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

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Abstract

The viral envelope glycoprotein (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 $\beta 1$, 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, we found that the steric shielding efficiency differed among filovirus strains and was correlated with the difference in their relative pathogenicities. While the highly glycosylated mucin-like region of GP was indispensable, the differential shielding efficiency did not necessarily depend on the primary structure of the mucin-like region, suggesting the importance of the overall properties (e.g., flexibility and stability) of the GP molecule for efficient shielding of host proteins.

Keywords: Ebola virus, Marburg virus, glycoprotein, steric shielding, integrin, MHC, pathogenicity

Introduction

Filoviruses (viruses of the genera *Marburgvirus* and *Ebolavirus* in the Family *Filoviridae*) are enveloped, negative-stranded RNA viruses. Filovirus infection causes severe hemorrhagic fever in human and non-human primates (Feldmann and Geisbert, 2011). To date, there is one known species in the genus *Marburgvirus* consisting of two distinct viruses, Marburg virus (MARV) and Ravn virus. In contrast, five viruses (Ebola virus, Sudan virus, Taï Forest virus, Bundibugyo virus, and Reston virus) are recognized within the genus *Ebolavirus*, representing the distinct virus species, *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*, respectively. Differential pathogenicity has been suggested among filoviruses (Geisbert and Hensley, 2004; Mahanty and Bray, 2004). Ebola virus (EBOV) within the species *Zaire ebolavirus* is thought to be most pathogenic among the viruses in the genus *Ebolavirus* with case-fatality rates up to 90%, whereas Reston virus has never caused lethal infection in humans. Among Marburg viruses, the Angola strain caused the largest outbreak with the highest case fatality rate (90%) among Marburg viruses. While there was another outbreak in Durba in 1998-2000 in which the case fatality rate was 83% (Bausch et al., 2006), it is noted that in most of earlier outbreaks of Marburg hemorrhagic fever case fatality rates did not exceed 50% (Smith et al., 1982; Bausch et al., 2008), and that macaques experimentally infected with the Angola strain died after a rapidly progressive

illness if compared with other viruses such as the strain Musoke (Musoke) tested in the previous experiments (Daddario-DiCaprio et al., 2006; Geisbert et al., 2007). Thus, it could be suggested that the Angola strain is more pathogenic than the Musoke strain, although statistically significant data are not available for the Musoke strain, due to low case numbers. However, the molecular basis explaining the differential pathogenicity of filoviruses remains elusive, although previous studies suggested that the viral envelope glycoprotein (GP) may play important roles (Geisbert and Hensley, 2004; Matsuno et al., 2010; Simmons et al., 2002; Takada et al., 2004; Yang et al., 2000).

Filovirus GP is the only spike protein and is responsible for virus entry into host cells. EBOV and MARV GP undergoes proteolytic cleavage by host proteases such as furin, resulting in production of two subunits, GP1 and GP2, which are linked by a single disulfide bond (Jeffers et al., 2002; Volchkov et al., 1998; Volchkov et al., 2000). GP1 contains a putative receptor-binding region and mucin-like region (MLR) that has a number of potential N- and O-linked glycosylation sites (Dube et al., 2009; Kuhn et al., 2006). GP2 has a transmembrane domain, cytoplasmic tail, and internal fusion loop (Sanchez et al., 2007; Weissenhorn et al., 1998).

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 (Chan et al., 2000; Takada et al.,

2000; Yang et al., 2000). Though this can be observed in various types of cells, the subsequent results of GP expression are different among cell types (Simmons et al., 2002). While human cardiac microvascular endothelial cells were reported to undergo anoikis and detachment-mediated apoptosis upon GP expression (Ray et al., 2004), transient expression of GP did not cause death in human embryonic kidney 293T cells (Simmons et al., 2002).

GP-mediated downregulation of the host cell surface proteins such as integrin $\beta 1$ was proposed to be the molecular mechanism for morphological changes of host cells (Simmons et al., 2002; Takada et al., 2000). However, a recent study demonstrated that these host proteins were indeed expressed but sterically masked by GP on the cell surface (Francica et al., 2010). 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 $\beta 1$, major histocompatibility complex class I (MHC I), and other immune molecules on the surface of GP-expressing cells, which might result in abrogation of cell adhesion and prevention of interaction between lymphocytes and infected cells.

In this study, to investigate the possible contribution of GP-mediated steric shielding to the pathogenicity of filoviruses, we compared the shielding effects among filovirus strains having different pathogenicities and found a correlation between the shielding effects and their

pathogenic potential. In addition, we mapped the GP regions responsible for the different shielding effects observed among the viruses tested.

