HOKKAIDO UNIVERSITY

Title	Studies on steric shielding of cell surface proteins by filovirus envelope glycoproteins
Author(s)	野依, 修
Degree Grantor	北海道大学
Degree Name	博士(獣医学)
Dissertation Number	甲第11284号
Issue Date	2014-03-25
DOI	https://doi.org/10.14943/doctoral.k11284
Doc URL	https://hdl.handle.net/2115/56169
Type	doctoral thesis
File Information	Osamu_Noyori.pdf

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Studies on steric shielding of cell surface proteins by filovirus envelope glycoproteins

（フィロウイルスエンベロープ糖蛋白質による
細胞表面分子の立体的遮蔽現象に関する研究）

Osamu NOYORI

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Abbreviations

APC	allophycocyanin
cDNA	complementary deoxyribonucleic acid
CT	cytoplasmic tail
DMEM	Dulbecco's modified Eagle's medium
EBOV	Ebola virus
eGFP	enhanced green fluorescent protein
FACS	fluorescence activated cell sorter
FasL	Fas ligand
FCS	fetal calf serum
FLICA	fluorochrome inhibitor of caspases
GC	glycan cap
GP	glycoprotein
HEK	human embryonic kidney
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IFL	internal fusion loop
MAB	monoclonal antibody
MARV	Marburg virus
MFI	mean fluorescence intensity
MHC I	major histocompatibility complex I
MLR	mucin-like region
PBS	phosphate buffered saline

PI	propidium iodide
PVDF	polyvinylidene difluoride
RBR	receptor-binding region
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	signal peptide
TM	transmembrane domain
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VSV	vesicular stomatitis virus

Preface

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 with high mortality rate up to 90% and over 1,900 people died in total. Since the first outbreak of Marburg virus (MARV) infection in nonhuman primates in 1967, sporadic outbreaks of filovirus infection have been reported mainly in central Africa (51). The severity, combined with the absence of treatment and vaccination options licensed for human use, makes filoviruses biosafety level-4 agents and biothreat pathogen of category A. While the transmission route of filoviruses to human and nonhuman primates remains inconclusive, extensive epidemiological studies have implicated fruit bat species as likely natural reservoir hosts of filoviruses (34, 45, 46, 61, 72). Indeed, MARVs were isolated from apparently healthy cave-dwelling Egyptian fruit bats (*Rousettus aegyptiacus*) in Uganda and recently suggested to circulate among these fruit bats (2, 63).

To date, there is one known species in the genus *Marburgvirus*, consisting of two distinct viruses, Marburg virus and Ravn virus. On the other hand, five viruses (Ebola virus, Sudan virus, Taï Forest virus, Bundibugyo virus, and Reston virus) are known within the genus *Ebolavirus*, representing the distinct virus species, *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*, respectively. Recently, a novel bat-derived filovirus, named Lloviu virus, was found in Cueva del Lloviu, Spain, and tentatively classified into the newly proposed genus *Cuevavirus* in the family *Filoviridae* (31, 42), but its pathogenicity for humans and/or nonhuman primates remains to be elucidated.

Filovirus particle consists of at least seven structural proteins, including the only envelope spike protein glycoprotein (GP). The GP precursor assembles as a trimer and is modified by

N-glycosylation in the endoplasmic reticulum. Trafficking of GP precursor to the Golgi apparatus leads to the refinement of N-glycosylation and the addition of O-glycans to the region described mucin-like region (MLR) that is highly divergent among filovirus GPs (8, 19). Subsequently, GP precursor undergoes proteolytic cleavage via ubiquitous host convertase furin and its family members in the trans-Golgi network, resulting in the production of two subunits, GP1 and GP2, which are linked by a single disulfide bond (28, 64, 66). GP1 contains a putative receptor-binding region, glycan cap containing N-glycans, and MLR that has a number of potential N- and O-linked glycosylation sites (18, 32, 33). GP2 has a transmembrane domain, cytoplasmic tail, and internal fusion loop (Fig. 1) (51). Following modification, GPs are transported to and compartmentalized with the other viral proteins at the cholesterol- and glycosphingolipids-rich microdomains designated as lipid raft (7). Despite extensive research, the molecular basis for the pathogenicity of filovirus remains elusive. Various studies have shown that while viral envelope GP is not the sole determinant of filovirus pathogenicity, it contributes significantly to the filovirus virulence (23, 24, 39, 54, 58, 70).

GPs have been known to be involved not only in filovirus replication cycle such as entry and budding, but in the activation of primary target cells as well (e.g., macrophages and dendritic cells) (13, 37, 68, 71). Furthermore, it has been reported that GP expression on the cell surface leads to loss of cell-cell interaction as well as cell rounding and detachment from the culture dish (15, 59, 70). Though these phenomena can be observed in various types of cells, the subsequent results of GP expression are different among cell types (54). While human cardiac microvascular endothelial cells were reported to undergo anoikis and detachment-mediated apoptosis upon GP expression (47), transient expression of GP did not cause death in human embryonic kidney cells (54). GP-mediated downregulation of the host cell surface proteins such as integrin β1 was proposed to be the molecular mechanism for morphological changes of host

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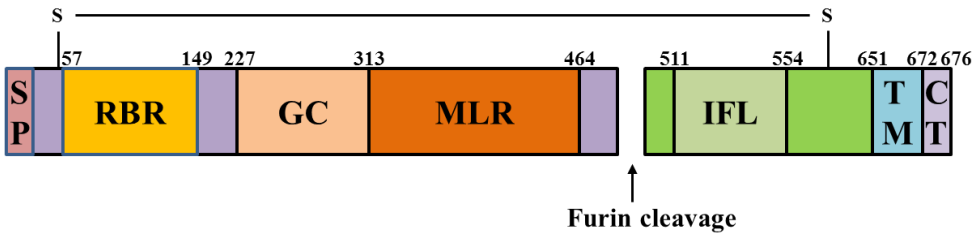

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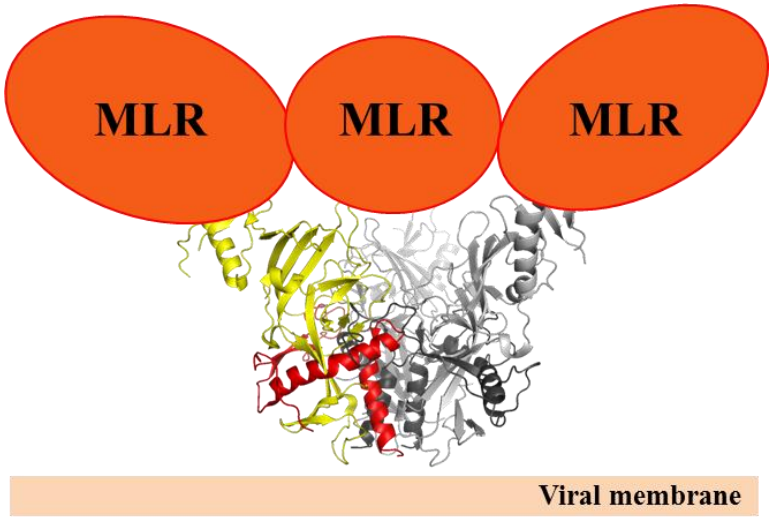

Fig. 1. Structure of Ebola virus (EBOV) envelope GP. Schematic diagram of EBOV GP is shown (A). SP; signal peptide, RBR; putative receptor-binding region, GC; glycan cap, MLR; mucin-like region, IFL; internal fusion loop, TM; transmembrane domain, CT; cytoplasmic tail. The crystal structure of trimeric EBOV GP (PDB code 3CSY) is prepared by using PyMOL (DeLano Scientific LLC) (B). The predicted position of MLR is shown in orange spheres. A hetero dimer composed of GP1 (yellow) and GP2 (Red) is shown.

cells described above (54, 59). However, a recent study demonstrated that these host proteins were indeed expressed but sterically masked by GP on the cell surface (21). It was proposed that the MLR of GP, which is highly glycosylated and spatially occupies a very large region, formed a steric shield over host proteins including integrin β1, major histocompatibility complex class I (MHC I), and other immune molecules on the surface of GP-expressing cells. Then, it was hypothesized that functional impairment of host molecules via steric shielding might be a mechanism determining the severity of filovirus infection.

The present thesis consists of two chapters. In chapter I, I compared the efficiency of steric shielding by GPs against MHC I and integrin β1 molecules among filoviruses and found that shielding efficiency was correlated with the differential pathogenic potential of the filovirus species or strains. It was also found that while MLR is indispensable for steric shielding, the overall properties (e.g., stability and flexibility) of GP molecule, but not the primary structure of MLR itself, was important for efficient shielding of host molecules. In chapter II, to investigate whether steric shielding indeed interferes with the function of host molecules on the cell surface, I analyzed the Fas-mediated apoptosis in cells expressing GPs and found that filovirus GPs formed a steric shield over Fas molecule and suppressed Fas-mediated apoptotic signal.

Chapter I

Differential potential for envelope glycoprotein-mediated steric shielding of host cell surface proteins among filoviruses

Introduction

Among filoviruses, the differential pathogenicity in human has been reported (23, 36) . In the genus *Marburgvirus*, the Angola strain caused the largest outbreak with the presumably highest case fatality rate (90%) among MARVs (5, 6, 16, 22, 55). 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%, whereas Reston virus has never caused lethal infection in human to date. However, the molecular basis explaining this differential pathogenicity of filoviruses remains elusive, although previous studies suggested that GP may play important roles (23, 24, 39, 54, 58, 70).

It was reported that the ability of MLR in GP to mask MHC I and other molecules on the cell surface, coupled with the inhibitory effect of MLR on cell-cell adhesion through the molecules like integrin β1, may be a strategy for avoiding CD8 T cell-mediated killing of EBOV infected cells (21). Then, it should be noted that the sequence of amino acids in MLR is highly divergent among species and strains of filoviruses. Therefore, the possibility is raised that this phenomenon designated steric shielding may be involved in the pathogenicity of filoviruses with the differential shielding efficiency among species and strains of filovirus.

