Processing of the Ebola virus glycoprotein by the proprotein convertase furin
(proteolytic processing)

VIKTOR E. VOLCHKOVT, HEINZ FELDMANN, VALENTINA A. VOLCHKova, AND HANS-DIETER KLENK
Institut für Virologie, Philipps-Universität Marburg, 35011 Marburg, Germany

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ABSTRACT In the present study, we have investigated processing and maturation of the envelope glycoprotein (GP) of Ebola virus. When GP expressed from vaccinia virus vectors was analyzed by pulse–chase experiments, the mature form and two different precursors were identified. First, the endoplasmic reticulum form preGPem, full-length GP with oligomannoside N-glycans, was detected. preGPem (110 kDa) was replaced by the Golgi-specific form preGP (160 kDa), full-length GP containing mature carbohydrates. preGP was finally converted by proteolysis into mature GP1,2, which consisted of two disulfide-linked cleavage products, the aminoterminal 140-kDa fragment GP1, and the carboxy-terminal 26-kDa fragment GP2. GP1,2 was also identified in Ebola virions. Studies employing site-directed mutagenesis revealed that GP was cleaved at a multibasic amino acid motif located at positions 497 to 501 of the ORF. Cleavage was blocked by a peptidyl chloromethylketone containing such a motif. GP is cleaved by the proprotein convertase furin. This was indicated by the observation that cleavage did not occur when GP was expressed in furin-defective LoVo cells but that it was restored in these cells by vector-expressed furin. The Reston subtype, which differs from all other Ebola viruses by its low human pathogenicity, has a reduced cleavability due to a mutation at the cleavage site. As a result of these observations, it should now be considered that proteolytic processing of GP may be an important determinant for the pathogenicity of Ebola virus.

Ebola and Marburg viruses, the two species within the family Filoviridae (1), are among the most pathogenic agents causing fulminant hemorrhagic fever in humans and nonhuman primates. Yet, we are only beginning to understand the interactions of these viruses with their hosts, and our knowledge on genetics, pathogenicity, and natural history is still limited. Although outbreaks have so far always been self-limiting, our ignorance concerning the natural reservoir and the lack of immunoprophylactic and chemotherapeutic measures makes these infections a matter of high concern in biomedical science. The chronology of human epidemics and epizootics in nonhuman primates proves that filoviruses are prototypes of emerging/re-emerging pathogens. The recent emergence of a new Ebola subtype in Cote d'Ivoire (2) and the re-emergence of Ebola subtype Zaire in former Zaire (3) and Gabon (4, 5) once again showed that these viruses have altered from exotic agents to pathogens of serious public health concerns.

The genomes of filoviruses display linear arranged genes on a single negative-stranded RNA molecule that encode the seven structural proteins in the order nucleocapsid protein, virion structural protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and RNA-dependent RNA polymerase (L) (6–8). In general, filoviral genes are transcribed into monocistronic subgenomic RNA species (mRNA) (6, 9). In contrast to all other filoviral genes, including the GP gene of Marburg virus (10), the organization and transcription of the fourth gene (GP) of EBOV is unusual and involves transcriptional editing, which is needed to express GP. A nonstructural small glycoprotein (sGP) is synthesized from the unedited GP mRNA, which is extensively secreted from infected cells (11, 12).

EBOV GP is a type I transmembrane glycoprotein 676 amino acids in length. The middle region of GP is variable, extremely hydrophilic, and carries the bulk of the glycosylation sites for N- and O-glycans that account for approximately one-third of the molecular weight (11, 13). Experimental data on GP function do not exist. However, the fact that GP is the only surface protein of virions suggests a function in receptor binding and fusion with cellular membranes.

The fusogenic properties of many viral glycoproteins require posttranslational proteolytic processing (14). Most of these glycoproteins are activated by subtilisin-like eukaryotic endoproteases (proprotein convertases) at multibasic cleavage sites (for review, see ref. 15). This type of processing has never been demonstrated with filovirus glycoproteins, and their functions were thought to be associated with noncleaved GP. We show here, however, that maturation of EBOV GP involves post-translational cleavage of a precursor at the C-terminal end of the sequence R-T-R-R501 into the disulfide-linked fragments GP1 and GP2. The proprotein convertase furin has been identified as a cleavage enzyme. Lower susceptibility to furin cleavage has been observed with the less pathogenic subtype Reston, suggesting that cleavability of GP may be a determinant of pathogenicity.

