



# Suppression of Fas-mediated apoptosis via steric shielding by filovirus glycoproteins



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## ABSTRACT

Apoptotic death of virus-infected cells is generally thought to be a defense mechanism to limit the spread of infectious virions by eliminating virus-producing cells in host animals. On the other hand, several viruses have been shown to have anti-apoptotic mechanisms to facilitate efficient viral replication and transmission. In this study, we found that the filovirus glycoprotein (GP) expressed on cell surfaces formed a steric shield over the Fas molecule and that GP-expressing cells showed resistance to cell death induced by a Fas agonistic antibody. These results suggest that filovirus GP-mediated steric shielding may interfere with the Fas-induced apoptotic signal transduction in infected cells and serve as an immune evasion mechanism for filoviruses.

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## 1. Introduction

Filoviruses (viruses of the genera *Marburgvirus* and *Ebolavirus* in the family *Filoviridae*) are enveloped, negative-stranded RNA viruses. These viruses are known to cause severe hemorrhagic fever in humans and/or nonhuman primates. To date, there is one known species in the genus *Marburgvirus*, consisting of two distinct viruses, Marburg virus (MARV) and Ravn virus. On the other hand, five distinct species are known in the genus *Ebolavirus*. The Ebola virus (EBOV) representing the species, *Zaire ebolavirus*, is thought to be the most pathogenic among the five known species in the genus *Ebolavirus*, with case-fatality rates of up to 90%. Among MARVs, strain Angola caused the largest outbreak in 2004–05 in Angola, with the highest mortality rate (90%) [1,2]. A novel bat-derived filovirus, named Lloviu virus, was recently found in Cueva del Lloviu, Asturias, Spain, and tentatively classified into the newly proposed genus *Cuevavirus* in the family *Filoviridae* [3,4].

The viral envelope glycoprotein (GP) is the only spike protein of filoviruses and thus responsible for virus entry into host cells. Filovirus GP undergoes proteolytic cleavage by host proteases such as furin, resulting in the two subunits, GP1 and GP2, which are linked by a single disulfide bond [5–7]. GP1 contains a putative receptor-binding region and mucin-like region (MLR) that has a number of

potential N- and O-linked glycosylation sites [8,9]. It is known that the expression of EBOV GP in cultured cells results in loss of cell–cell interaction as well as cell rounding and detachment of cells from the substrate [10–12]. It was also proposed that the MLR of GP, which spatially occupies a very large region, might abrogate cell adhesion and/or prevent the interaction between lymphocytes and infected cells by forming a steric shield over host proteins such as integrin  $\beta$ 1 and major histocompatibility complex class I (MHC I) on the surfaces of GP-expressing cells [13]. We further reported that the shielding effect was not only observed for EBOV GP but also GPs of other ebolaviruses in different species and MARVs, and that the steric shielding efficiency was correlated with the difference in their relative pathogenicities, suggesting that better shielding effects may possibly be related to higher pathogenicities of particular filovirus strains [14].

In general, apoptotic death of virus-infected cells is an important host defense mechanism to limit viral spread. Apoptosis via the extrinsic pathway is induced by members of the tumor necrosis factor (TNF) family such as Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). FasL, a type II membrane protein, is dominantly expressed on activated T cells and natural killer cells, whereas Fas, a type I membrane protein and a receptor of FasL, is expressed on the surface in diverse cell population [15–17]. The binding of apoptosis-inducing ligands to their receptors results in signal transduction in cells and the formation of the death-inducing signaling complex followed by the activation of caspases that ultimately induce cell death [18]. Following induction of apoptosis, morphologic features such as cell shrinkage, nuclear fragmentation, and apoptotic body formation are observed [19].