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 β 1 and MHC I on the surface of GP-expressing cells (Francica et al., 2010). To compare this effect between viruses with differential pathogenicity, HEK293T 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 β 1 (MAB17-029) and MHC I (Fig. 1). 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 β 1 was observed on Zaire GP-expressing cells compared with that of Reston GP (Fig. 1A, 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. 1B). We 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. 1C). It should be noted that integrin β 1 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. 1A, right panel), confirming that the reduced detection level of cell surface integrin β 1 by

MAB17-029 was not due to reduced expression (i.e., down-regulation) of this molecule. Furthermore, we confirmed by Western blotting that overall intracellular expression levels of these host proteins were not affected by the expression of GPs (Fig. 1D).

Comparison of the shielding effects between MARV strains. To examine whether the shielding effect was similarly observed upon MARV GP expression, we selected two strains, Angola and Musoke, which likely have differential pathogenicity for humans and nonhuman primates (Daddario-DiCaprio et al., 2006; Geisbert et al., 2007). These MARV GPs also shielded integrin β 1 and MHC I molecules on cell surfaces, suggesting that the steric shielding effect is a common phenomenon in filovirus GP-expressing cells (Fig. 2A, B, and C). Consistent with the expression of Zaire and Reston GPs, the MHC I molecule was more markedly shielded than integrin β 1. 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. 2D).

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, we constructed

MLR-deletion mutant GPs (Z Δ muc and A Δ muc) 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. 3). We 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 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, we constructed chimeric mutant GPs whose MLRs were swapped between viruses (ZRZ, RZR, AMA, and MAM) (Fig. 4A and B) and analyzed their shielding effects together with wild-type GPs (Fig. 4C and D). As compared with wild-type Zaire GP, slightly decreased and comparable effects for integrin β 1 and MHC I, respectively, were observed on cells expressing ZRZ. Unexpectedly, only small shielding effects on integrin β 1 and MHC I were 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. 5A and B). Then the efficiency of steric shielding caused by these mutant GPs was compared with the condition in wild-type GPs (Fig. 5C and D). While ZZR showed a shielding effect on integrin $\beta 1$ 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 (Matsuno et al., 2010) 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.

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, we 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. We constructed 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) (Fig. 6A and B). The shielding effects of these mutant GPs were compared (Fig. 6C and D). 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.

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 (Andersson et al., 1985). 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 (Blagoveshchenskaya, et al., 2002; Ishido et al., 2000), 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 (Francica et al., 2010). 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 (Chan et al., 2000; Simmons et al., 2002; Takada et al., 2000; Yang et al., 2000). 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 (Alazard-Dany et al., 2006). These phenomena caused by EBOV GP expression are likely due to a distinctive mechanism called "steric shielding" that was recently

proposed (Francica et al., 2010; Reynard et al., 2009). 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 (Francica et al., 2009; Simmons et al., 2002; Sullivan et al., 2005; Takada et al., 2000; Yang et al., 2000). 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 (Francica et al. 2010; Reynard et al., 2009). Since amino acid sequences of the MLR vary among filoviruses, we 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 our expectation, the shielding effect was not simply dependent on the primary structure of MLR itself. Our 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, we 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 $\alpha\beta$ 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 (Harrison et al., 2012; Matsuno et al., 2010). 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 (Lee et al., 2008). 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. 1 and 2, GPs of all filoviruses tested masked MHC I more efficiently than integrin β 1. Although epitopes of probing antibodies might be one of the primary factors as shown by the differential recognition of integrin β 1 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 β 2-microglobulin (45 and 12 kDa, respectively), is much smaller than integrin β 1 (130 kDa). To investigate this

hypothesis, we tested another adhesion molecule, CD151 (32kDa). 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 (Bavari et al., 2002), 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) (Bene et al., 2004; Wadehra et al., 2003). On the other hand, tetraspanins including CD151 form microdomains known as tetraspanin-enriched microdomains, which are different from the lipid raft (Blumenthal et al., 2012). These reports may support our 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 (Alazard-Dany et al., 2006; Volchkov et al., 2001). Moderate expression levels of GP did not affect the surface expression of MHC I and integrin β 1 (Alazard-Dany et al., 2006). 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 (Groseth et al., 2012). 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, we 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 (Simmons et al., 2002) which are major target cells whose infection might be directly involved in the pathogenesis of filovirus infection (Davis et al., 1997; Geisbert and Hensley, 2004; Schnittler and Feldmann, 1999). 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.