In this study, to investigate the possibility that the steric shielding by GP can be a factor determining the pathogenicity of filovirus, I compared the shielding efficiency against MHC I

and integrin β1 among species and strains of filoviruses having different pathogenicities. It was found that shielding efficiency against integrin β1 was significantly correlated with the differential pathogenic potential of filoviruses. In addition, I mapped a single amino acid involved in the difference of shielding efficiency between MARV strains.

Materials and Methods

Plasmids

For GP expression, complementary DNAs (cDNAs) encoding full-length GPs of strains Mayinga-76 (Zaire), Reston-89 (Reston), Angola, and Musoke were used. By using the primers containing the desired sequences and the class IIS restriction enzyme (BsmBI), the MLR-deletion mutant (ZΔmuc and AΔmuc), chimeric GP constructs (ZZR, ZRR, RRZ, RZZ, AAM, AMM, MMA, and MAA), and mutant GPs with a single substitution (A/H504Y, A/G547V, A/A596T, A/R618K, M/Y504H, M/V547G, M/T596A, and M/K618R) were generated as described previously (39). Wild-type and all mutant GPs were cloned into the mammalian expression plasmid pCAGGS and then used for transfection. All cloned genes were sequenced to ensure that no errors were introduced.

Transfection

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C with 5% CO_2. HEK 293T cells were plated in 6-well plates one day before transfection. Cells were transfected with 2 μg of plasmids by TransIT (Mirus) according to the manufacturer's directions. For analyses of the shielding effects of wild-type Zaire, Reston, and their mutant GPs, cells were cotransfected with 2 μg of plasmids encoding enhanced green fluorescent protein (eGFP) and GPs. For analyses of the shielding effects of MARV GPs, cells were transfected with plasmids encoding wild-type or mutant MARV GPs. Forty hours posttransfection, cells were collected, washed once in fluorescence activated cell sorter (FACS) buffer containing 0.5% fetal calf serum (FCS) and 0.05% sodium azide in

phosphate buffered saline (PBS), and used for flow cytometric analyses.

Monoclonal antibodies to GPs

A mouse monoclonal antibody (MAB) 42/3.7 (IgG1) that broadly binds to GP of all known viruses within the genus *Ebolavirus* (39), ZGP662/1.1 (IgG2a), which recognizes amino acid positions 171-190 (YRGTTFAEGVVAFLILPQAK) in GP1 of Zaire GP (57), MARV GP-specific mouse monoclonal antibody MGP14-22 (IgG1), which recognizes amino acid positions 445-465 (FPFLDGLINAPIDFDPVPNTK) on GP2, and AΔM16-2-13 (IgM) (29) were generated according to a standard procedure reported previously (41, 60), and purified from mouse ascites using protein A agarose columns (Bio-Rad). Purified MGP14-22 was labeled with Alexa Fluor 488 using an Alexa Fluor 488 Protein Labeling Kit (Invitrogen).

Flow cytometry

For detection of integrin β1 and MHC I, allophycocyanin (APC)-conjugated anti-human integrin β1 antibody MAB17-029 (eBioscience) and phycoerythrin-Cy5-conjugated anti-human human leukocyte antigen (HLA)-ABC antibody (eBioscience) were used, respectively. Cells were incubated on ice with these antibodies for 30 min. To detect integrin β1 on GP-expressing cells, MAB1965 (Chemicon) was also used. Transfected cells were washed three times with FACS buffer and then incubated on ice for 30 min with Alexa 647-labeled goat anti-mouse IgG (Invitrogen). To gate Zaire and Reston GP-expressing cells, I first tried to stain the cells with MAB ZGP42/3.7, which recognizes an epitope shared by Zaire and Reston GPs (39). However, Zaire and Reston GPs were stained differently (i.e., the reactivity to Zaire GP was lower than that to Reston GP), most likely due to self-shielding effects against the epitope on GP (21), which might be different between Zaire and Reston GPs. Thus, HEK 293T cells were

cotransfected with plasmids each expressing eGFP or GP (Zaire or Reston), and GP-expressing cells were analyzed by gating the GFP-positive cells. To verify that eGFP-positive cells also express GP, cells transfected with these plasmids in the same conditions were stained with MABs (ZGP746/16.2 or ZGP42/3.7) (57), and I confirmed that at least 70% of cells expressing eGFP also expressed GP. On the other hand, MARV (Angola and Musoke) GPs were similarly stained on the cells by using MAB MGP14-22, and GP-positive cells were gated for the detection of integrin β1 and MHC I, with the exception of staining of MARV GP-expressing cells with MAB MAB1965. For these cells, GFP-positive cells were gated following cotransfection with eGFP- and MARV GP-expressing plasmids. Following gating viable cells by forward and side scatter, 7000 to 10000 eGFP-gated or 4000 to 7000 GP-gated events were accumulated and analyzed for the detection of integrin β1 and MHC I with Becton Dickinson FACS Canto and FlowJo software (Tree Star, Inc.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Expression levels of wild-type and mutant GPs in transfected cells were verified by SDS-PAGE and Western blotting (Fig. 2). Cells expressing GP were lysed with Laemmli sample buffer (Bio-Rad) and the insoluble fraction was removed by centrifugation. Solubilized proteins were separated by SDS-PAGE, and blotted on a polyvinylidene difluoride (PVDF) membrane (Millipore). Non-specific binding to the membrane was blocked with 3% skim milk in PBS. ZGP42/3.7, MGP14-22, ZGP662/1.1, AΔM16-2-13, and an anti-β-actin antibody (AC-15; Abcam) were used as primary antibodies. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson Immuno Research), or anti-mouse IgM (Kirkegaard & Perry Laboratories) followed by visualization with Immobilon Western

chemiluminescent horseradish peroxidase (HRP) substrate (Millipore).

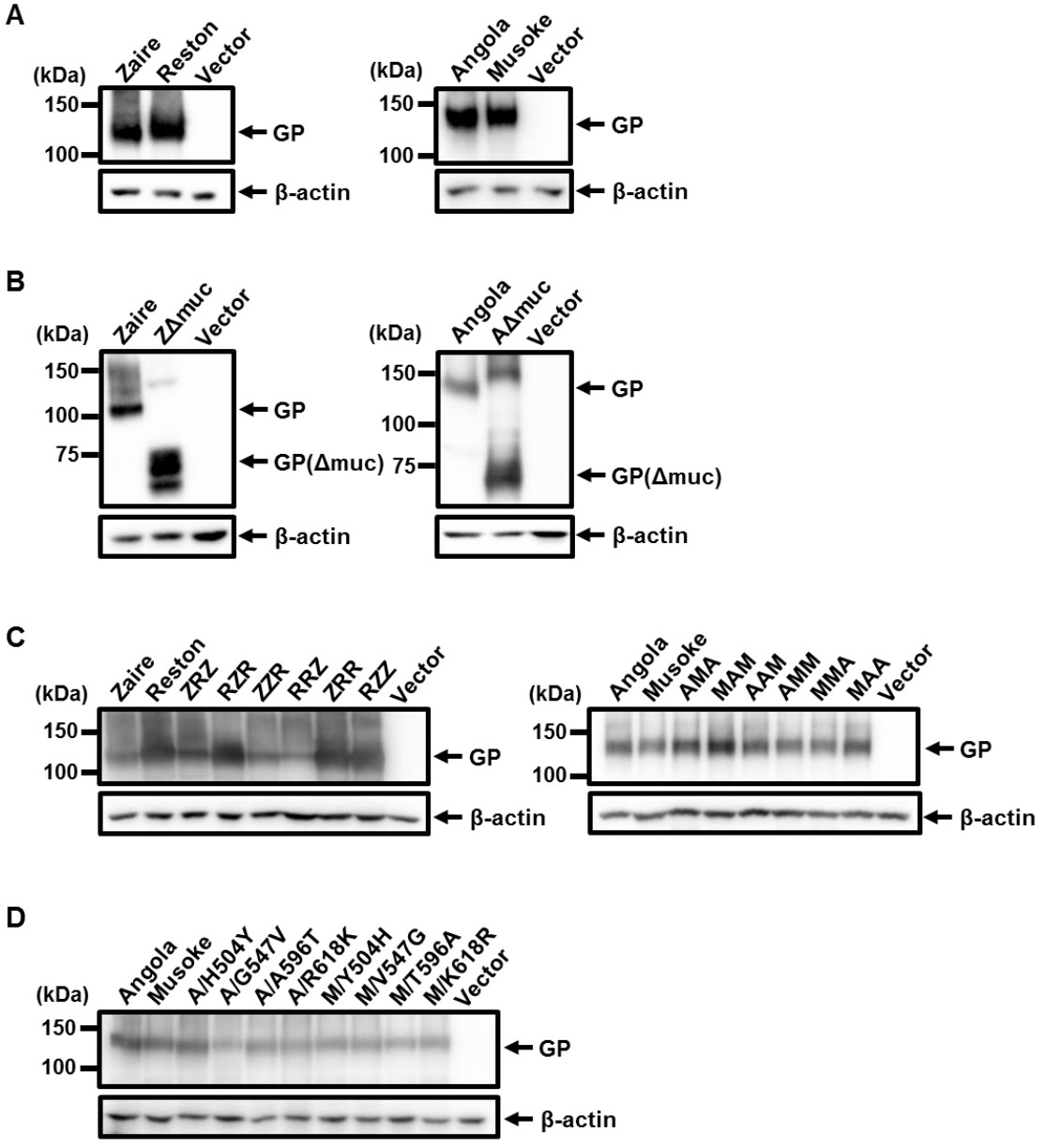

Figure 2. Amounts of GPs expressed in HEK 293T cells. HEK 293T cells transfected with wild-type (A) or mutant GPs (B, C, and D) were analyzed by SDS-PAGE under nonreducing conditions and Western blotting. Wild-type Zaire, Reston GPs, and their chimeric mutants were detected with ZGP42/3.7 (A and C, left panels). For the detection of ZΔmuc, ZGP622/1.1 that bound to ZΔmuc more efficiently than ZGP42/3.7 was used (B, left panel). Wild-type Angola and Musoke GPs, and their mutants were detected with MGP14-22 or AΔM16-2-13. Since AΔmuc lacks the epitope of MGP14-22, another MAB (AΔM16-2-13) that bound to AΔmuc was used (B, right panel).

