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Involvement of viral envelope GP2 in Ebola virus entry into cells expressing the macrophage galactose-type C-type lectin

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ABSTRACT

Ebola virus (EBOV) infection is initiated by the interaction of the viral surface envelope glycoprotein (GP) with the binding sites on target cells. Differences in the mortality among different species of the Ebola viruses, *i.e.*, *Zaire ebolavirus* (ZEBOV) and *Reston ebolavirus* (REBOV), correspond to the *in vitro* infectivity of the pseudo-typed virus constructed with the GPs in cells expressing macrophage galactose-type calcium-type lectin (MGL/CD301). Through mutagenesis of GP2, the transmembrane-anchored subunit of GP, we found that residues 502–527 of the GP2 sequence determined the different infectivity between VSV-ZEBOV GP and -REBOV GP in MGL/CD301-expressing cells and a histidine residue at position 516 of ZEBOV GP2 appeared essential in the differential infectivity. These findings may provide a clue to clarify a molecular basis of different pathogenicity among EBOV species.

1. Introduction

Ebola virus (EBOV) belongs to the family *Filoviridae* and causes severe hemorrhagic fever in humans and nonhuman primates [1,2]. Four species have been identified taxonomically to date: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Cote d'Ivoire ebolavirus* (CEBOV), and *Reston ebolavirus* (REBOV). ZEBOV shows the highest mortality (up to 90%) in humans, whereas REBOV has never been associated with human disease. The molecular basis for virulence of EBOV remains unknown.

EBOV is an enveloped, non-segmented negative-strand RNA virus that contains at least seven structural proteins [3]. One of these gene products, the surface glycoprotein (GP), apparently engages with viral attachment and entry into cells [4,5]. GP is synthesized as a precursor protein GP0, and cleaved into the GP1 and GP2 subunits by furin [6]. GP1 is a surface-exposed subunit, which is thought to interact with cell surface receptors. GP1 also has a mucin-like domain that is highly *N*- and *O*-glycosylated. Many C-type lectins that preferentially recognize *N*- and *O*-glycans (asialoglycoprotein receptor, DC-SIGN, L-SIGN, MGL/CD301, and LSECtin) were reported to function as cellular factors to promote filovirus infection [7–10]. We previously showed that MGL/CD301, enhanced EBOV infection and the infectivity of pseudo-typed virus bearing ZEBOV GP in MGL/CD301-expressing K562 cells (MGL/CD301-K562) was higher than that bearing REBOV GP, suggesting that GP could be a pivotal molecule that determines the different pathogenicity between ZEBOV and REBOV [10].

The other transmembrane-anchored subunit, GP2, forms trimers on the viral envelope and is involved in fusion between viral and cellular membranes through a fusion peptide motif during the initial step of infection [11]. This fusion occurs after cleavage of GP1 by two endosomal cysteine proteases, cathepsin B and cathepsin L, in the endosome [12,13] and the viral genome is released into the cytoplasm, followed by viral replication. However, it remains unknown whether GP2 is involved in the different infectivity of ZEBOV and REBOV in C-type lectin-expressing cells.

In the present study, we used MGL/CD301-K562 to examine the different infectivity between ZEBOV and REBOV. Infectivity of vesicular stomatitis virus (VSV) pseudo-typed with ZEBOV mutant GP whose GP2 was replaced with REBOV GP2 was dramatically reduced down to the extent to match the infectivity of VSV with REBOV GP. By contrast, replacement of REBOV GP2 with ZEBOV GP2 enhanced infectivity of VSV with REBOV GP up to the level of VSV with ZEBOV GP. These results suggest that GP2 has an important role in determining the different infectivity of ZEBOV and REBOV in MGL/CD301-K562. By a mutagenesis approach, we further characterized the importance of the residues 502–527, especially a histidine residue at position 516, of ZEBOV GP2. These findings provide a better understanding of the differences in EBOV pathogenesis between ZEBOV and REBOV and may inform new therapeutic strategies against EBOV infection.

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2. Materials and methods

2.1. Cells

HEK293T cells and human chronic myelogenous leukemia (K562) cells were grown in Dulbecco's modified Eagle Medium–High Glucose (DMEM-HG) and RPMI 1640 medium, respectively, supplemented with 10% FBS. Plasmids encoding MGL/CD301 were transfected using the calcium phosphate method. MGL/CD301-expressing cells were selected with G418 (Nacalai Tesque).