Materials and Methods

Plasmids

For GP expression, 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 (Z Δ muc and A Δ muc), 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 (Matsuno et al., 2010). 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 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% CO₂. 293T cells were plated in 6-well plates one day before transfection. Cells were transfected with 2 μ g 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 FACS buffer (0.5% FCS and 0.05% sodium azide in 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* (Nakayama et al., 2010), ZGP662/1.1 (IgG2a), which recognizes amino acid positions 171-190 (YRGTTF AEGVVAFLILPQAK) in GP1 of Zaire GP (Takada et al., 2007), MARV GP-specific mouse monoclonal antibody MGP14-22 (IgG1), which recognizes amino acid positions 445-465 (FPFLDGLINAPIDFDPVNTK) on GP2, and AΔM16-2-13 (IgM) (Kajihara et al., 2012) were generated according to a standard procedure reported previously (Takada et al., 2001; Nakayama et al., 2011), 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 β 1 and MHC I, APC-conjugated anti-human integrin β 1 antibody MAB17-029 (eBioscience) and PE-Cy5-conjugated anti-human HLA-ABC antibody (eBioscience) were used, respectively. Cells were incubated on ice with these antibodies for 30 min. To detect integrin β 1 on GP-expressing cells, MAb 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, we first tried to stain the cells with MAb ZGP42/3.7, which recognizes an epitope shared by Zaire and Reston GPs (Nakayama et al., 2010). 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 (Francica et al., 2010), which might be different between Zaire and Reston GPs. Thus, 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) (Takada et al., 2007), and we 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 β 1 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 (Fig. 2A, right panel). 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 β 1 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 (Supplementary Figure 1). 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, AAM16-2-13, 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 (Millipore).

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Figure captions

Fig. 1. Differential steric shielding efficiency between Zaire and Reston GPs. 293T cells transfected with pCAGGS expressing wild-type Zaire GP, Reston GP, or pCAGGS alone (Vector) were stained for integrin β 1 (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 mean 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 β 1 and MHC I were examined by Western blotting (D).

Fig. 2. Differential steric shielding efficiency between Angola and Musoke GPs. 293T cells transfected with pCAGGS expressing wild-type Angola GP or Musoke GP, or pCAGGS alone (Vector) were stained for integrin β 1 (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. 1 (*, $P < 0.05$;

******, $P < 0.01$). Intracellular expression levels of integrin $\beta 1$ and MHC I were examined by Western blotting (D).

Fig. 3. 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). 293T cells transfected with pCAGGS expressing wild-type Zaire GP, Angola GP, Z Δ muc, A Δ muc, or pCAGGS alone (Vector) were stained for integrin $\beta 1$ (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. 1 and 2.

Fig. 4. 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). 293T cells transfected with wild-type GPs, chimeric mutant GPs, or pCAGGS alone (Vector) were stained for integrin $\beta 1$ (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. 1 and 2. Statistical significance were analyzed for integrin $\beta 1$ (*, $P < 0.05$; **, $P < 0.01$).

Fig. 5. 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). 293T cells transfected with wild-type GPs or chimeric mutant GPs, or pCAGGS alone (Vector) were stained for integrin $\beta 1$ (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. 1 and 2. Statistical significance were analyzed for integrin $\beta 1$ (*, $P < 0.05$; **, $P < 0.01$).

Fig. 6. 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). 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 $\beta 1$ (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. 1 and 2. Statistical significance were analyzed for integrin $\beta 1$ (*, $P < 0.05$; **, $P < 0.01$).

Supplementary Figure 1. Amounts of GPs expressed in 293T cells. 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 Z Δ muc, ZGP622/1.1 that bound to Z Δ muc 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 A Δ M16-2-13. Since A Δ muc lacks the epitope of MGP14-22, another MAb (A Δ M16-2-13) that bound to A Δ muc was used (B, right panel).