Results

Comparison of the shielding effects between viruses of the genus *Ebolavirus*.

It has been demonstrated that the MLR forms a steric shield over integrin β1 and MHC I on the surface of GP-expressing cells (21). To compare this effect between viruses with differential pathogenicity, HEK 293T cells were transiently transfected with plasmids expressing GP of a strain Mayinga-76 (Zaire) or a strain Reston-89 (Reston) and analyzed by flow cytometry with probing antibodies to integrin β1 (MAB17-029) and MHC I (Fig. 3). The steric shielding was expected to be observed as decreased cell surface expression levels of these host proteins due to the sterically hindered antibody access to the proteins. A prominent shielding effect for integrin β1 was observed on Zaire GP-expressing cells compared with that of Reston GP (Fig. 3A, left panel). On the other hand, MHC I was comparably shielded by Zaire and Reston GPs and this molecule was almost undetectable on the cells expressing these GPs (Fig. 3B). I quantified the expression levels of both host proteins by calculating the relative means of fluorescence intensity (MFI) and confirmed the significantly different shielding effects between Zaire and Reston GPs (Fig. 3C). It should be noted that integrin β1 was detected normally on the surface of GP-expressing cells when cells were stained with another antibody (MAB1965) whose epitope is likely different from that of MAB17-029 (Fig. 3A, right panel), confirming that the reduced detection level of cell surface integrin β1 by MAB17-029 was not due to reduced expression (i.e., down-regulation) of this molecule. Furthermore, I confirmed by Western blotting that overall intracellular expression levels of these host proteins were not affected by the expression of GPs (Fig. 3D).

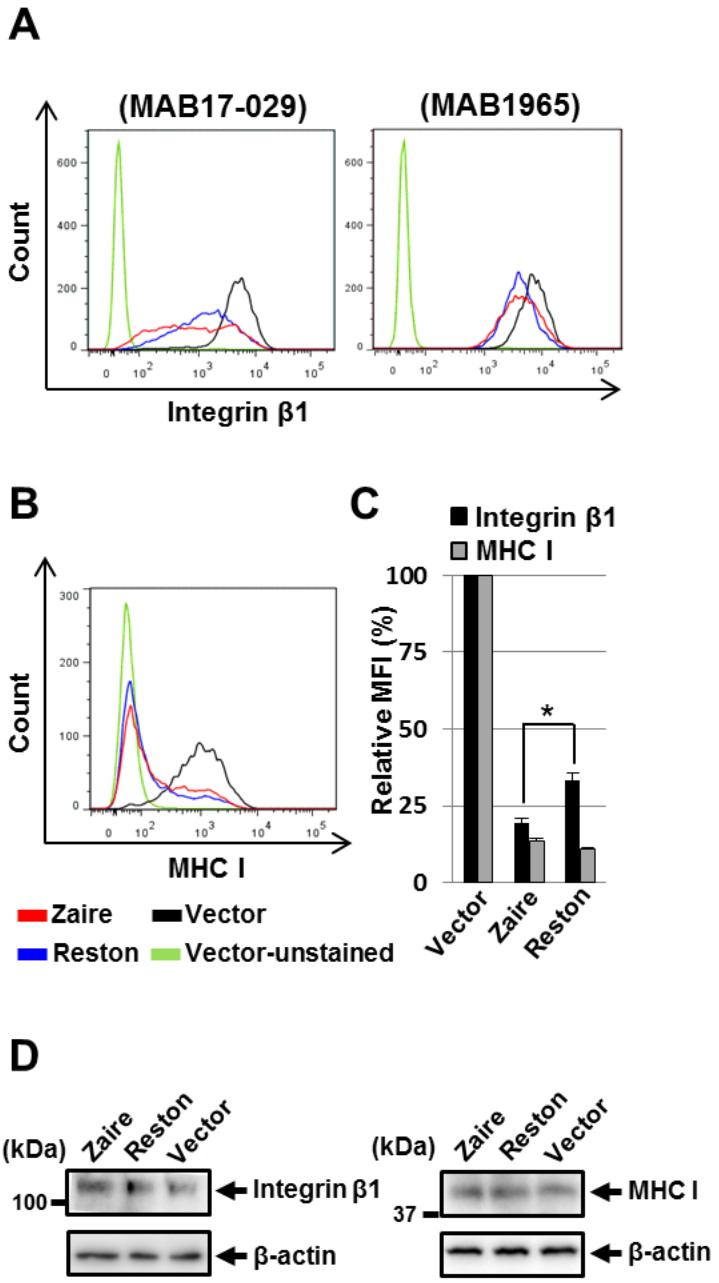

Fig. 3. Differential steric shielding efficiency between Zaire and Reston GPs. HEK 293T cells transfected with pCAGGS expressing wild-type Zaire GP, Reston GP, or pCAGGS alone (Vector) were stained for integrin β1 (MAB17-029 or MAB1965) (A) or MHC I (B) and analyzed by flow cytometry. Data are representative of five independent experiments. To quantify the shielding effects, the relative means of fluorescence intensity (MFI) of detected host proteins was calculated according to the formula [(MFI of GP expressing cells - MFI of unstained cells)/(MFI of vector transfected cells - MFI of unstained cells) x 100] (C). Bars represent the averages of MFI and standard deviations of five independent experiments. Statistical significance was determined using Student's *t*-test (*, $P < 0.05$). Intracellular expression levels of integrin β1 and MHC I were examined by Western blotting (D).

Comparison of the shielding effects between MARV strains.

To examine whether the shielding effect was similarly observed upon MARV GP expression, I selected two strains, Angola and Musoke, which likely have differential pathogenicity for humans and nonhuman primates (5, 6, 16, 22, 55). These MARV GPs also shielded integrin β1 and MHC I molecules on cell surfaces, suggesting that the steric shielding effect is a common phenomenon in filovirus GP-expressing cells (Fig. 4A, B, and C). Consistent with the expression of Zaire and Reston GPs, the MHC I molecule was more markedly shielded than integrin β1. Interestingly, Angola GP showed more prominent shielding effects for both proteins than Musoke GP. Similarly to Zaire and Reston GP-expressing cells, intracellular expression levels of these host proteins were not affected by the expression of MARV GPs (Fig. 4D).

Role of the MLR structure in the steric shielding.

To ascertain whether the highly glycosylated MLR played an essential role for GP-mediated shielding effects, MLR-deletion mutant GPs (ZΔmuc and AΔmuc) were constructed and analyzed their shielding effects by comparing them with the respective wild-type GPs. As expected, reduced shielding effects were observed on the cells expressing MLR-deletion GPs (Fig. 5). I then focused on the role of the MLR in different shielding effects between filovirus strains. Amino acid sequence comparison between Zaire and Reston GPs indicates that the similarity of their MLRs is approximately 16%. Although there is a relatively high amino acid similarity in MLRs between Angola and Musoke GPs (86%), the numbers of potential O-glycosylation sites vary between these GPs (i.e., Angola GP has more potential O-glycosylation sites than Musoke GP), suggesting that the steric shielding effect is potentially dependent on the primary structure of the MLR. To address this hypothesis, I constructed chimeric mutant GPs whose MLRs were swapped between viruses