2.2. Viruses

Plasmids encoding wild-type ZEBOV (Mayinga), REBOV (Pennsylvania), and chimeric GP were transfected into human embryonic kidney (HEK293T) cells using Trans-IT LT1 (Takara). Twenty-four hours after transfection, the cells were infected with pseudo-typed virus bearing VSV glycoprotein (VSV G), in which GFP gene is inserted instead of VSV G gene (VSVΔG∗-VSV G) [4] at one multiplicity of infection for 1 h at 37 °C. Cells were then washed with serum-free DMEM-HG twice, and then once with DMEM-HG with 10% fetal bovine serum; then, medium was added. After a 24-h incubation at 37 °C in a CO_2 incubator, the culture fluid was collected and centrifuged to remove cells. Each virus stock was stored at −80 °C until use.

2.3. Virus-like particles (VLPs)

HEK293T cells (4×10^6 cells/10 cm dish) were transfected with pCAGGS-GP and pCAGGS-VP40 using Trans-IT LT1. Ten micrograms of plasmid DNA and 40 μl transfection reagent were mixed in 0.5 ml of OPTI-MEM (Gibco), then added to the cells, and incubated at 37 °C for 48 h. The culture medium was harvested and centrifuged at 7000 rpm for 20 min to remove cellular debris. The supernatants were then layered on a 25% sucrose cushion, and ultra-centrifuged at 27,000 rpm for 1.5 h at 4 °C using Beckman SW28. The supernatant was discarded, and the pellet containing VLPs was resuspended in PBS and stored at −20 °C until use.

2.4. Viral infection

Infectivity of VSVΔG∗ complemented with various GPs was determined by counting GFP-positive cells using flow cytometry. Each viral titer was standardized to infected K562-mock cells with the same amount of virus (i.e., both virus stocks were diluted to give $0.4–1 \times 10^3$ infectious unit/ml in K562-mock cells) [4]. The ratio of infectivity in MGL/CD301-K562 cells to K562-mock cells was determined for each pseudo-typed virus. The relative infectivity (percentage) of infected cells was calculated based on the number of infected K562-mock cells according to the following formula.

$$\text{Relative infectivity} = \frac{\text{number of GFP-positive K562-MGL/CD301 cells infected with one pseudo-typed virus}}{\text{number of GFP-positive K562-mock cells infected with one pseudo-typed virus}} \times 100$$