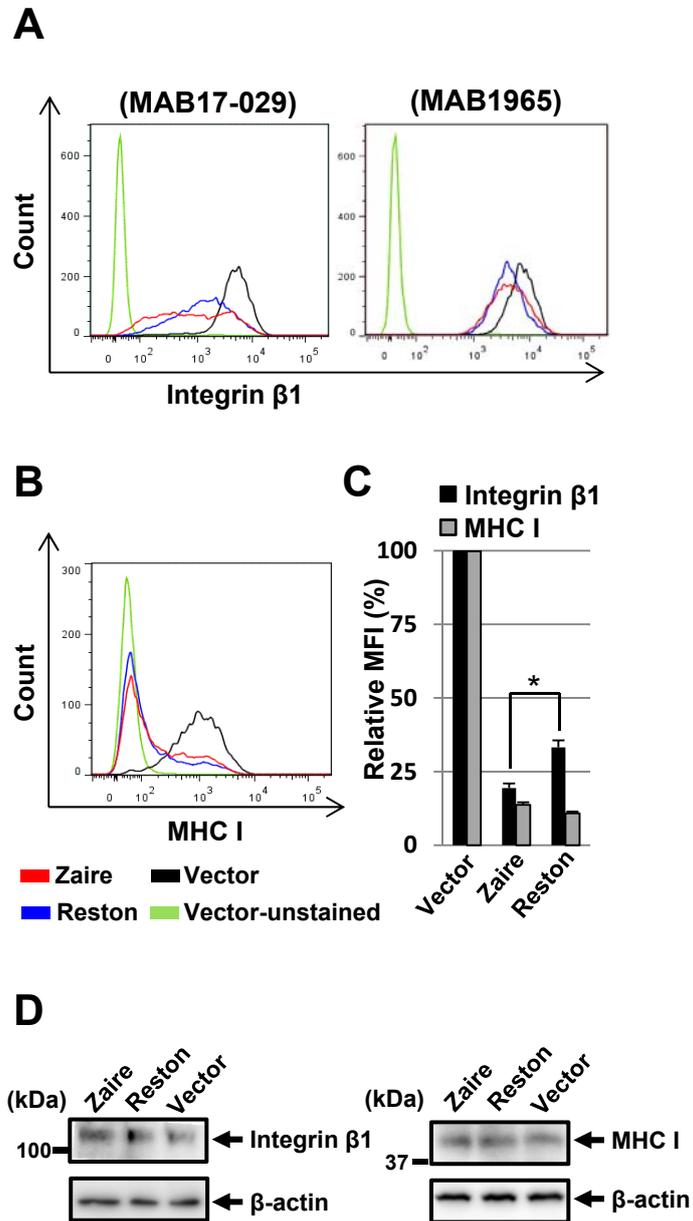


Fig. 1

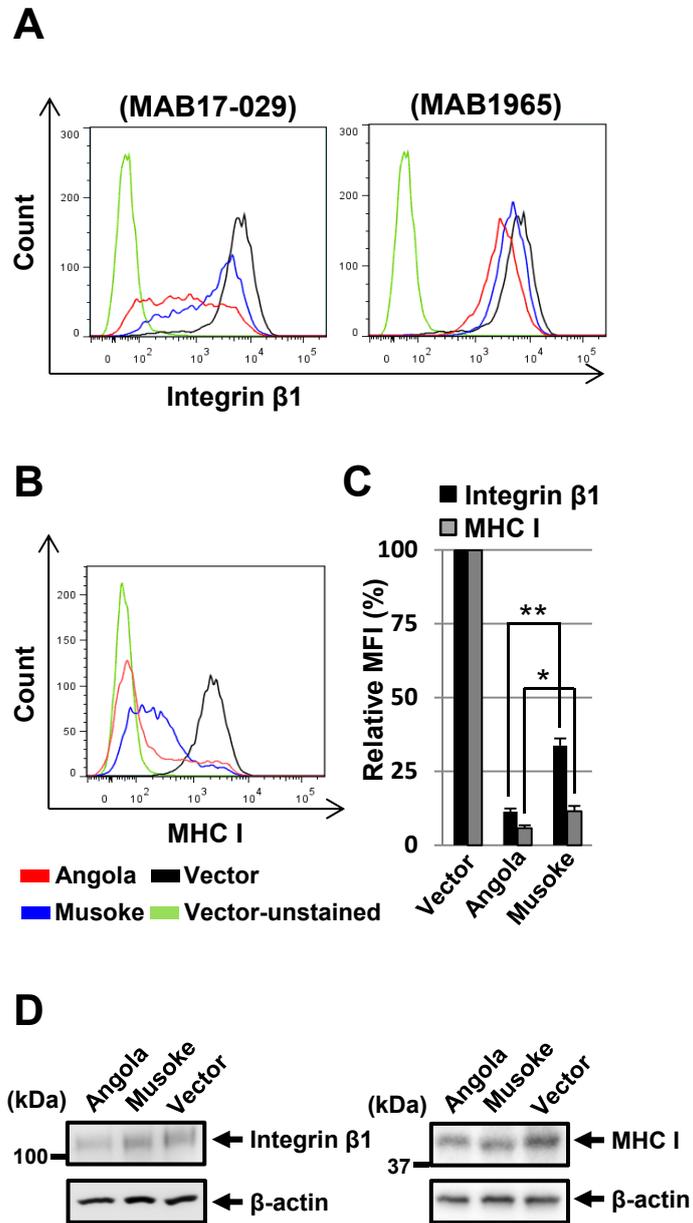


Fig. 2

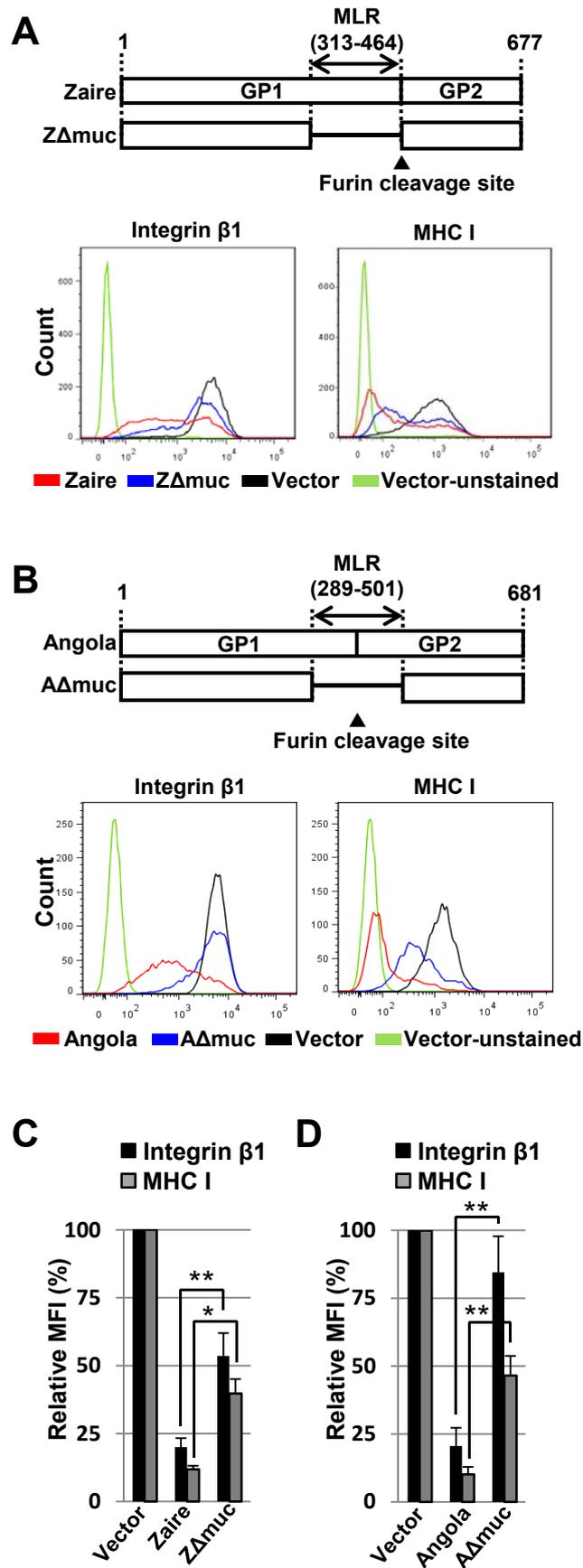


Fig. 3