MATERIALS AND METHODS

Viruses and Cell Cultures. Strain Eckron of the Zaire subtype (provided by Institute Voor Tropische Geneeskunde, Antwerp, Belgium) and strain Pennsylvania of the Reston subtype of EBOV (provided by A. Sanchez, CDC, Atlanta, GA) were propagated on Vero-E6 cells (ATCC CRL 1586) and purified from tissue culture medium as described previously (16). The recombinant vaccinia viruses vTF7-3, expressing T7 polymerase (provided by B. Moss. National Institutes of Health, Bethesda, MD), VVHfur, expressing human furin (provided by G. Thomas, Oregon Health Sciences University, Portland, OR), and vSCGP8, expressing EBOV GP (11), were

Abbreviations: EBOV, Ebola virus; GP, glycoprotein; moi, multiplicity of infection; pfu, plaque-forming unit; VP, virion structural protein.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF034645).

†To whom reprint requests should be addressed at: Institut für Virologie, Philipps-Universität Marburg, Postfach 2360, 35011 Marburg, Germany, e-mail: volchkov@mailer.uni-marburg.de.

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propagated in Vero-E6 and/or CV-1 cells. CV-1, HeLa, RK13, and Vero-E6 cells were maintained in Dulbecco’s medium (GIBCO) containing 10% fetal calf serum (GIBCO). LoVo cells (human colon adenocarcinoma cells, ATCC CCL 229) were maintained in F-12 HAM medium (Sigma) containing 10% fetal calf serum.

**Metabolic Labeling.** Vero-E6 cells (75-cm² flasks) were inoculated with EBOV strain E2crk at concentrations of 25 or 80 with the decanoylated peptidyl chloromethylketone during starvation, pulse, and chase periods either without or in inhibition studies, cells infected with vSCGP8 were incubated without or with mutated sequences were verified by sequence determination. The cDNA fragment containing the edited GP-ORF of the Pennsylvania strain of EBOV GP was inserted into the expression vector plasmid DNA [9 μg; 15 μl of lipofectin (BRL)]. Cells were washed 3–4 days postinfection with Dulbecco’s medium containing recombinant plasmid DNA (9 μg; 15 μl of lipofectin (BRL)). Cells were washed 6 h postinfection, starved in methionine-cysteine-free medium for 1 h, and labeled with 100 μCi/ml [35S]Promix (Cys-Met) (Amersham) or 500 μCi/ml [3H]glucosamine (Amersham), respectively. Virus was harvested 12 h postlabeling and partially purified from culture medium by centrifugation through a 20% sucrose cushion (120 min at 100,000 × g). Pelleted virus was resuspended in PBS and lysed with 2% SDS, 10% Nonidet P-40, and 0.4% deoxycholate.

**Pulse–Chase Experiments.** HeLa or RK13 cells (3 × 10⁶) were infected with either vSCGP8 or vTF7-3 (transient expression) at an moi of 10 pfu/cell. For transient expression, the inoculum was replaced after 1 h by transfection medium containing recombinant plasmid DNA [9 μg; 15 μl of lipofectin (BRL)]. Cells were washed 6 h postinfection, starved in methionine-cysteine-free medium for 1 h, and labeled with 100 μCi/ml [35S]Promix (Cys-Met) (Amersham). After a 20-min pulse, the inoculum was replaced with medium for chase. Labeled cells were lysed immediately in 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 iodoacetamide/1 mM phenylmethylsulfonyl fluoride) at 4°C. Immunoprecipitation was performed using goat anti-EBOV Igs.

**Generation of EBOV Reston GP.** A cDNA fragment containing the edited GP-ORF of the Pennsylvania strain of subtype Reston was generated from GP-specific mRNA by reverse transcriptase-PCR using strain Reston-derived oligonucleotides from a sequence determined previously (EVU23152, GenBank). The cDNA product was cloned into pGEM 3Zf(+) and is referred to as pGEM-RP8. The sequence was determined and submitted to GenBank (AF034645).