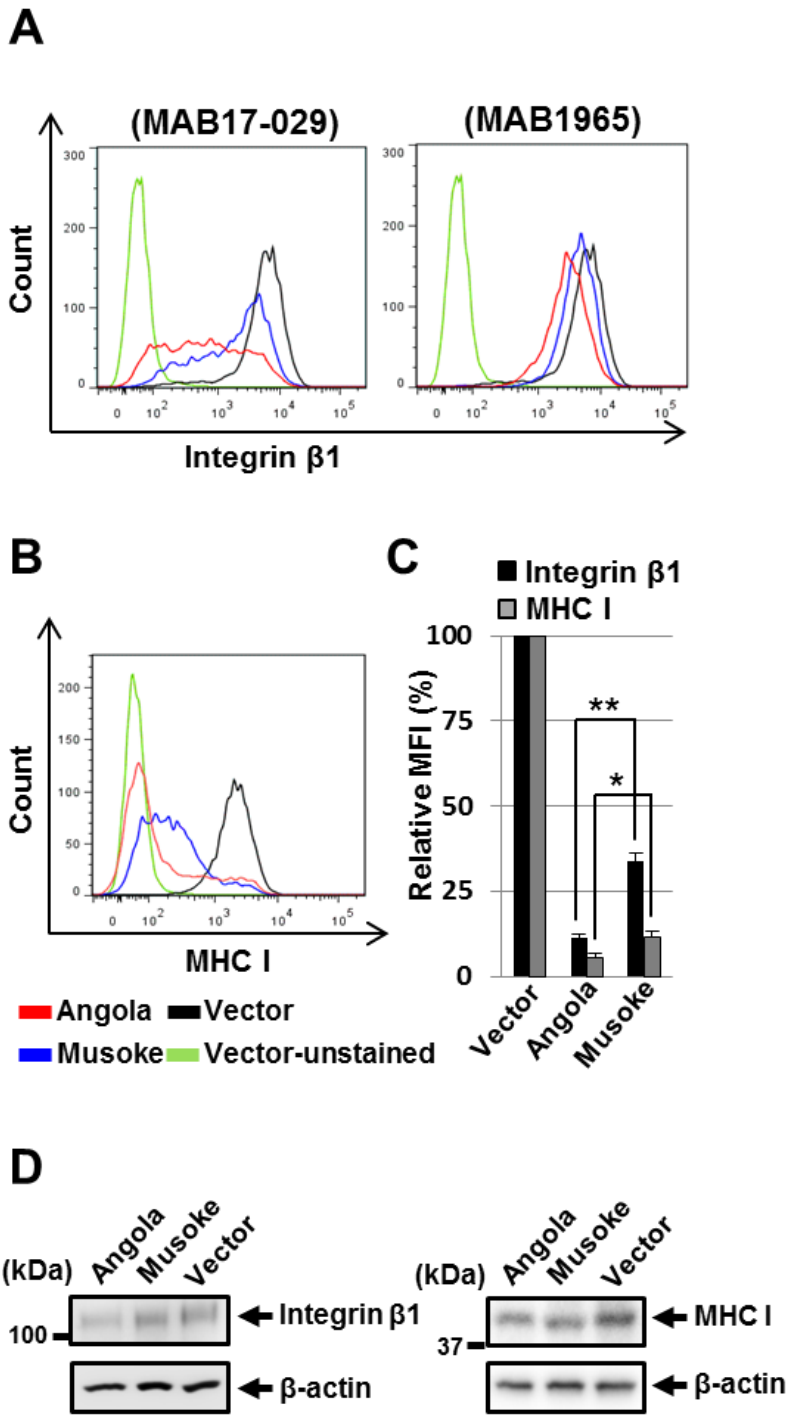

Fig. 4. Differential steric shielding efficiency between Angola and Musoke GPs. HEK 293T cells transfected with pCAGGS expressing wild-type Angola GP or Musoke GP, or pCAGGS alone (Vector) were stained for integrin β1 (MAB17-029 or MAB1965) (A) or MHC I (B) and analyzed by flow cytometry. Data are representative of four independent experiments. MFI and statistical significance were analyzed (C) as described in the legend of Fig. 3 (*, $P < 0.05$; **, $P < 0.01$). Intracellular expression levels of integrin β1 and MHC I were examined by Western blotting (D).

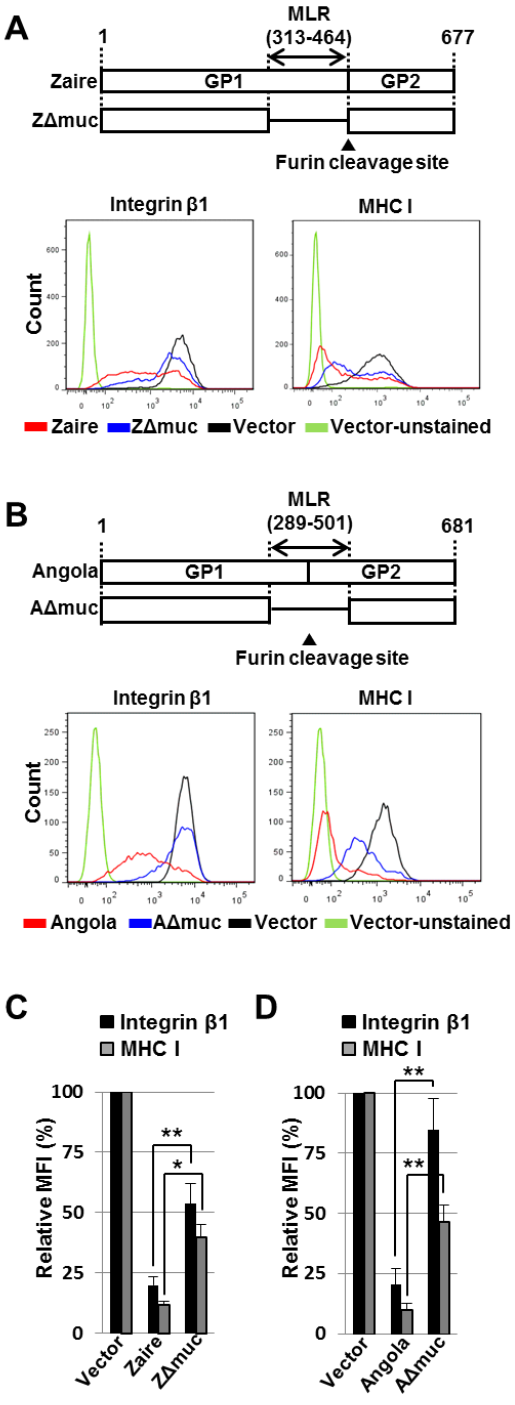

Fig. 5. Reduced steric shielding efficiency after the deletion of the MLR. The names of the mutant GPs and relevant amino acid positions are shown in the schematic (A and B, upper). HEK 293T cells transfected with pCAGGS expressing wild-type Zaire GP, Angola GP, ZΔmuc, AΔmuc, or pCAGGS alone (Vector) were stained for integrin β1 (MAB17-029) or MHC I and analyzed by flow cytometry (A and B, lower). Data are representative of 4 or more independent experiments. MFI (C and D) and statistical significance were analyzed as described in the legends of Figs. 3 and 4.

(ZRZ, RZR, AMA, and MAM) (Fig. 6A and B) and analyzed their shielding effects together with wild-type GPs (Fig. 6C to F). As compared with wild-type Zaire GP, slightly decreased and comparable effects for integrin β1 and MHC I, respectively, were observed on cells expressing ZRZ. Unexpectedly, only small shielding effects on integrin β1 and MHC I was observed on cells expressing RZR. Similarly, swapping of the MLR of MARV GPs (AMA and MAM) did not reverse the phenotype. Taken together, these results indicated that the MLR was required for efficient steric shielding, but its primary structure was not essential for the differential effects between Zaire and Reston or Angola and Musoke GPs.

Identification of the GP region required for efficient steric shielding.

To further investigate which region of GP was involved in the efficiency of steric shielding, chimeric mutants between Zaire and Reston GPs (ZZR, ZRR, RRZ, and RZZ) or Angola and Musoke GPs (AAM, AMM, MMA, and MAA) were constructed (Fig. 7A and B). Then the efficiency of steric shielding caused by these mutant GPs was compared with the condition in wild-type GPs (Fig. 7C to F). While ZZR showed a shielding effect on integrin β1 as strong as that of wild-type Zaire GP, the other chimeric mutants between Zaire and Reston GPs showed comparable or lesser shielding effects compared to wild-type Reston GP. MHC I was almost undetectable on cells expressing these chimeric mutant GPs (ZZR, ZRR, RRZ, and RZZ) as was the case with wild-type Zaire and Reston GPs. On the other hand, all chimeric mutant MARV GPs with the GP2C region (39) derived from Angola GP (i.e., AMA, MAA, and MMA) had shielding effects on both proteins that were as efficient as wild-type Angola GP, indicating that the MARV GP2C region played a critical role in the optimal steric shielding effect.

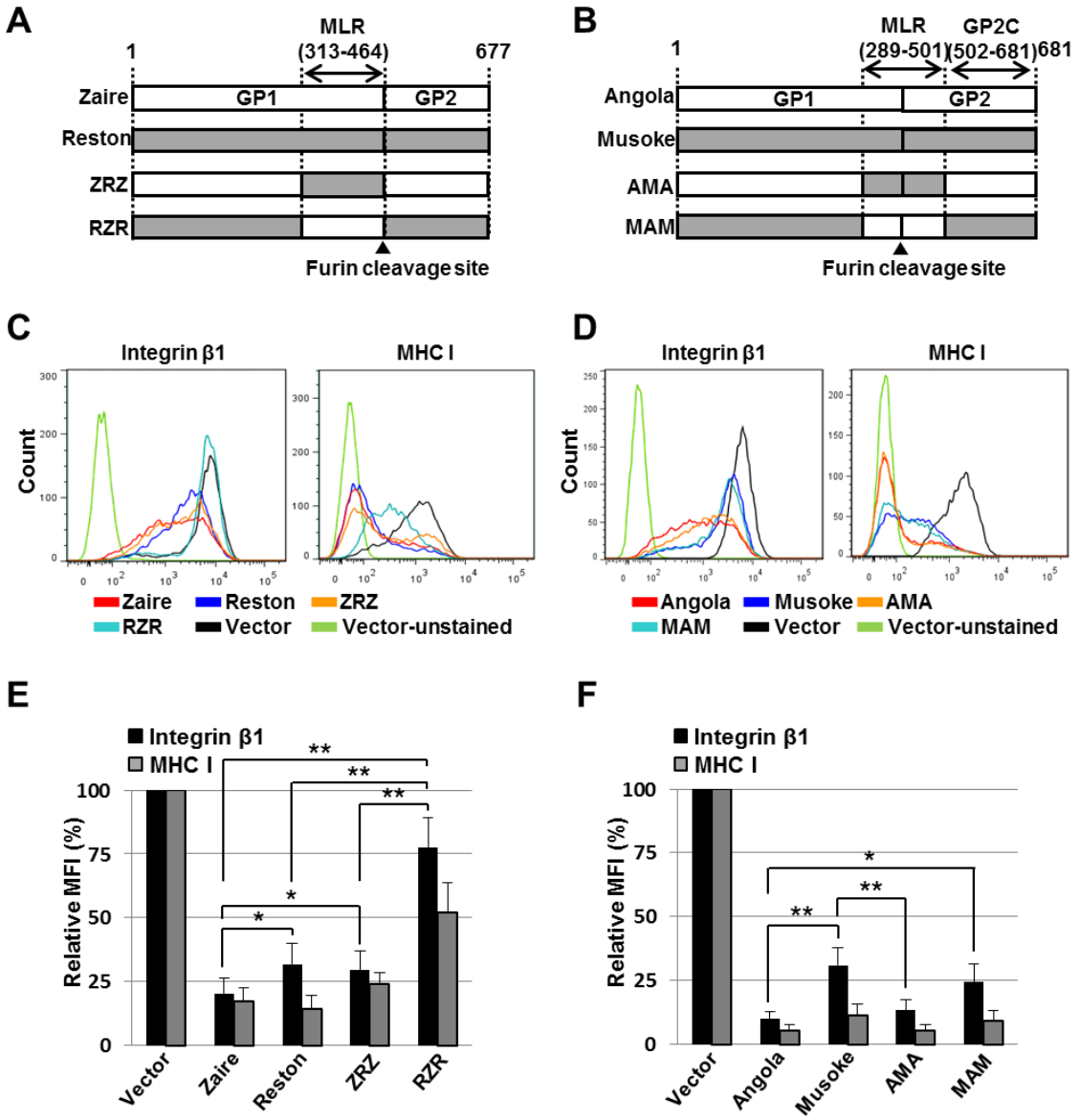

Fig. 6. Limited effects of MLR swapping on the shielding efficiency. Names of the mutant GPs and relevant amino acid positions are shown in the schematic (A and B). HEK 293T cells transfected with wild-type GPs, chimeric mutant GPs, or pCAGGS alone (Vector) were stained for integrin β1 (MAB17-029) or MHC I and analyzed by flow cytometry (C and D). Data are representative of 4 or more independent experiments. MFIs (E and F) were analyzed as described in the legends of Figs. 3 and 4. Statistical significance were analyzed for integrin β1 (*, $P < 0.05$; **, $P < 0.01$).