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To counteract the induction of apoptosis, some viruses have evolved multiple mechanisms that interfere with the death signal in infected cells [20]. Although upregulation of TRAIL and Fas molecules associated with EBOV infection of humans and experimentally infected animals has been reported [21–25], it was shown that EBOV did not naturally induce apoptosis in infected cells in vitro. However, no suppressive effect on the TRAIL-induced apoptotic signal has been observed in EBOV-infected cells [25]. In this study, we focused on Fas-mediated signaling, which is one of the major extrinsic pathways for the induction of apoptosis, and found that filovirus GPs formed a steric shield on the cell surface and that GP-expressing cells showed resistance to Fas-induced cell death, suggesting that interference with apoptotic signal transduction may serve as an immune evasion mechanism of filoviruses.

## 2. Materials and methods

### 2.1. Plasmids

For expression of EBOV and MARV GPs, cDNAs encoding full-length GPs of strains Mayinga-76 (Zaire) and Angola (Angola), respectively, were used [14]. Coding regions of the Lloviu virus GP were synthesized in pBS II SK vector (FASMAC) based on the nucleotide sequence of the Lloviu GP [26]. After digestion by restriction enzymes, each gene was cloned into the mammalian expression vector pCAGGS.

### 2.2. Transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM)(Gibco) supplemented with 10% fetal bovine serum at 37 °C with 5% CO<sub>2</sub>. The cells were transfected with plasmids using Lipofectamine 2000 (Mirus) according to the manufacturer's directions. Six hours posttransfection, the culture medium was changed to fresh DMEM supplemented with 10% fetal bovine serum. The cells were collected and washed once with FACS buffer (0.5% FCS and 0.05% sodium azide in PBS), and used for flow cytometric analyses.

### 2.3. Monoclonal antibodies to GPs

Mouse monoclonal antibodies (MAbs) were generated according to a standard procedure reported previously [27,28]. Zaire GP-specific MAb ZGP746/16.2 (IgG2a), which recognizes amino acid positions 391–410 (TPVYKLDISEATQVEQHRR) in GP1 [29], MARV GP-specific MAb MGP14-22 (IgG1), which recognizes amino acid positions 445–465 (FPFLDGLINAPIDFDPVNTK) in GP2, Lloviu GP-specific MAb LGP14-2 (IgG1), and a vesicular stomatitis virus (VSV) G protein-specific MAb (VSV-G(N)1-9) [28] were purified from mouse ascites using protein A agarose columns (Bio-Rad). Purified ZGP746/16.2, MGP14-22, and LGP14-2 were labeled with Alexa Fluor 488 using an Alexa Fluor 488 Protein Labeling Kit (Invitrogen) for the analysis of shielding effects against Fas (CD95).

### 2.4. Induction of apoptosis and flow cytometry

To detect cell surface Fas molecules on GP-expressing cells, HeLa cells transfected with GP-expressing plasmids or pCAGGS alone (vector) were stained with an allophycocyanin-conjugated anti-human Fas antibody (DX2; eBioscience) and Alexa Fluor 488-labeled MAbs ZGP746/16.2, MGP14-22, or LGP14-2. Following forward and side scatter gating, more than 7000 cells were accumulated and analyzed for the detection of Fas and GP with a Becton Dickinson FACS Canto flow cytometer and FlowJo software (Tree Star, Inc.). To analyze the Fas-induced apoptotic signal and cell

death, HeLa cells transfected with GP-expressing plasmids or the vector alone were incubated for 36 h and treated with 100 ng/ml of an agonistic anti-Fas/Apo-1 MAb (IgM) (CH-11; Medical & Biological Laboratories) or isotype control MAb APH159-1-3 (IgM) that recognizes influenza virus hemagglutinin, and then processed for apoptotic cell detection according to the manufacturer's instructions. To detect activated caspases, cells harvested after 3-h incubation with CH-11 or APH159-1-3 were stained with each GP-specific MAb and Alexa 647-labeled goat anti-mouse IgG (Invitrogen), resuspended in fluorochrome inhibitor of caspases (FLICA) (ImmunoChemistry Technologies), which irreversibly binds to caspases 1, 3, 4, 5, 6, 7, 8, and 9, incubated for 1 h at 37 °C in a 5% CO<sub>2</sub> atmosphere, and stained with propidium iodide (PI). To monitor cell viability, cells cultured in the presence of CH-11 or APH159-1-3 were collected at 6 and 12 h and stained with GP- or VSV G-specific MAbs and Alexa 647-labeled goat anti-mouse IgG (Invitrogen) and PI. Following forward and side scatter gating, more than 7000-gated events (GP-, or VSV G-positive) were accumulated and analyzed for the detection of FLICA and/or PI by flow cytometry. Cells transfected with the vector alone were not gated for GP.