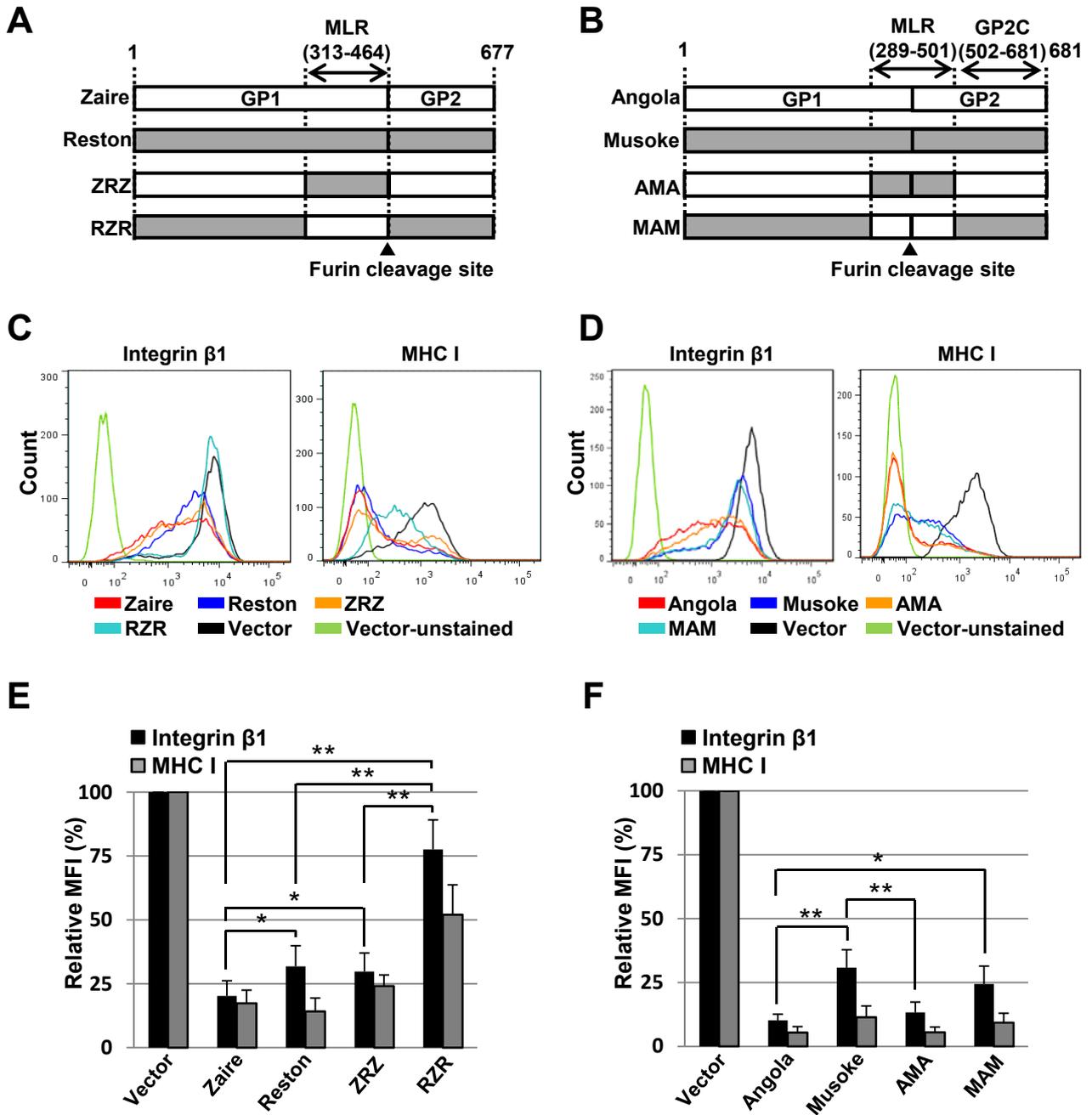


Fig. 4

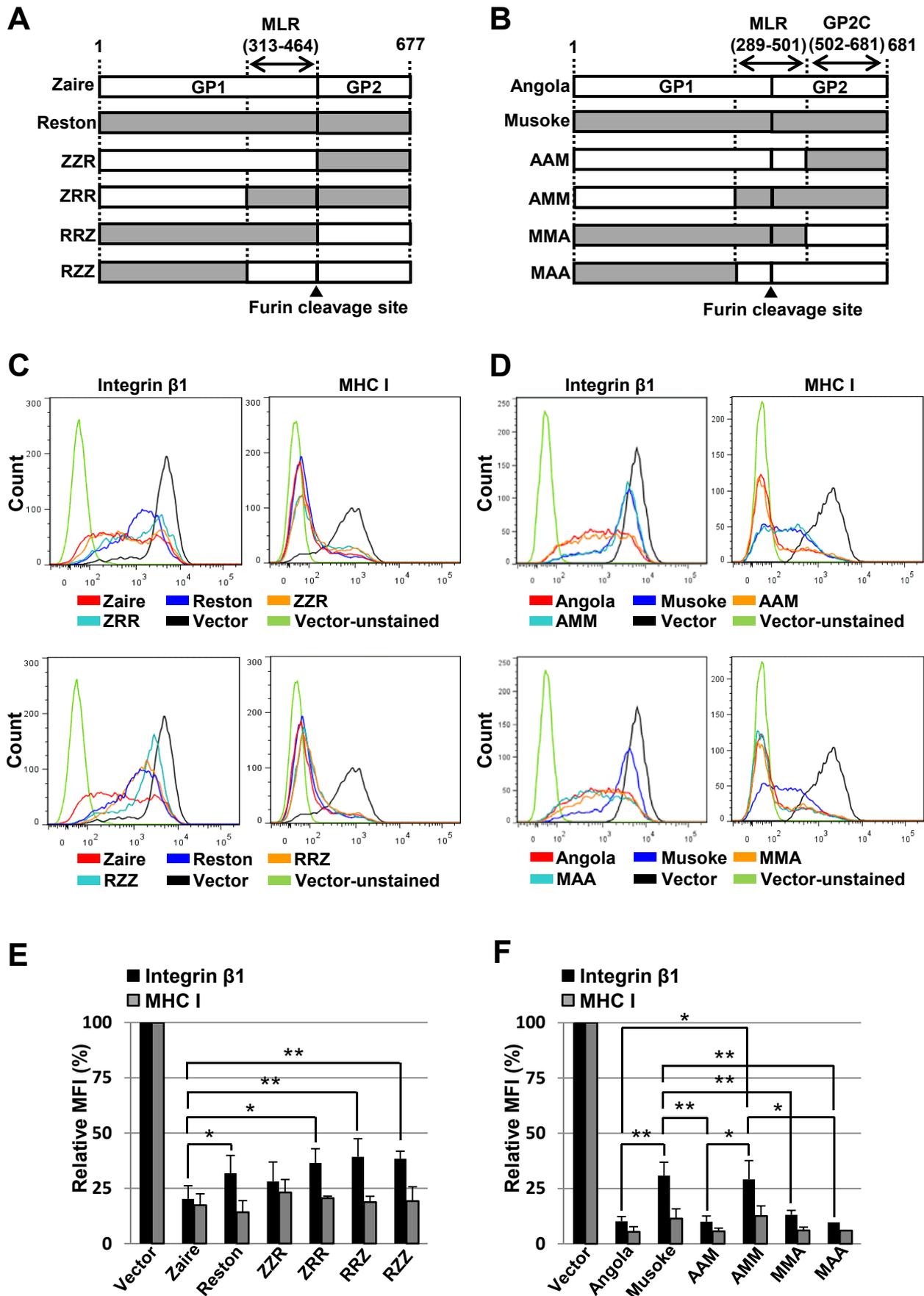


Fig. 5

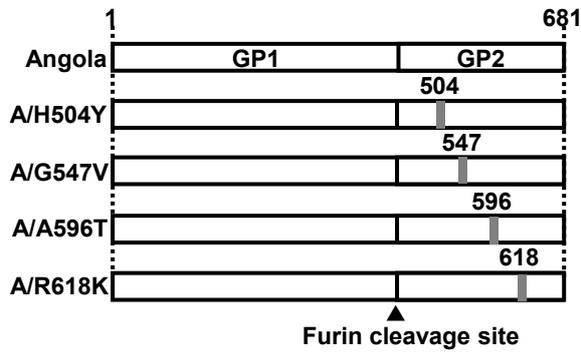
