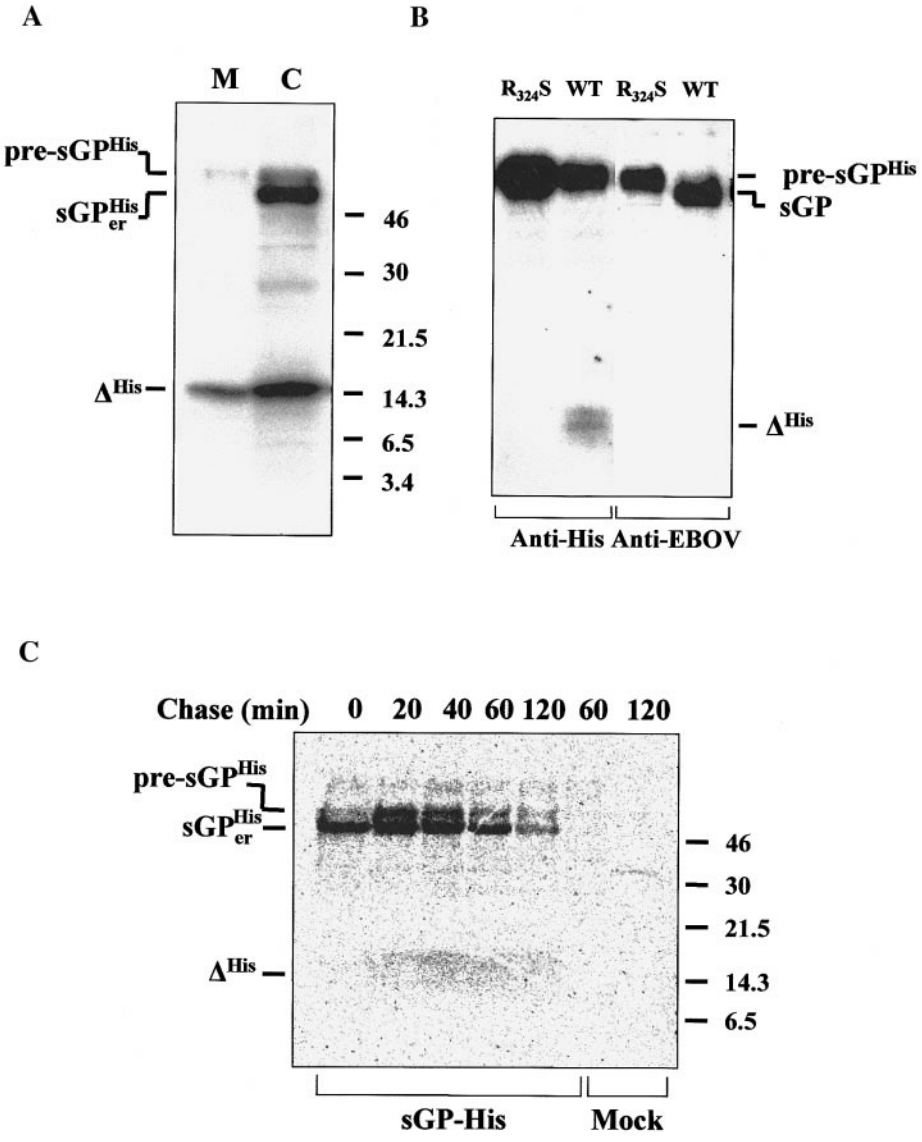

FIG. 4. Intracellular processing of mutant sGP-His. HeLa cells were infected with vTF7-3 and transfected with pGEM-mGP7/His expressing sGP elongated by six carboxy-terminal histidine residues, or with pGEM-mGP7/His-$R_{324}S$ expressing the sGP cleavage site mutant with a 6xHis tail. At 18 h posttransfection, lysed cells and/or culture supernatants (B) were separated by 15% SDS–PAGE under reducing conditions and analyzed for the presence of sGP-specific proteins by immunoblotting using either mouse anti-His antibodies (A and B) or goat anti-EBOV immunoglobulins (B). (C) Pulse-chase analysis of sGP/His processing. HeLa cell monolayers were infected with vTF7-3 followed by transfection with pGEM-mGP7/His. At 4 h posttransfection, cells were labeled with [^{35}S]cysteine for 20 min and subsequently chased for various times as indicated. Labeled proteins from cell lysates were immunoprecipitated using mouse anti-His antibodies, separated on 15% SDS–PAGE under reducing conditions, and analyzed by fluorography. Mock control represents HeLa cells infected with vaccinia virus VTF7-3 and transfected with plasmid expressing wild-type sGP. M, culture medium; C, lysed cells.

immunoprecipitation analyses were carried out as described previously (Volchkov *et al.,* 1995; Volchkov *et al.,* 1998a).