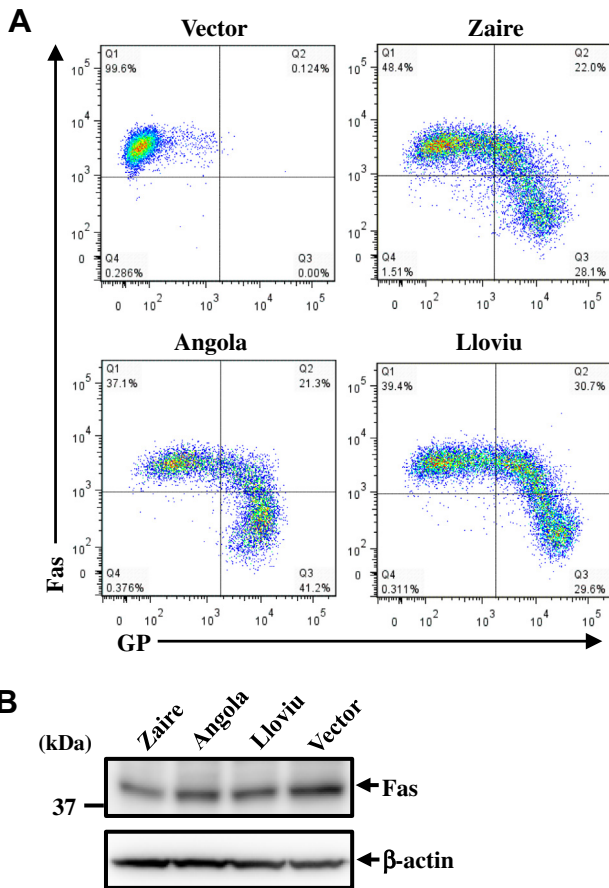
### 2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Cells were lysed with Laemmli sample buffer (Bio-Rad) and 1% NP-40 under reducing conditions, and then the insoluble fraction was removed by centrifugation. Solubilized proteins were separated by SDS-PAGE and blotted on a polyvinylidene difluoride membrane (Millipore). Non-specific binding to the membrane was blocked with 3% skim milk in PBS. An anti-CD95 antibody (EPR5700; Abcam) and an anti  $\beta$ -actin antibody (AC-15; Abcam) were used as primary antibodies. The bound antibodies were detected with peroxidase-conjugated goat anti-rabbit IgG (H + L) (Kirkegaard & Perry Laboratories) or anti-mouse IgG (Jackson ImmunoResearch) followed by visualization with Immobilon Western (Millipore).

## 3. Results and discussion

In general, it is believed that a death signal through Fas–FasL interaction on the cell surface is involved in the elimination of virus-infected cells, providing an important defense mechanism against viral infections. It has been demonstrated that filovirus GPs expressed on the cell surface form a steric shield over host proteins and disrupt their interaction with extracellular matrices and immune cells [13,14,30]. Thus, we first investigated whether filovirus GPs showed the steric shielding effect against cell surface Fas molecules. HeLa cells were transiently transfected with plasmids expressing Zaire, Angola, or Lloviu GPs and analyzed by flow cytometry with probing antibodies to Fas (Fig. 1). The steric shielding effect was expected to be observed as decreased detection of the Fas molecule on the cell surface due to the sterically hindered antibody access to the relevant host proteins [13,30]. We found that a prominent shielding effect for the cell surface Fas molecule was commonly observed on Zaire, Angola, and Lloviu GP-expressing cells (Fig. 1A). Overall intracellular protein expression levels of Fas were not affected by the expression of these GPs (Fig. 1B). These results suggested that Fas molecules on GP-expressing cells might be masked by the steric shielding effect. This striking shielding effect is likely due to the relatively small molecular size of Fas (approximately 45 kDa) and its localization to lipid rafts together with GP [31,32].