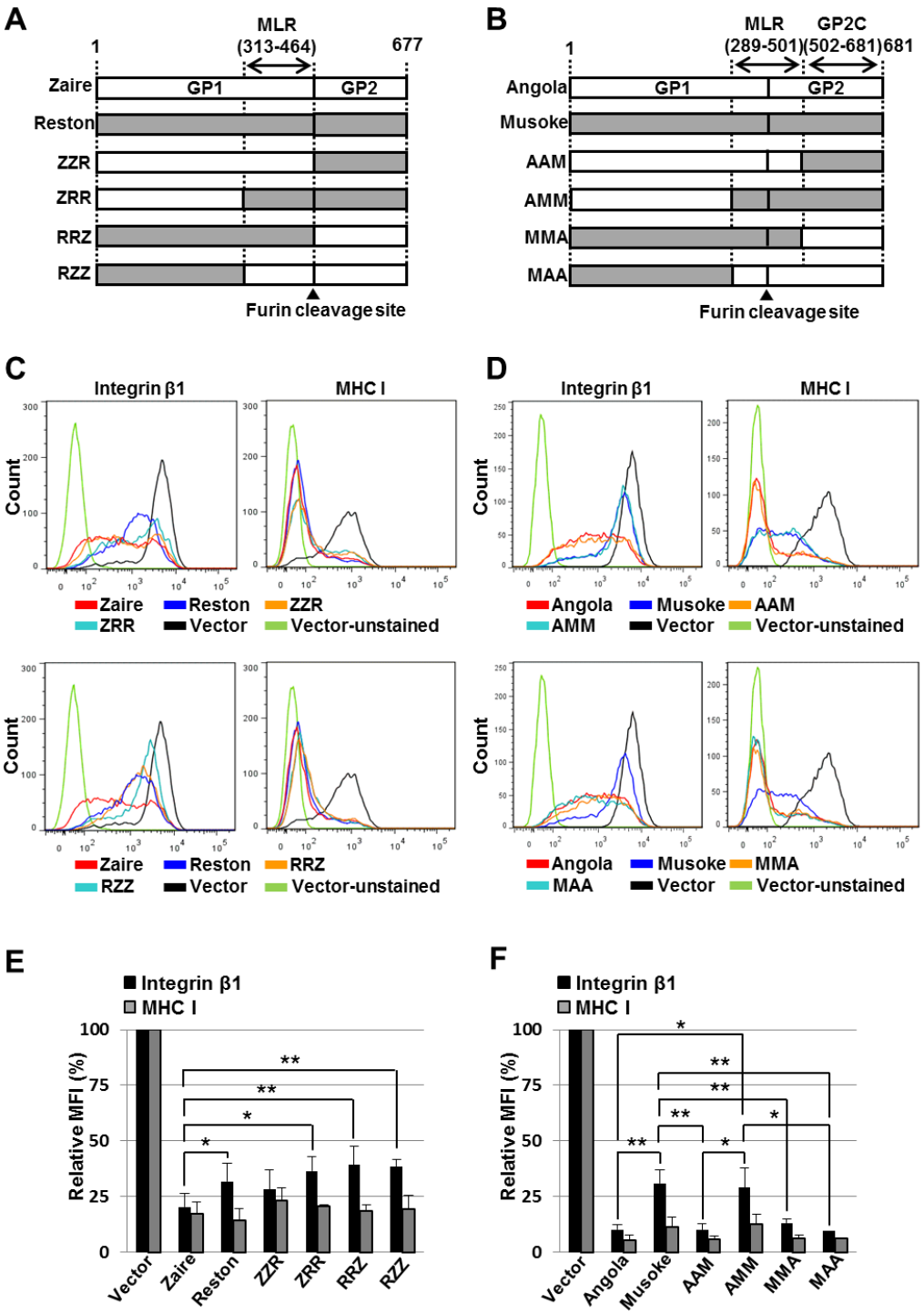

Fig. 7. Importance of the GP2 region of MARV GP for efficient steric shielding. Names of the mutant GPs and relevant amino acid positions are shown in the schematic (A and B). HEK 293T cells transfected with wild-type GPs or chimeric mutant GPs, or pCAGGS alone (Vector) were stained for integrin β1 (MAB17-029) or MHC and analyzed by flow cytometry (C and D). Data are representative of 4 or more independent experiments. MFIs (E and F) were analyzed as described in the legends of Figs. 3 and 4. Statistical significance were analyzed for integrin β1 (*, $P < 0.05$; **, $P < 0.01$).

Importance of the amino acid residue at position 547 for the efficient steric shielding by MARV GP.

Since the difference between MARV GPs was more prominent in the shielding effect, albeit with a much smaller amino acid sequence difference than between Zaire and Reston GPs, I then sought to identify the amino acid residues responsible for the differential ability to produce steric shielding between Angola and Musoke GPs. There are four different amino acids in the GP2C region between Angola and Musoke GPs. Eight mutant GPs containing a single-amino acid substitution: four Angola-based mutant GPs (A/H504Y, A/G547V, A/A596T, and A/R618K) and four Musoke-based mutant GPs (M/Y504H, M/V547G, M/T596A, and M/K618R) were constructed (Fig. 8A and B). The shielding effects of these mutant GPs were compared (Fig. 8C to F). All Angola-based mutant GPs showed shielding effects comparable to that of wild-type Angola GP. Similarly, amino acid substitutions at positions 504, 596, and 618 in Musoke-based mutant GP (M/Y504H, M/T596A, and M/K618R, respectively) did not affect their steric shielding abilities. Interestingly, however, the substitution at position 547 of Musoke GP (M/V547G) resulted in an enhanced shielding effect comparable to that of wild-type Angola GP.

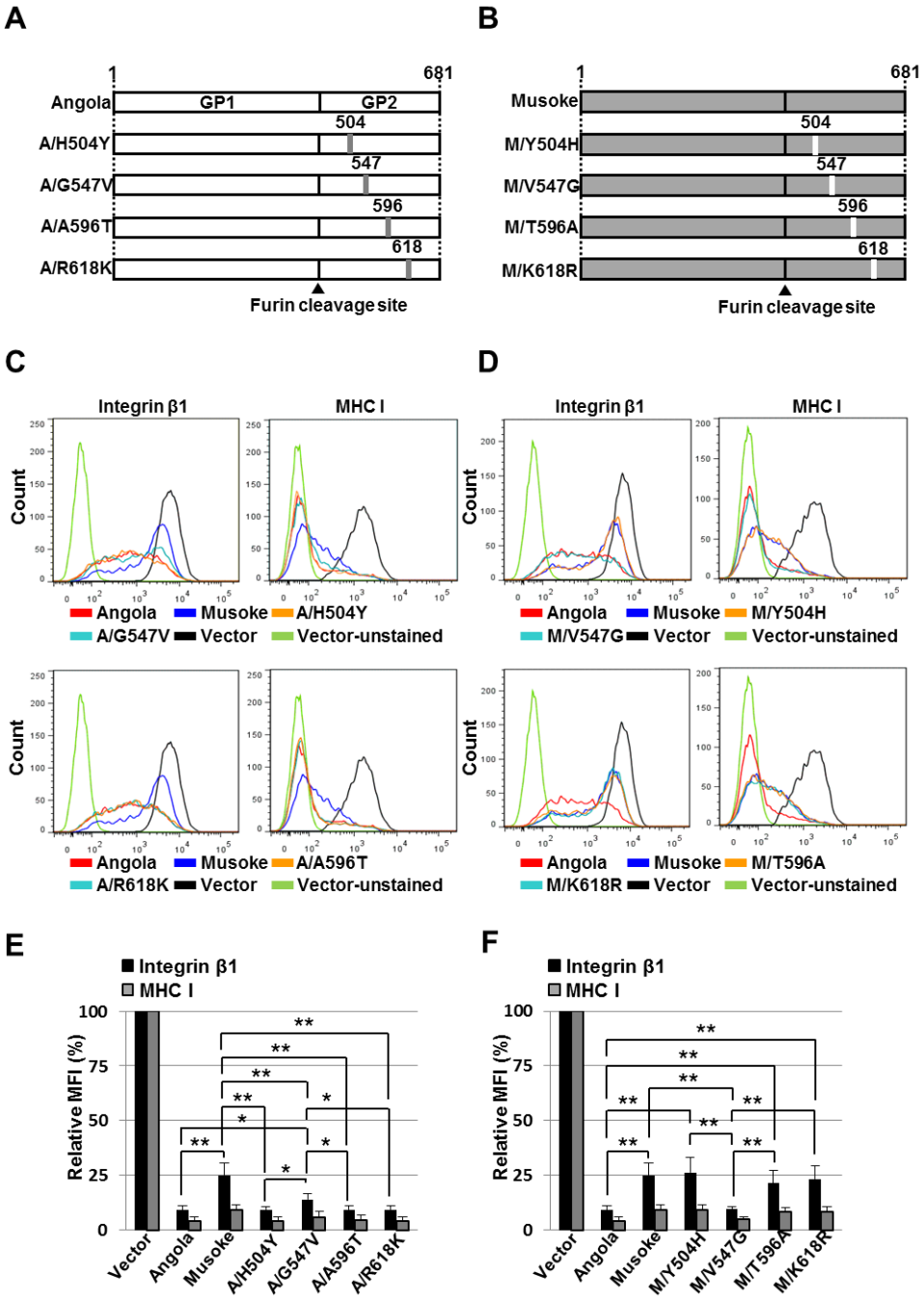

Fig. 8. Importance of the amino acid at position 547 for the shielding efficiency of MARV GP. Names of the mutant GPs and relevant amino acid positions are shown in the schematic (A and B). HEK 293T cells transfected with pCAGGS expressing wild-type Angola GP, Musoke GP, Angola-based mutant GPs (A/H504Y, A/G547V, A/A596T, and A/R618K), or Musoke-based mutant GPs (M/Y504H, M/V547G, M/T596A, or M/K618R), or pCAGGS alone (Vector) were stained for integrin β1 (MAB17-029) or MHC I and analyzed by flow cytometry (C and D). Data are representative of 4 or more independent experiments. MFIs (E and F) were analyzed as described in the legends of Figs. 3 and 4. Statistical significance were analyzed for integrin β1 (*, $P < 0.05$; **, $P < 0.01$).