**Oligonucleotide-Directed Mutagenesis.** PCR mutagenesis using the Quick-change kit (Stratagene) according to the instructions of the manufacturer was performed to generate the Zaire (Z) and Reston (R) GP cleavage site mutants (pGEM-ZF1, pGEM-ZF2, pGEM-R/K, pGEM-R/R) and the Zaire GP tail elongation mutant (pGEM-GPLT). Clones with mutated sequences were verified by sequence determination.

**Endoproteolytic Cleavage Inhibition Assay.** For cleavage inhibition studies, cells infected with vSCGP8 were incubated during starvation, pulse, and chase periods either without or with the decanoylated peptide chloromethylketone decRVKR-cmk at concentrations of 25 or 80 μM.

**Glycosidase Treatment.** Analyses were performed on recombinant GP expressed from vTF7-3-infected and plasmid (pGEM-GP8 or pGEM-GPLT)-transfected HeLa cells (1 × 10⁶). Immunoprecipitated proteins were treated under reducing and denaturing conditions according to the instructions of the supplier (BioLabs, Germany).

**RESULTS**

**EBOV GP Undergoes Posttranslational Proteolytic Cleavage into Two Disulfide-Linked Fragments.** EBOV GP is a complex membrane glycoprotein. To better understand the sequence of maturation steps, pulse–chase labeling experiments were performed on vector-expressed GP. Cells infected with the recombinant vaccinia virus vSCGP8 were pulse-labeled for 20 min with [35S]methionine/cysteine and chased for different time intervals prior to lysis; proteins were then immunoprecipitated using anti-EBOV Igs. Three high molecular weight forms of GP could be discriminated (Fig. 1A).

Immediately after pulse labeling, GP was detected in a 110-kDa form that had previously been identified as the precursor present in the endoplasmic reticulum due to its endo H

![Image](https://example.com/fig1.png)

**FIG. 1.** Synthesis, processing, and transport of EBOV GP. Pulse–chase analyses of EBOV GP were done in RK13 cells infected with vSCGP8 (A) or in HeLa cells infected with vTF7-3 and transfected with pGEM-GPLT (B). At 6 h postinfection, cells were pulse-labeled for 20 min (lane 0) and chased for the indicated time intervals. Lysed cells were immunoprecipitated with goat anti-EBOV Igs. Precipitated proteins were separated on 15% (A, C) or 8% (B) polyacrylamide gels, and proteins were visualized by fluorography. (D) Endoglycosidase digestion of the tail elongation mutant GPLT expressed in HeLa cells. Cells were infected with vTF7-3 and subsequently transfected with pGEM-mGP8 expressing wild-type GP (GP) or pGEM-GPLT expressing GPLT. At 18 h posttransfection, cells were lysed and subjected to endoglycosidase treatments. Digests were separated by SDS/PAGE and blotted onto a poly(vinylidene difluoride) membrane. Detection of GP-specific proteins was performed with goat anti-EBOV Igs. The positions of the precursors (preGP × and preGP) and the proteolytically cleaved subunits GP and preGP × are indicated.
polyacrylamide gels under reducing and under nonreducing conditions. The positions of the mature GP (GP1,2), and the two cleavage subunits GP1 and GP2, are indicated.

Fig. 2. Virion GP of EBOV consists of two cleavage subunits. EBOV GP metabolically labeled with [3H]glucosamine or with [35S]methionine-cysteine was immunoprecipitated from purified virions grown in Vero E6 cells and analyzed by 15% SDS/PAGE under reducing or nonreducing conditions. The positions of the mature GP (GP1,2), and the two cleavage subunits GP1 and GP2, are indicated.

conditions (Fig. 2). After treatment with β-mercaptoethanol, the 140- and 26-kDa bands of GP1 and GP2, respectively, were detected. Labeling with [3H]glucosamine and immunoprecipitation with a GP-specific antiserum proved that GP2 is distinct from VP24, which has a similar molecular weight. In the absence of β-mercaptoethanol, a band designated GP1,2 was detected, indicating that GP1 and GP2 form a disulfide-linked complex under nonreducing conditions. Taken together, these data indicate that GP undergoes proteolytic processing after vector expression as well as after EBOV infection and that GP is present in virions in the cleaved form.