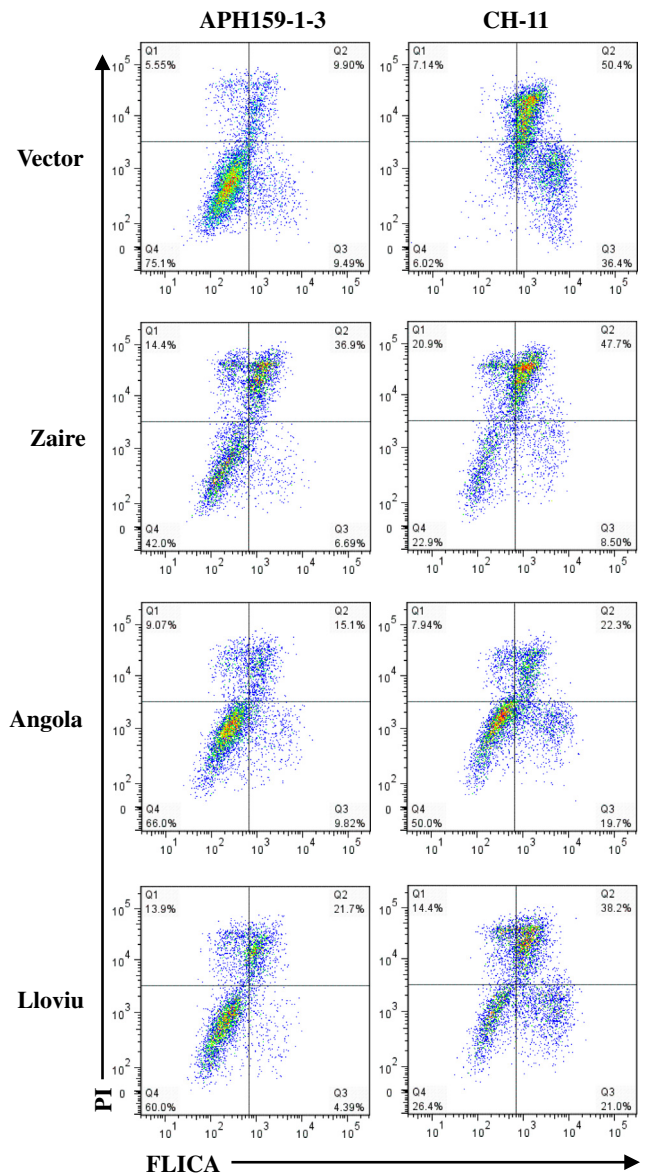
To investigate whether GP-mediated steric shielding functionally blocks subsequent apoptotic signaling through Fas–FasL



**Fig. 1.** Intensive steric shielding effects against Fas on filovirus GP-expressing cells. HeLa cells transfected with pCAGGS expressing Zaire GP, Angola GP, Lloviu GP, or pCAGGS alone (Vector) were stained with an anti-Fas antibody and analyzed by flow cytometry. (A) Intracellular expression levels of Fas were examined by Western blotting. (B) Data are representative of three independent experiments.

interaction, HeLa cells transfected with GP-expressing plasmids or the vector alone were treated with a Fas agonistic MAb (CH-11) or control MAb (APH159-1-3). After 3-h incubation with the MAbs, cells were stained with cell-permeable FLICA and PI to observe caspase induction and cell viability, respectively (Fig. 2). In control (vector-transfected) cells, 75.1% of cells treated with APH159-1-3 were viable, but only 6.0% of the CH-11-treated cells were viable. Instead, the numbers of late and early apoptotic CH-11-treated cells were remarkably elevated (from 9.9% to 50.4% and from 9.49% to 36.4%, respectively), indicating that the CH-11 treatment intensively induced apoptosis in the control cells. Interestingly, however, the induction of apoptosis was clearly attenuated in Zaire GP-expressing cells treated with CH-11, as indicated by limited increases of the numbers of early and late apoptotic cells and limited decreases of viable cells compared to those of cells treated with APH159-1-3. Similarly, a reduction in apoptotic signals and limited decrease of viability were observed in CH-11-treated cells expressing the other filovirus GPs tested. These results suggested that the death signal triggered via Fas/FasL interaction on the cell surface was hindered by steric shielding effects caused by filovirus GPs on the cell surface.