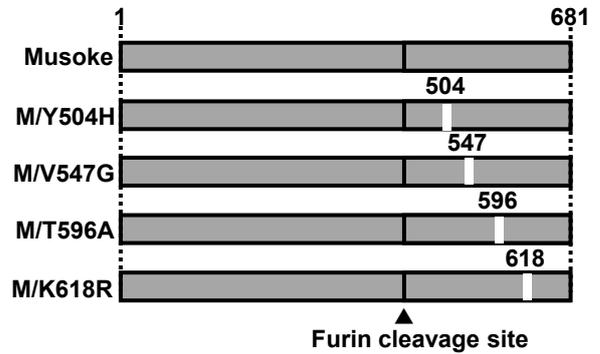
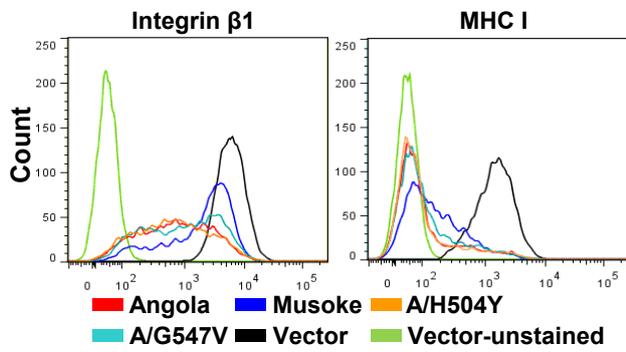
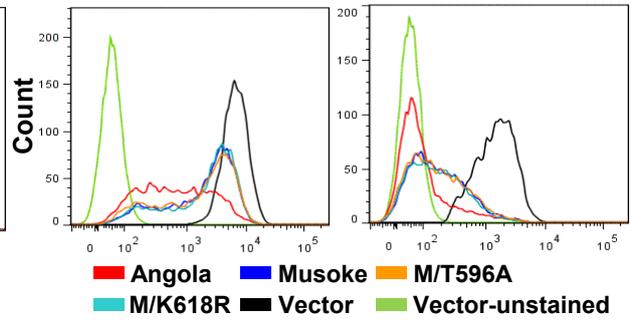
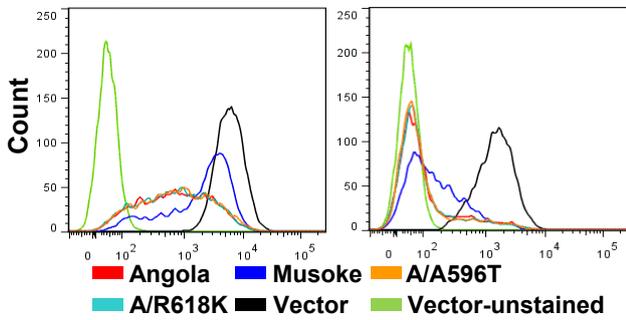