EBOV GP Is Cleaved by Furin. EBOV GP contains the sequence R-R-T-R-R$_{500}$, which is a potential proprotein convertase recognition motif. To test whether this motif is indeed used as a cleavage site, the sequence was modified by site-specific mutagenesis. In mutant Z/F1, arginine at position 501 was substituted by lysine (R-R-T-R-R $\rightarrow$ R-R-T-R-K), and in mutant Z/F2 the arginine residues at positions 500 and 501 were changed to asparagine and methionine, respectively (R-R-T-R-R $\rightarrow$ R-R-T-N-M) (Fig. 3). Both mutants were evaluated for proteolytic processing by transient expression in VTF7-3-infected HeLa cells. Unlike wild-type GP, which was processed into subunits GP1 and GP2, both mutants expressed only the uncleaved preGP. This result indicates that GP is cleaved at the carboxyl-terminal side of arginine 501 and that the cleavage site has the classical consensus sequence R-X-K/R-R recognized by ubiquitous proprotein convertases, such as furin. The observation that mutant Z/F1 displaying the consensus sequence R-X-X-R$_{500}$ is not cleaved may be explained by the dislocation of the motif within the GP sequence or by the fact that this motif is less frequently used as a cleavage site (17). In addition, the results obtained with these mutants indicate that another multibasic sequence RKIR$_{302}$ is not used as a cleavage site of the EBOV GP.

Intracellular processing of the cleavage site mutants Z/F1 and Z/F2. HeLa cells were infected with VTF7-3 and transfected with the plasmids pGEM-mGP8 (wild-type GP, WT), pGEM-Z/F1 (mutant Z/F1), and pGEM-Z/F2 (mutant Z/F2). At 6 h postinfection, cells were pulse-labeled for 20 min and chased for 240 min. Immunoprecipitated proteins were separated under reducing conditions on 8% (Upper gel) or 15% (Lower gel) polyacrylamide gels. The positions of the noncleaved precursor (preGP) of both mutants and the cleavage subunits GP1 and GP2 are indicated. The sequences at the cleavage sites are shown at Top.
furin by covalently blocking the substrate binding site of the enzyme (15). It was therefore of interest to find out if such inhibitors were able to prevent cleavage of preGP. Experiments were performed on RK13 cells infected with vSCGP8 (Fig. 4). Six-hour postinfection cells were treated with 25 or 80 µM of decRVKR-cmk, pulse-labeled, and incubated for a 4-h chase. Proteins were immunoprecipitated from cell lysates using anti EBOV Ig, treated with 2% SDS with or without 

\[ \beta \]-mercaptoethanol, and subsequently analyzed on SDS/PAGE. The inhibitor completely blocked cleavage at both concentrations as indicated by the persistence of preGP and the absence of GP1 and GP2 (Fig. 4). An interesting observation was made when uncleaved GP that had accumulated after expression in the presence of decRVKR-cmk was compared with cleaved GP by electrophoresis under nonreducing conditions. Whereas GP1,2 showed the expected molecular mass of about 160 kDa, uncleaved GP had a significantly reduced electrophoretic mobility with an apparent molecular mass well above 220 kDa (*preGP). preGPer was partly also present in a conformation of uncleaved GP differs from that of cleaved GP. The most likely explanation for these differences in migration are variations in protein folding. It therefore seems that the conformation of uncleaved GP differs from that of cleaved GP.

To further support the concept that GP is cleaved by furin, we have expressed GP in LoVo cells, which lack the active form of this enzyme (18). Unlike HeLa (Figs. 1B and 3), RK13 (Figs. 1A and 4), and Vero cells (Fig. 2), LoVo cells were unable to cleave GP (Fig. 5A, lanes 1–6). To definitely prove that GP is cleaved by furin, LoVo cells were co-infected with recombinant vaccinia viruses expressing GP (vSCGP8) and human furin (VVhfur). As also shown in Fig. 5A (lanes 7–12), GP was now cleaved in LoVo cells. This experiment demonstrates clearly that GP is cleaved by furin but not by other proprotein convertases expressed in LoVo cells, such as PACE4 or PC7. Thus, it seems that furin plays a prime role in GP cleavage when analyzed under nonreducing conditions, the *preGP1 and *preGP2 forms of uncleaved GP were also detected in LoVo cells (Fig. 5B), again supporting the concept that uncleaved and cleaved GP differ in conformation.