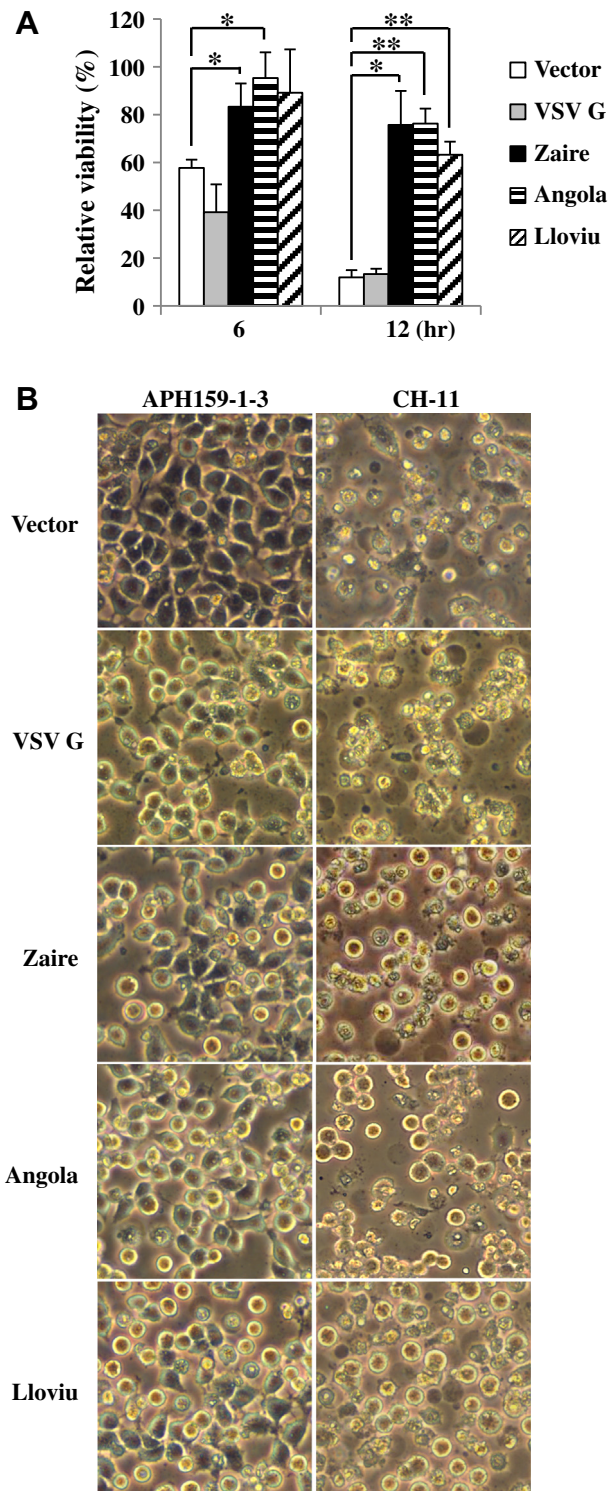
Upon incubation with CH-11, viabilities of VSV G-expressing and vector-transfected cells were drastically decreased at 6 and 12 h, and more than 80% of the cells died during the incubation period. In contrast, more than 60% of the cells expressing filovirus GPs survived even after 12-h incubation (Fig. 3A). Cell rounding and detachment, most likely due to the disrupted function of adhesion molecules (e.g., integrins) by GP-mediated steric