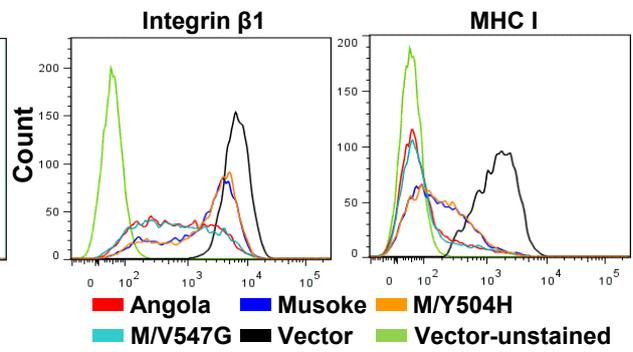
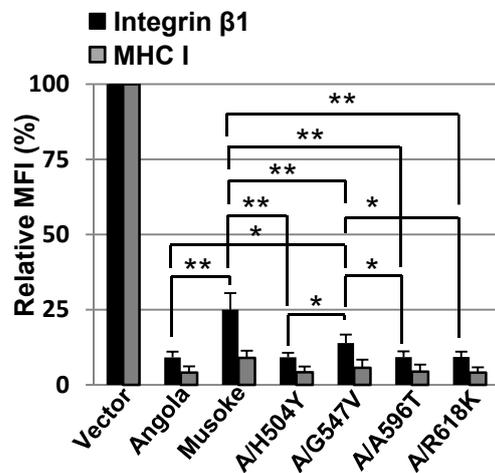
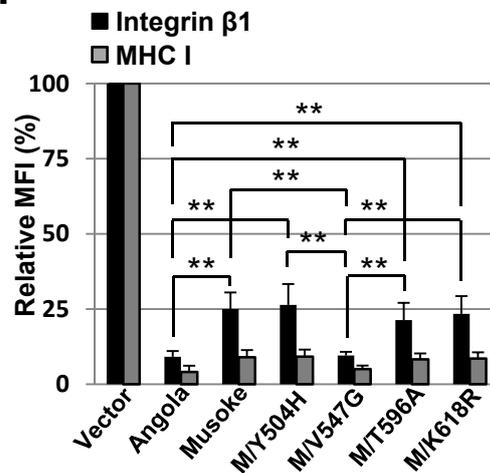
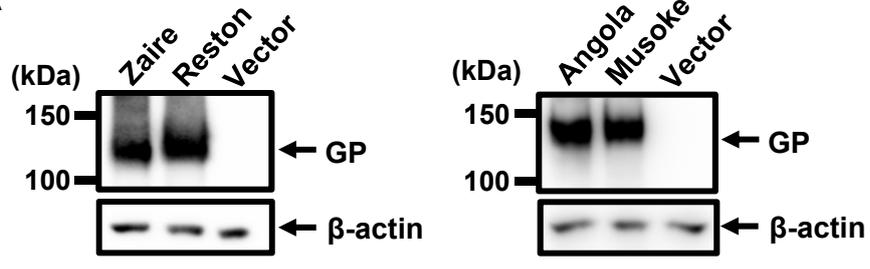