Discussion

Many viruses have developed strategies to evade host immunity. One of the well-documented mechanisms is interference with the expression of host proteins involved in immune reactions. For example, adenovirus E19 protein prevents MHC I transport to the plasma membrane (3). Cell surface MHC I molecules are internalized upon expression of some viral proteins such as human immunodeficiency virus Nef and Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8 K3/K5 proteins (11, 27), which might result in reduced contact with cytotoxic T lymphocytes. Similarly, it was recently shown that interaction between MHC I and T-cell receptors was blocked upon EBOV GP expression (21). Furthermore, morphological changes (i.e., cell rounding, detachment from the culture dish) induced by dysfunction of cellular adhesion proteins (e.g., integrins) are observed in GP-expressing cells (15, 54, 59, 70). These morphological changes are consistently observed in cells expressing GPs of viruses within all known filovirus species (unpublished data) and indeed also in EBOV-infected cells (1). These phenomena caused by EBOV GP expression are likely due to a distinctive mechanism called “steric shielding” that was recently proposed (21). The present study further indicated that this GP function was common in filoviruses, including MARV.

It is well documented that the MLR of EBOV GP plays a critical role in the morphological changes of GP-expressing cells, likely caused by steric shielding effects (20, 54, 56, 59, 70). It was also reported that the MLR and sugar chains on the GP molecule were important for epitope shielding, suggesting that the MLR plays a crucial role in the steric shielding effect (21, 49). Since amino acid sequences of the MLR vary among filoviruses, I initially assumed that the difference in the steric shielding effects observed among filoviruses was due, at least in part, to the difference of amino acid sequences and glycan structures in the MLR of GPs. However,

contrary to my expectation, the shielding effect was not simply dependent on the primary structure of MLR itself. My data obtained with chimeric mutant GPs suggested the importance of the overall properties (e.g., glycosylation pattern, flexibility, and/or stability) of the GP molecule for the efficiency of steric shielding for host proteins.

In MARV GP, I found that the amino acid residue at position 547 in GP2 was important for the efficiency of the steric shielding. Since the glycine at 547 is presumed to form a stable αβ strand included in the internal fusion loop that wraps around the outside of the GP trimer, it may affect the flexibility of GP and/or the efficiency of conformational change as discussed previously (25, 39). Interestingly, GP mutants, AAM and A/G547V, also showed shielding effects comparable to that of wild-type Angola GP despite their amino acid at the position of 547 being derived from Musoke, suggesting another important factor(s) for the higher shielding capacity of Angola GP. It has been shown that EBOV GP1 has a glycan cap containing N-glycans in the GP1 head subdomain (33). Since this domain is fully exposed on the upper and outer surfaces of GP1, it could also be assumed that these conformations, as well as the GP2 region, contribute significantly to the high shielding efficiency. If Angola GP also has a domain with a similar function, it might compensate for the negatively affecting substitutions in these mutant GPs (i.e., AAM and A/G547V). Structural information on MARV GP may be needed to fully understand the determinants for the differential shielding efficiency between MARVs.

As shown in Figs. 3 and 4, GPs of all filoviruses tested masked MHC I more efficiently than integrin β1. Although epitopes of probing antibodies might be one of the primary factors as shown by the differential recognition of integrin β1 between MAB17-029 and MAB1965, the efficiency of steric shielding was presumed to also be affected by the molecular size of the host protein. Indeed, MHC I consisting of two polypeptide chains, α and β2-microglobulin (45 and 12 kDa, respectively), is much smaller than integrin β1 (130 kDa). To investigate this

hypothesis, I tested another adhesion molecule, CD151 (29 kDa). However, all tested GPs showed only moderate shielding effects for CD151, despite this molecule having smaller molecular weight than MHC I (data not shown), suggesting that other factors might be involved in the efficiency of steric shielding. Since EBOV and MARV GPs might be compartmentalized in the lipid raft during viral assembly and budding (7), host proteins colocalized with GP in the lipid raft are expected to be masked predominantly. Accordingly, MHC I is expressed in the lipid raft in some cell lines (e.g., uveal melanoma and malignant variant of DAC) (9, 67). On the other hand, tetraspanins including CD151 form microdomains known as tetraspanin-enriched microdomains, which are different from the lipid raft (12). These reports may support my hypothesis that the GP-mediated shielding efficiency is dependent on not only molecular size but also the localization of host proteins.

Previously, it was shown that the expression levels of GP were tightly controlled in infected cells and cytotoxic effects occurred late in infection (1, 65). Moderate expression levels of GP did not affect the surface expression of MHC I and integrin β1 (1). Thus, it might be possible that GP-mediated steric shielding may be only observed when GP is expressed at high density on the cell surface. In addition, a recent report suggests that GP is not the sole determinant for the different pathogenicity between Zaire and Reston viruses and other viral proteins also play a significant role (24). Further studies with reverse genetics approaches using infectious recombinant viruses should be needed to clarify the importance of the steric shielding effect in the pathogenesis in infected animals.

In this study, I demonstrated the differential capacity to form steric shielding among filovirus GPs, which might be correlated with their different pathogenicities. It should be noted that Zaire and Reston GPs showed differential efficiency in cell rounding of macrophages (54) which are major target cells whose infection might be directly involved in the pathogenesis of

filovirus infection (17, 23, 53). If GP-mediated steric shielding plays important roles in the evasion of host immune responses initiated by the various signaling factors mounting correct host responses (e.g., Fas, CD80, and CD86), it may be one of the critical determinants for the pathogenesis of filovirus infection. Thus, it is necessary to clarify the importance of the shielding effect on those signaling factors on immune cells such as macrophages and dendritic cells.

Summary

The viral envelope GP is thought to play important roles in the pathogenesis of filovirus infection. It is known that GP expressed on the cell surface forms a steric shield over host proteins such as major histocompatibility complex class I and integrin β1, which may result in the disorder of cell-to-cell contacts and/or inhibition of the immune response. However, it is not clarified whether this phenomenon contributes to the pathogenicity of filoviruses. In this study, I found that the steric shielding efficiency differed among filovirus strains and was correlated with the difference in their relative pathogenicities. While the highly glycosylated MLR of GP was indispensable, the differential shielding efficiency did not necessarily depend on the primary structure of the MLR, suggesting the importance of the overall properties (e.g., flexibility and stability) of the GP molecule for efficient shielding of host proteins.

Chapter II

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

Introduction

It was 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 β1 and MHC I on the surfaces of GP-expressing cells (21). In chapter I, I 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 (43).

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 (35, 40, 52). 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 (62). Following induction of apoptosis, morphologic features such

as cell shrinkage, nuclear fragmentation, and apoptotic body formation are observed (26).

To counteract the induction of apoptosis, some viruses have evolved multiple mechanisms that interfere with the death signal in infected cells (10). Although upregulation of TRAIL and Fas molecules associated with EBOV infection of humans and experimentally infected animals has been reported (4, 44, 48, 50, 69), 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 (44).

In this study, the impact of steric shielding against Fas-mediated signaling, which is one of the major extrinsic pathways for the induction of apoptosis, was investigated. I found that filovirus GPs formed a steric shield over Fas molecule 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 evasion mechanism of host defense by filoviruses.

Materials and Methods

Plasmids

For expression of EBOV and MARV GPs, cDNAs encoding full-length GPs of strains Mayinga-76 (Zaire) and Angola (Angola), respectively, were used. Coding regions of the Lloviu virus GP were synthesized in pBS II SK vector based on the nucleotide sequence of the Lloviu GP (38). Vesicular stomatitis virus (VSV) G protein was used as a negative control. After digestion by restriction enzymes, each gene was cloned into the mammalian expression vector pCAGGS.

Transfection

HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. The cells were transfected with plasmids using Lipofectamine 2000 (Life Technologies) 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, and used for flow cytometric analyses.

Monoclonal antibodies to GPs

Mouse MABs were generated according to a standard procedure reported previously (41, 60). Zaire GP-specific MAB ZGP746/16.2 (IgG2a), which recognizes amino acid positions 391-410 (TPVYKLDISEATQVEQHHRR) in GP1 (57), MARV GP-specific MAB MGP14-22 (IgG1), Lloviu GP-specific MAB LGP14-2 (IgG1), and a VSV G protein-specific MAB [VSV-G(N)1-9] (41) 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).

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 APC-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 hours and treated with 100 ng/ml of an agonistic MAB anti-Fas/Apo-1 (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-hour 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 hour at 37°C in a 5% CO2 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 hours 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.

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 PVDF 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 β-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 chemiluminescent HRP substrate (Millipore).

Results and discussion

In general, it is thought that a death signal through Fas-FasL interaction on the cell surface is involved in the elimination of virus-infected cells (30), 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 (21, 43, 49). Thus, I 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. 9). 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 (21, 49). I found that a prominent shielding effect for the cell surface Fas molecule was commonly observed on Zaire, Angola, and Lloviu GP-expressing cells (Fig. 9A). Overall intracellular protein expression levels of Fas were not affected by the expression of these GPs (Fig. 9B). 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 (7, 14).