Oligonucleotide-directed mutagenesis

The construction of the plasmid pGEM-mGP7 was described previously (Volchkov *et al.,* 1995). PCR mutagenesis using the QuikChange-mutagenesis kit (Stratagene, Germany) according to the instructions of the manufacturer was performed to generate sGP cleavage site mutant $R_{324}S$ (pGEM-mGP7/$R_{324}S$), sGP/His mutant containing six histidine residues at the carboxy-terminal end of the sGP open reading frame (pGEM-mGP7/His), and a double mutant containing a 6xHis tag and a mutated cleavage site (pGEM-mGP7/His-$R_{324}S$). Clones with mutated sequences were verified by sequence determination.

Endoproteolytic cleavage inhibition assay

For cleavage inhibition studies, Vero E6 cells infected with EBOV strain Eckron were washed 3 to 4 days postin-

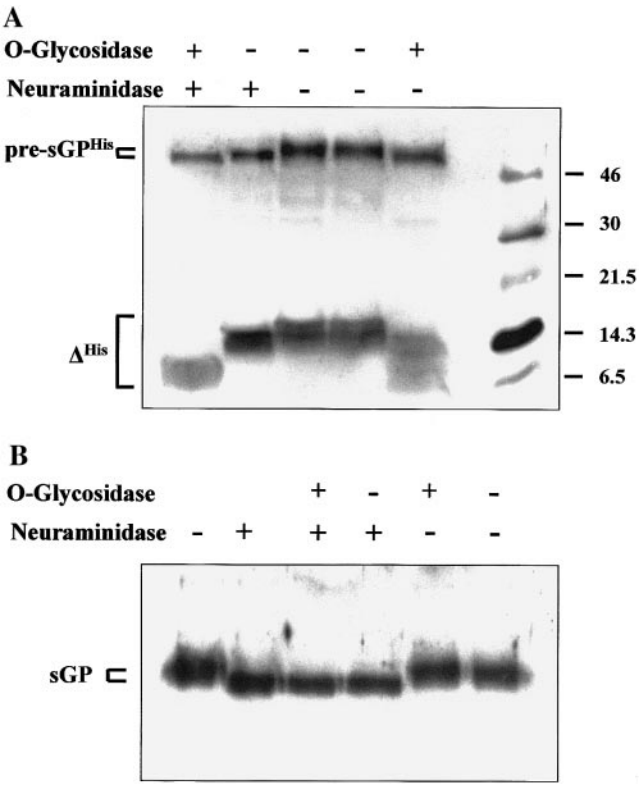

FIG. 5. Neuraminidase and O-glycosidase digestion of EBOV sGP. HeLa cells were infected with vTF7-3 followed by transfection either with pGEM-mGP7/His (A) or pGEM-mGP7 (B). At 22 h posttransfection, culture supernatants were collected and subjected to neuraminidase and/or O-glycosidase treatment. Digested samples were separated either by 15% (A) or 10% (B) SDS–PAGE under reducing conditions and analyzed by immunoblot using either mouse anti-His antibodies (A) or anti-EBOV immunoglobulins (B).

fection with Dulbecco's medium and incubated without or with the decanoylated peptidyl chloromethyl ketone decRVKR-cmk (kindly provided by W. Garten, Institute of Virology, Marburg, Germany) at concentrations of 25 or 80 μM for the next 6 h. For expression of recombinant sGP, HeLa cells infected with vSCGP7, were incubated during starvation, pulse, and chase periods either without or with the inhibitor at a concentration of 30 or 60 μM.

In vitro cleavage by furin

sGP expressed in the presence of inhibitor was immunoprecipitated as described earlier. Protein A–sepharose with bound sGP was washed two times with 100 mM Hepes buffer (pH 7.5), and finally treated with purified recombinant furin in the presence of 10 mM $CaCl_2$ at 37°C for 2 h (Hallenberger *et al.,* 1997). The samples were subsequently treated by SDS (2% end concentration) for 10 min at 37°C. Prior to loading onto 15% 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).

Glycosidase treatment

Analyses were performed on recombinant sGP expressed from HeLa cells that were infected with vTF7-3 and transfected with plasmid (pGEM-mGP7 or pGEM-mGP7/His). Culture supernatants were treated according to the instruction of the suppliers of glycosidases. In the case of neuraminidase, reaction mixtures contained 50 mM Na acetate, pH 5.5, and 1 mU of neuraminidase (Behring, Germany). After incubation at 37°C for 2 h, samples were either subjected to analysis or were additionally treated with O-glycosidase. In the case of O-glycosidase, reaction mixtures contained 50 mM Na phosphate, pH 7.4, and 1 mU of O-glycosidase (Boeringer Mannheim, Germany). Samples were treated at 37°C overnight. Control samples were incubated under the same conditions but without the addition of glycosidases. After digestions, samples were incubated in buffer containing 2% SDS and 5% β-mercaptoethanol for 10 min at 37°C and subsequently separated on 10 or 15% polyacrylamide gels, followed by immunoblot analysis using anti-His antibodies or anti EBOV immunoglobulins.

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