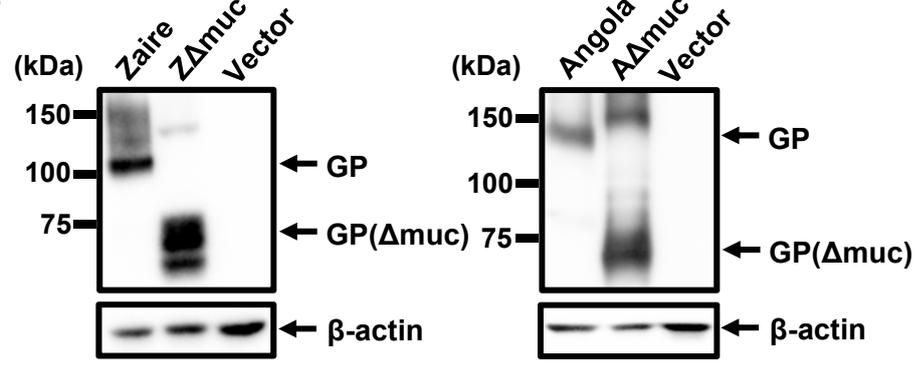
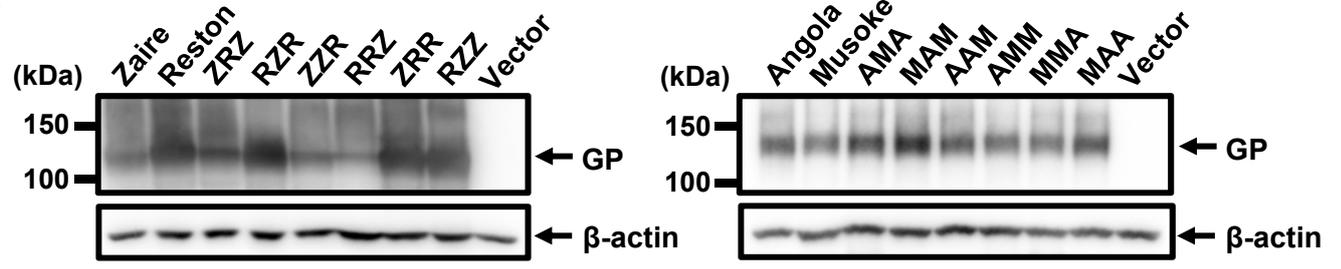
A**B****C****D****E****F**

Fig. 6

A**B****C****D**