**Subtype Reston GP Shows Reduced Cleavability.** Subtype Reston differs from subtype Zaire by an altered cleavage site displaying the sequence R-K-Q-K-R (EVU23152, EV23416, EV23417; GenBank), which is not a classical furin recognition motif. To analyze the effect of this sequence variation on processing, the GP gene of the Pennsylvania strain of EBOV Reston was cloned, modified by site-directed mutagenesis at the cleavage site, and expressed using the vaccinia virus T7 system. As shown in Fig. 6, both precursors as well as the cleaved subunits GP1 and GP2 were indicated that cleavage kinetics were significantly decreased with Reston GP. The results obtained with mutant R/K that has a classical furin cleavage site similar to Zaire GP (lanes 5 and 6) indicated that cleavage kinetics were significantly decreased with Reston GP. The results obtained with mutant R/K demonstrated that cleavage was even further reduced when R at position -1 was replaced by K (lanes 1 and 2). Thus, we could show that proteolytic processing of EBOV GP can be modulated by altering the cleavage site and that Reston GP has a lower cleavability than Zaire GP.

**DISCUSSION**

Maturation of the EBOV GP involves a complex sequence of co- and posttranslational processing events. In the present

![Fig. 4](image48x133to272x431)

**Fig. 4.** Effect of decanoylated R-V-K-R chloromethylketone on cleavage of EBOV GP. RK13 cells were infected with vSCGP8. At 6 h postinfection, cells were pulse-labeled for 20 min and chased for various times in the absence (0 µM) or presence (25 µM, 80 µM) of inhibitor. Immunoprecipitated samples were separated by PAGE under reducing conditions on 15% gels (Top) and 8% gels (Middle) and under nonreducing conditions on 8% gels (Bottom). The positions of the precursors (preGP1 and preGP2) as well as the cleavage subunits GP1 and GP2 are indicated. Under nonreducing conditions, high Mr forms of both precursors are seen (*preGP and *preGPfr).
study, mature GP and two different precursor forms have been identified. The first precursor form is detected after pulse labeling as a 110-kDa glycoprotein that is converted by endo H treatment into a 75-kDa polypeptide (11). The data indicate that this precursor is full-length GP containing oligomannosidic N-glycans. It represents precursor GP present in the endoplasmic reticulum and has therefore been designated preGP<sub>pr</sub>. The second precursor, designated preGP<sub>pr</sub>, is first observed after a 10–20-min chase period as an endo H-resistant glycoprotein with a molecular mass of about 160 kDa. This form represents full-length GP containing mature carbohydrates and is present in the Golgi apparatus. Mature GP is GP<sub>1,2</sub>, which consists of two disulfide-linked cleavage products, the amino-terminal 140-kDa fragment GP<sub>1</sub> and the carboxy-terminal 26-kDa fragment GP<sub>2</sub> (Fig. 7). GP<sub>1,2</sub> is the virion form of GP, and it is also present in cells, presumably in the trans Golgi network and on the cell surface. These observations demonstrate that the filovirus glycoprotein is modified by extensive N-glycosylation and O-glycosylation when transported through the exocytic pathway. We show here that the glycoprotein of a filovirus undergoes posttranslational proteolytic cleavage by a proprotein convertase.

Although it has been known for some time from sequence analyses that EBOV GP contains a potential recognition site for proprotein convertases (7, 19), it has not been observed until now that cleavage does indeed occur. The failure to detect cleavage seems to be largely due to the fact that preGP<sub>pr</sub>, GP<sub>1,2</sub>, and GP<sub>1</sub> have very similar migration rates on polyacrylamide gels. Furthermore, GP<sub>2</sub> tends to escape detection because it contains relatively little carbohydrate (Fig. 7) and co-migrates with VP24, another structural protein of EBOV. The use of the tail elongation mutant proved therefore to be very important in the present study because it allowed a clear discrimination between the precursor and the large cleavage fragment. It should be mentioned that Marburg virus GP has also a multibasic cleavage site at position 432 to 435, and preliminary data show that it is cleaved, too (unpublished data).