**Fig. 2.** Reduced Fas-mediated apoptosis in filovirus GP-expressing cells. HeLa cells transfected with pCAGGS expressing Zaire GP, Angola GP, Lloviu GP, or pCAGGS alone (Vector) were incubated with APH159-1-3 or CH-11 for 3 h, stained with FLICA and PI, and analyzed by flow cytometry. Live cells were assumed to be neither FLICA nor PI positive (FLICA<sup>-</sup>/PI<sup>-</sup>). Early apoptotic cells are stained with FLICA but unstained with PI (FLICA<sup>+</sup>/PI<sup>-</sup>). Late apoptotic/secondary necrotic cells are positive for both FLICA and PI (FLICA<sup>+</sup>/PI<sup>+</sup>). Data are representative of three independent experiments.

shielding, were observed in filovirus GP-expressing cells but not in VSV G-transfected or vector-transfected cells (Fig. 3B left panels). Consistent with the reduced viability of VSV G- and vector-transfected cells, intensive cell death characterized by progressive cell shrinkage through apoptosis was observed in these cells (Fig. 3B right panels). In contrast, such morphological changes were only slightly observed in GP-expressing cells (i.e., rounded cells) and apoptotic death was exclusively observed in GP-untransfected cells in the same well (Fig. 3B right panels). These results indicated that GP-expressing cells were more resistant to cell death induced by Fas-mediated apoptosis than VSV G- or vector-transfected cells.

In this study, we found a reduction in apoptotic signals and higher viability in filovirus GP-expressing cells, suggesting that the steric shielding effect against the Fas molecule might protect



**Fig. 3.** Increased viability of filovirus-expressing cells after Fas stimulation. HeLa cells transfected with pCAGGS expressing Zaire GP, Angola GP, Lloviu GP, or pCAGGS alone (Vector) were incubated in the presence of APH159-1-3 or CH-11, collected at 6 and 12 h, and stained with PI. PI-negative cells were counted by flow cytometry. (A) The relative viabilities of cells transfected with each plasmid were calculated according to the following formula [(number of viable cells transfected with each plasmid after treatment with CH-11)/(number of viable cells transfected with each plasmid after treatment with APH159-1-3)  $\times$  100]. Experiments were performed three times, and averages and standard deviations are shown. Statistical significance was analyzed by Student's *t*-test for comparison to cells transfected with the vector alone ( $*P < 0.05$ ,  $**P < 0.01$ ). Morphological changes of HeLa cells transfected with each plasmid were observed after 12-h incubation with APH159-1-3 or CH-11 (B).

filovirus GP-expressing cells from the Fas-mediated apoptotic signal. It is noteworthy that the GP of Lloviu virus, which has not been biologically characterized since no infectious virus has been isolated, also had the potential to suppress Fas-mediated apoptosis, like other filoviruses that cause lethal diseases in humans and nonhuman primates. Moreover, it was confirmed that integrin  $\beta$ 1 and MHC I were also masked by the Lloviu virus GP (unpublished data). These observations may provide useful information concerning the pathogenic potential of Lloviu virus. This study suggests that the ability to interfere with functions of host cell surface proteins is likely a common property among the GPs of all filoviruses thus far known. On the other hand, it was shown previously that the expression levels of EBOV GP were tightly controlled in infected cells, with cytotoxic effects occurring late in infection [33,34], and that moderate levels of GP expression might not affect the detection of MHC I and integrin  $\beta$ 1 on the cell surface [34]. Thus, we cannot exclude the possibility that GP-mediated steric shielding of the Fas molecule may only be observed when GP is expressed with high density on the cell surface.

Although further investigations using infectious filoviruses are needed to clarify the importance of the steric shielding effect in the pathogenesis in infected animals, this study suggests that GP-mediated steric shielding may play important roles in immune evasion from host responses initiated by various signaling factors, including Fas-mediated apoptosis. Interestingly, selective apoptosis induced by a dsRNA-dependent caspase recruiter strongly inhibited virus replication, suggesting that apoptosis might be capable of playing an important role in overcoming filovirus infection [25]. Thus, modulation of the signaling machinery including the Fas/FasL interaction may therefore be a useful strategy for the development of antiviral therapeutics.

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