To investigate whether GP-mediated steric shielding functionally blocks subsequent apoptotic signaling through Fas-FasL 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-hour incubation with the MABs, cells were stained with cell-permeable

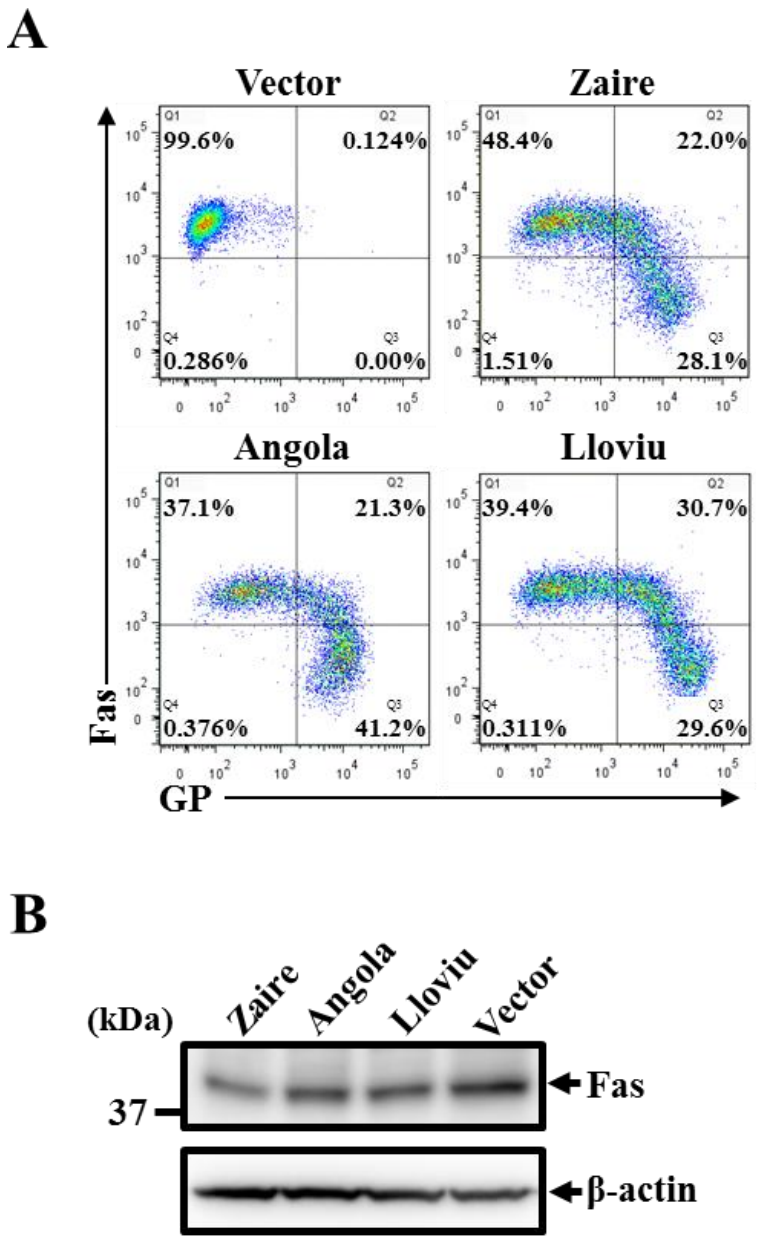

Fig. 9. Intensive steric shielding effects against Fas on filovirus GP-expressing cells. HeLa cells transfected with pCAGGS expressing Zaire GP, Angola GP, Lloviu GP, or pCAGGS alone (Vector) were stained with an anti-Fas antibody and analyzed by flow cytometry (A). Intracellular expression levels of Fas were examined by Western blotting (B). Data are representative of three independent experiments.

FLICA and PI to observe caspase induction and cell viability, respectively (Fig. 10). 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 hours, 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-hour incubation (Fig. 11A). Cell rounding and detachment, most likely due to the disrupted function of adhesion molecules (e.g., integrins) by GP-mediated steric shielding, were observed in filovirus GP-expressing cells but not in VSV G-transfected or vector-transfected cells (Fig. 11B, 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. 11B, 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. 11B, right panels). These results indicated that GP-expressing cells were more resistant to cell death

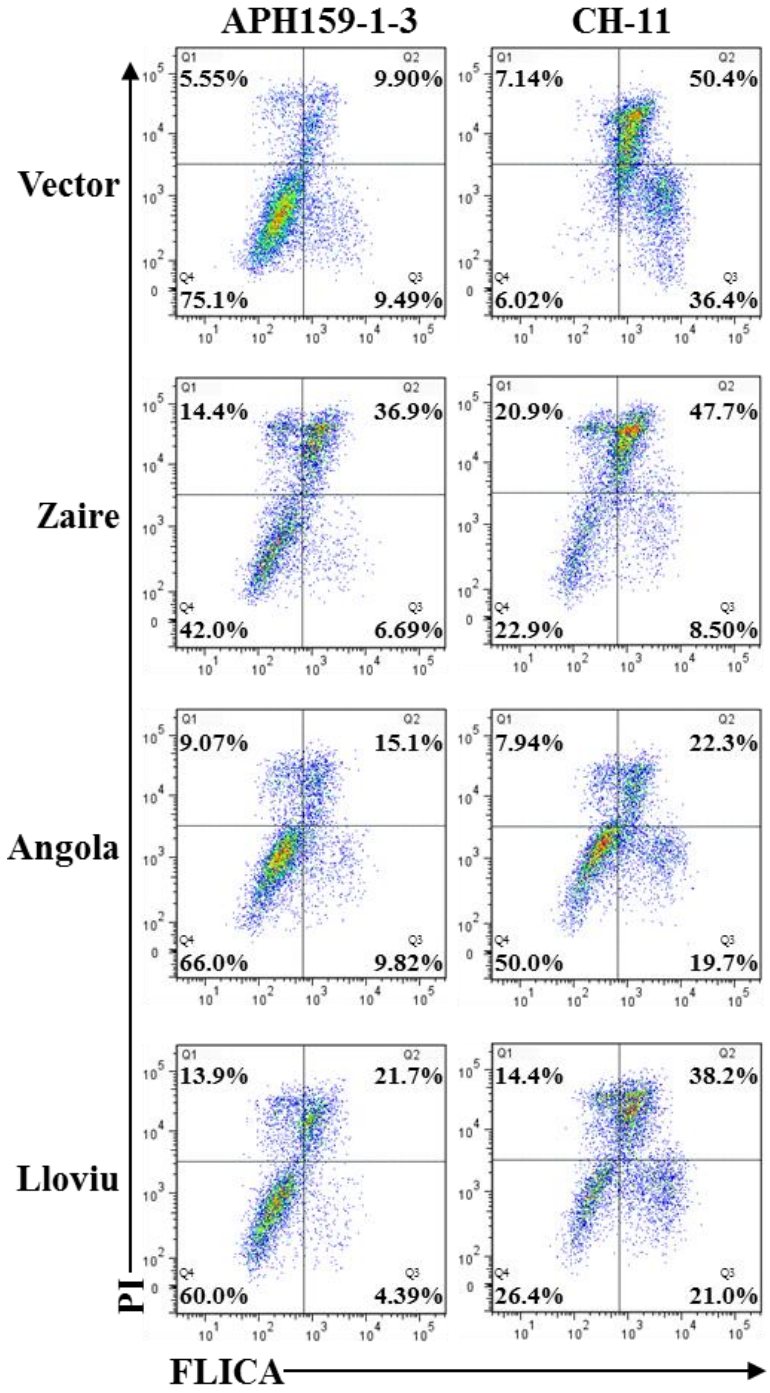

Fig. 10. Reduced Fas-mediated apoptosis in filovirus GP-expressing cells. HeLa cells transfected with pCAGGS expressing Zaire GP, Angola GP, Lloviu GP, or pCAGGS alone (Vector) were incubated with APH159-1-3 or CH-11 for 3 hours, stained with FLICA and PI, and analyzed by flow cytometry. Live cells were assumed to be neither FLICA nor PI positive (FLICA-/PI-). Early apoptotic cells are stained with FLICA but unstained with PI (FLICA+/PI-). Late apoptotic/secondary necrotic cells are positive for both FLICA and PI (FLICA+/PI+). Data are representative of three independent experiments.

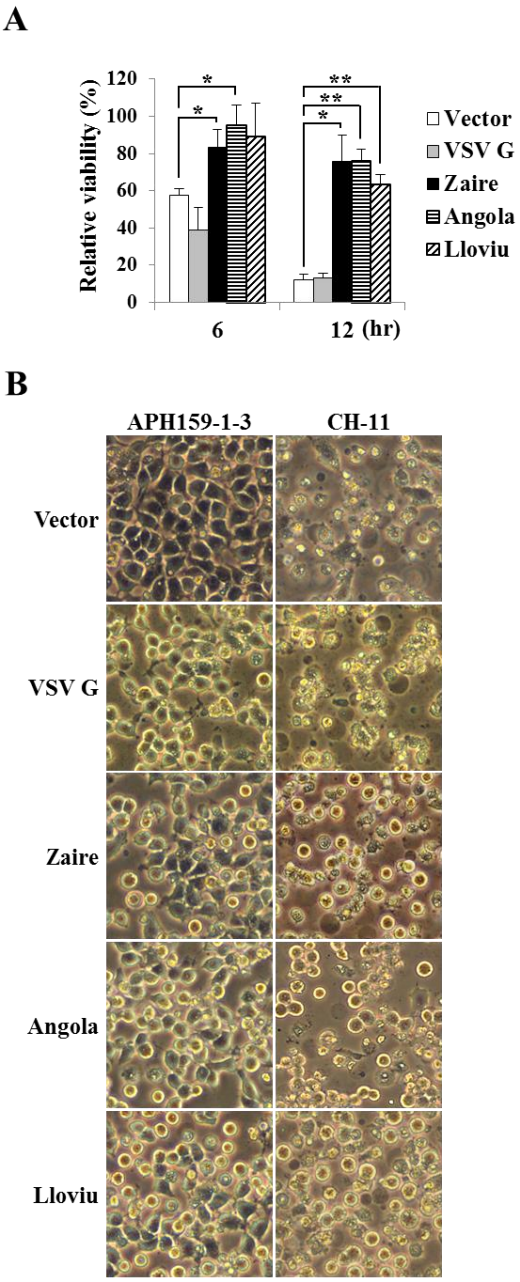

Fig. 11. Increased viability of filovirus-expressing cells after Fas stimulation. HeLa cells transfected with pCAGGS expressing Zaire GP, Angola GP, Lloviu GP, VSV G, or pCAGGS alone (Vector) were incubated in the presence of APH159-1-3 or CH-11, collected at 6 and 12 hours, and stained with PI. PI-negative 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) x 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-hour incubation with APH159-1-3 or CH-11 (B).

induced by Fas-mediated apoptosis than VSV G- or vector-transfected cells.