EBOV GP is cleaved by furin. This is indicated by the observation that cleavage did not occur when GP was expressed in the furin-defective LoVo cell line but that it was restored in these cells by vector-expressed furin. The finding that cleavage was inhibited by a sequence-specific peptidyl chloromethylketone or by mutation of the cleavage site supports this concept. Furin belongs to the proprotein convertases, a family of subtilisin-like eukaryotic endoproteases that includes also PC1/PC3, PC2, PC4, PACE4, PC5/PC6, and LPC/PC7 (20). These enzymes are differentially expressed in cells and tissues, and they display similar but not identical specificity for basic motifs, such as R-X-K/R-R, at the cleavage site of their substrates. Furin seems to be expressed in most cells. It is a processing enzyme of the constitutive secretory pathway, as seems to be the case with PACE4, PC5/PC6, and LPC/PC7. The expression of PC1/PC3 and PC2 is restricted to the regulated secretory pathway of neuroendocrine cells. Furin is localized predominantly in the trans Golgi network (21, 22), but it is also secreted from cells in a truncated form (23, 24). Proprotein convertases activate numerous cellular proteins (25) and surface proteins of enveloped viruses. Furin seems to be the key enzyme in virus activation (26), but PC5/PC6 (27) and LPC/PC7 (28) are also involved. Thus, LPC/PC7 may be responsible for cleavage of the HIV glycoprotein in the furin-deficient LoVo cells. The observation that EBOV GP is not cleaved in these cells is interesting in this context. It is also noteworthy that furin, although ubiquitous, is particularly rich in hepatocytes and endothelial cells, which are both prime targets of Ebola virus (29). These observations stress the importance of furin as a processing enzyme of GP, but it remains to be seen in future studies if other proprotein convertases can substitute as cleaving enzymes.

Processing by proprotein convertases is an important control mechanism for the biological activity of viral surface proteins (26, 30). Cleavage occurs often next to a protein domain involved in fusion, and it has long been known that in these cases proteolytic cleavage is necessary for fusion activity. Proteolytic cleavage is the first step in the activation of these fusion proteins and is followed by a conformational change resulting in the exposure of the fusion domain (31–33). The conformational change may be triggered by low pH in endosomes, as is the case with influenza virus (34), or by the interaction with a secondary receptor protein at the cell surface, as is the case with HIV (35). We have so far not been able to demonstrate that cleavage of GP has an effect on fusion activity or on infectivity of EBOV. However, it is interesting to see that GP<sub>2</sub> contains a sequence of 16 uncharged and hydrophobic amino acids at a short distance (22 amino acids) from the cleavage site, which bears some structural similarity to the fusion peptides of retroviruses and has therefore been thought to play a role in EBOV entry (19). Furthermore, it seems from the present study that cleaved and uncleaved GP differ in folding, as indicated by their differential electro-
phoretic mobilities under nonreducing conditions. These observations are compatible with the view that proteolytic cleavage is a priming mechanism that renders GP susceptible for a conformational change required for fusion.

Finally, it has to be pointed out that proteolytic activation of viral glycoproteins is an important determinant for pathogenicity. Cleavage by furin and other ubiquitous proprotein convertases has been shown to be responsible for systemic infection caused by highly pathogenic strains of avian influenza and Newcastle disease virus (36). It is therefore tempting to speculate that cleavage by furin is also an important factor for the pantropism of EBOV and its rapid dissemination through the organism. Furthermore, variations at the cleavage site of GP may account for differences in the pathogenicity of EBOV. Our data show that the pathogenic strains Zaire, Sudan, and Ivory Coast, which have the canonical furin motif R-X-K/R-R at the cleavage site, are highly susceptible to cleavage, whereas the Reston strain, which seems to be apathogenic for humans and only moderately pathogenic for at least some monkey species (37), has reduced cleavability because of the suboptimal cleavage site sequence K-Q-K-R. That highly pathogenic variants may suddenly emerge from Reston-like strains by mutations restricted to the cleavage site is an intriguing hypothesis. On the other hand, it may be possible to obtain EBOV mutants with even lower cleavability than the Reston strain, and such viruses may have a potential as life vaccines. Because furin cleavage can be inhibited not only by peptidyl chloromethylketones as described here but also by less toxic components (38), inhibition of proteolytic cleavage may be a novel concept for treatment of EBOV infections.

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