In this study, I 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 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 β1 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 (1, 65), and that moderate levels of GP expression might not affect the detection of MHC I and integrin β1 on the cell surface (1). Thus, I 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 (44). Thus, modulation of the signaling machinery including the Fas/FasL interaction may therefore be a useful strategy for the development of

antiviral therapeutics.

Summary

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, I found that the filovirus 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.

Conclusions

Filovirus infection causes severe hemorrhagic fever in human and nonhuman primates. Despite extensive research, the molecular basis for the pathogenicity of filoviruses remains elusive. Previous studies showed that the viral envelope GP is involved in the pathogenicity of filoviruses. Recently, a novel GP function designated steric shielding, by which cell surface molecules are sterically shielded and functionally impaired on the surface of GP-expressing cells, has been reported. In this study, I investigated the possible contribution of GP-mediated steric shielding to the pathogenicity of filoviruses and the impact of the steric shielding against Fas-mediated apoptosis.

In chapter I, I found that the steric shielding efficiency differed among filovirus strains and the difference was correlated with their relative pathogenicities. While the GP MLR that is known to be the functional domain for the steric shielding was indispensable, the difference of shielding efficiency among filovirus GPs did not depend on the primary structure of the MLR. In addition, I mapped the GP regions responsible for the different shielding effects observed among the MARV strains tested and found that the amino acid residue at position 547 in GP2 was important for the efficiency of the steric shielding.

In chapter II, I found that the Fas molecule was prominently shielded on the surface of GP-expressing cells, resulting in the suppression of Fas-mediated apoptotic signaling and the increase of viability in GP-expressing cells. This apoptosis suppressive effect via steric shielding by GPs was observed in all tested filoviruses, representative of each genus of the family *Filoviridae*, suggesting that GPs of all filoviruses thus far known may have a common ability of the steric shielding to interfere with host proteins.

The present study suggests that the steric shielding by GPs may play important roles in

impairing the functions of cellular molecules and be involved in the pathogenicity of filoviruses.

Hence, information on the impact of the steric shielding effect against cell surface molecules important for host-cell homeostasis and/or antiviral activity may be helpful in developing antiviral therapeutics.

Acknowledgements

I would like to express my deepest gratitude to my boss, Prof. Ayato Takada (Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan). He gave me innumerable accurate advice and guidance during my course.

I sincerely appreciate supports and guidance for my research and studies from Prof. Yasuhiko Suzuki (Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan), Prof. Hirofumi Sawa (Division of Molecular Pathobiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan), Prof. Hideaki Higashi (Division of Infection and Immunity, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan).

I'm deeply grateful to the Specially Appointed Assistant Prof. Reiko Yoshida (Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan). She gave me several technical and intellectual supports.

I would like to thank Dr. Keita Matsuno (Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Disease, National Institutes of Health, Rocky Mountain Laboratories, Hamilton, MT, USA), Dr. Masahiro Kajihara (Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan), Dr. Eri Nakayama (Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan), Mr. Junki Maruyama (Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan), Assistant Prof. Manabu Igarashi (Division of

bioinformatics, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan), Associate Prof. Norikazu Isoda (Unit of Risk Analysis and Management, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan) for their technical and intellectual cooperation.

I would like to thank Associate Prof. Kimihito Ito (Division of bioinformatics, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan), Dr. Rashid Manzoor, Mr. Makoto Kuroda, Dr. Kazumasa Yokoyama, Ms. Hiroko Miyamoto (Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan) for their technical advice and supports. And also thank to Mr. Hiroki Yamaguchi, Ms. Mieko Muramatsu, Ms. Mari Ishijima, Mr. Naganori Nao, Ms. Wakako Furuyama, Ms. Akina Mori, and Ms. Ichiko Sawada (Division of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan) for their help and encouragement during my research and studies.

Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science has supported my research works.

Finally, I take pleasure in expressing my gratitude to my family, and the all members of Hokkaido University Research Center for Zoonosis Control for their kind and warm supports.

和文要旨

エボラウイルス (EBOV) およびマールブルグウイルス (MARV) はフィロウイルス科に属し、ヒトを含む霊長類に重篤な出血熱を引き起こす。種あるいは株間でヒトに対する病原性が異なっており、病原性を示さないウイルスが存在する一方で 90%もの高い致死率を示すウイルスも存在する事が知られている。しかしながら、フィロウイルスの病原性発現メカニズムについてはまだ不明な点が多く、有効な予防・治療法は確立されていない。フィロウイルスのエンベロープ糖蛋白質 (GP) はウイルス粒子表面に露出している唯一の膜蛋白質である。GP は宿主の全身組織に存在する酵素フューリンおよびその類似蛋白質によって GP1 および GP2 の二つのサブユニットに開裂される。また EBOV GP では GP1 に、MARV GP では GP1 および GP2 にまたがるように、高度に糖鎖付加を受け空間的に非常に大きな領域を占める mucin-like region (MLR) が存在する。近年、フィロウイルス GP 発現細胞の膜表面において、MLR が major histocompatibility complex class I (MHC I) および integrin β1 分子を立体的に覆い、リンパ球の活性化や細胞の接着作用を阻害する現象 (立体的遮蔽効果) が報告された。本研究では、ヒトに対し病原性が異なると考えられているフィロウイルス種および株間で GP の立体的遮蔽効率を比較し、本現象の病原性への関与を検討した。さらに、Fas 分子を介するアポトーシス誘導シグナルに対する立体的遮蔽現象の影響を解析した。

第一章では、ヒトに対して病原性が異なると考えられているフィロウイルス種及び株間で、MHC I および integrin β1 分子に対する GP の遮蔽効率を比較した。また、GP の遮蔽効率に寄与する領域を特定するため、遮蔽効率の異なる種または株間で MLR、GP1 および GP2 領域を入れ換えた変異体 GP を作成し、両宿主因子に対する遮蔽効率を比較した。その結果、解析に用いた全てのフィロウイルス GP は両宿主因子に対して立体的遮蔽効果を示すことが分かった。この時 integrin β1 と比較して MHC I 分子に対し

てより強力な立体的遮蔽効果が観察された。また、MHC I 分子に対しては種及び株間の遮蔽効率の違いはほとんど見られなかった。これは MHC I 分子の分子量が integrin β1 の分子量と比較して非常に小さい事が要因の一つであると考えられた。一方、integrin β1 に対しては、EBOV 属のうち、ヒトに病原性を示さないと考えられている Reston 種よりも、病原性が最も高いとされる Zaire 種の GP の方が高い遮蔽効率を示した。また、MARV においても同様に、流行時の致死率が最も高かった Angola 株 GP の方が Musoke 株 GP よりも両宿主因子に対し遮蔽効率が高いことが分かった。GP による遮蔽効率とフィロウイルスの病原性との間で相関がみられたことから、立体的遮蔽効果はフィロウイルスの病原性発現メカニズムの一つとして機能している可能性が示唆された。MLR は GP が立体的遮蔽効果を示す上で重要な領域であると考えられており、実際に MLR 欠失 GP では遮蔽効率の顕著な減弱が観察された。しかし、興味深いことに、遮蔽効率の異なる種または株間で互いに MLR を入れ換えたキメラ GP においては遮蔽効率の逆転は観察されなかった。その一方で、MARV GP の GP2 サブユニットに存在する 547 番目のアミノ酸を Angola 株と Musoke 株で置換した変異体 GP において、integrin β1 に対する遮蔽効率の逆転が観察された。以上の結果より、MLR は GP が宿主分子を立体的に遮蔽する上で必須であるが、その構造はウイルス種及び株間の遮蔽効率の違いには影響していないことが分かった。MARV GP において、遮蔽効率を左右する 547 番目のアミノ酸は、3 量体を形成する GP の外側を覆うように存在する internal fusion loop を構成するアミノ酸の一つである。従って、GP の遮蔽効率は MLR のみではなく、GP 分子全体の構造安定性によって総合的に決定されると考えられた。

第 2 章では、Fas 分子を介するアポトーシスシグナルに対する立体的遮蔽効果の影響を解析した。Zaire 種および Angola 株の GP 発現細胞において Fas 分子に対する立体的遮蔽効果が観察された。さらに、GP 発現細胞においてアポトーシス誘導性の抗 Fas 抗体刺激によるアポトーシスシグナルが減弱することが分かった。一般に、宿主は感染防

御機構の一つとして感染細胞にアポトーシスを誘導し、ウイルスの拡散を制御していると考えられている。本研究の結果から、フィロウイルスは感染細胞膜表面に GP を発現し、Fas 分子を立体的に遮蔽する事でナチュラルキラー細胞や細胞傷害性 T リンパ球等によるアポトーシス誘導から回避している可能性が示唆された。さらに、近年コウモリから検出された EBOV 様フィロウイルスである Lloviu virus の GP 発現細胞においても同様に Fas 分子に対する立体的遮蔽効果が観察された。従って、GP による立体的遮蔽効果はフィロウイルス感染細胞に広く共通する現象であると考えられた。

本研究により、GP は細胞膜表面において広範囲にわたる宿主因子に対して立体的遮蔽効果を示し、それらの機能を阻害する事でフィロウイルスの病原性に関与している可能性が示唆された。感染防御機構および細胞の恒常性維持に関わる宿主因子に対する立体的遮蔽効果の詳細な解析によって、抗ウイルス薬開発のための重要な知見が得られるものと期待される。

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