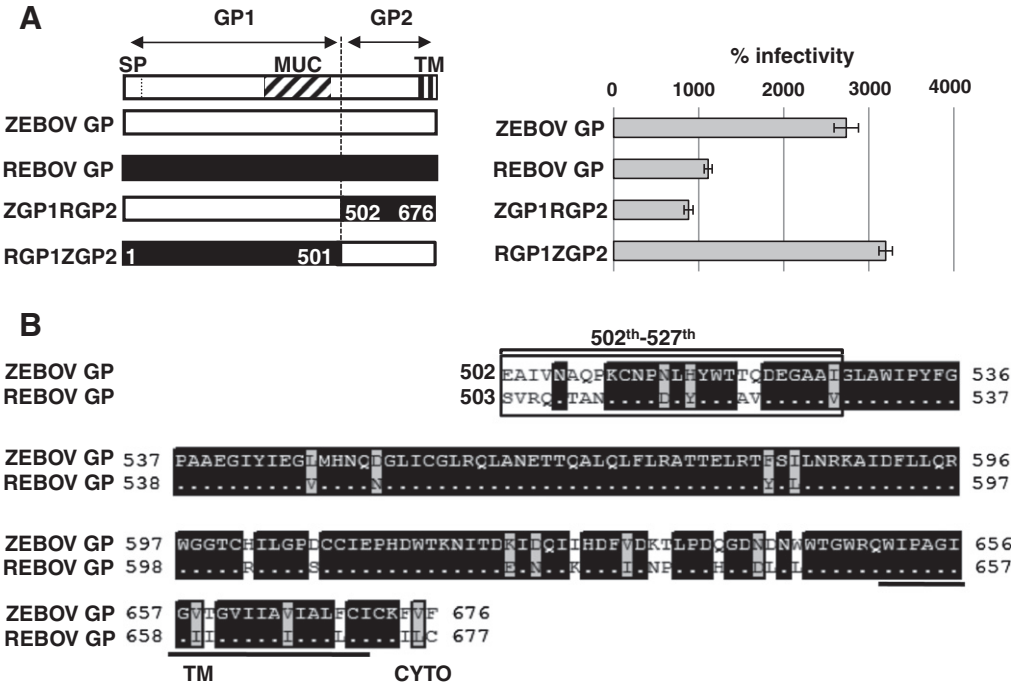

Fig. 1. The importance of GP2 on the infectivity between ZEBOV and REBOV to cells expressing MGL/CD301. (A) MGL/CD301-K562 cells (1×10^5 cells) were infected with VSV pseudo-typed with GPs of ZEBOV, REBOV, and chimeric GPs. The infected cells were counted using a flow cytometer. The titers were standardized to infected K562-mock cells with the same amount of viruses (i.e., both virus stocks were diluted to give $0.4–1 \times 10^3$ infectious units/ml in K562-mock cells) [4]. The ratio of infectivity of MGL/CD301-K562 cells to infectivity of K562-mock cells was determined for each pseudo-typed virus. All experiments were performed three times, and representative results are shown. (B) Comparison of primary structures of GP2 between ZEBOV and REBOV. TM: transmembrane domain and CYTO: cytoplasmic domain.

The relative infectivity represents the value corresponding to the single step infection because the pseudo-typed virus does not replicate or produce virus. Therefore, the actual difference in the infectivity is estimated to be more than orders of magnitude when fold increases are observed in the infectivity of pseudo-typed virus measured by this method.

2.5. GP modeling

Modeling of GP, including the loop lacking as shown by X-ray crystallography structure analyses (the residues 190–213 of GP1), was performed using the protocol from Loop Refinement (MODELLER) in Discovery Studio2.1 (Accerlys). After selecting the best model according to DOPE and PDF scores, model structure was evaluated by Profiles-3D and PROCHECK. Modeling of GP, in which the loop was estimated to be of helix structure, was removed. MolFeat v3.0 (FiatLux) was used for molecular representation [14].

3. Results

3.1. GP2 determines the different infectivity between ZEBOV and REBOV

During the processing of EBOV GP, nascent GP is known to be cleaved by furin into the GP1 (surface-exposed) and GP2 (membrane-anchored) subunits. The GP2 subunit is known to contain a fusion peptide that is involved in fusion of the GP complex with endosomes. To investigate the effect of the structural features of the GP2 on the different infectivity of ZEBOV and REBOV, we constructed VSV pseudo-typed with chimeric GPs whose GP2 were swapped each other (VSV-ZGP1RGP2 and VSV-RGP1ZGP2), and examined their infectivities (Fig. 1A). The infectivity of VSV-ZGP1RGP2 was significantly lower than that of virus with wild-type ZEBOV GP. Infectivity of VSV-RGP1ZGP2 was significantly higher than that of virus with wild-type REBOV GP. These results suggest that GP2 has a pivotal role in determining the efficiency to infect MGL/CD301-K562 cells. We then examined which region of GP2 is important for their different infectivity.

3.2. The amino acid residues between positions 502 and 527 of GP2 play an important role in determining EBOV infectivity

GP2 (amino acid residues at 502–670 of GP) contains the following functional regions: a fusion peptide motif (residues 528–539), an immunosuppressive motif (residues 584–600) [15–17], a transmembrane domain (residues 651–670) and a cytoplasmic domain (residues 671–676) (Fig. 1B). To investigate whether these GP2 regions are involved in the difference of infectivity of pseudo-typed viruses in MGL/CD301-K562 cells, we constructed VSV with chimeric GPs by swapping these regions corresponding to residues 528–676, 651–676, or 671–676 of ZEBOV GP2 and REBOV GP2 and assessed the infectivity of the viruses in MGL/CD301-K562 cells (Fig. 2A and B). All pseudo-typed viruses bearing these chimeric ZEBOV GP2 showed similar infectivity to that of VSV pseudo-typed with wild-type ZEBOV GP. These results suggest that the GP2 regions consisting of residues at 528–676, 651–676, or 671–676 were not involved in the different infectivity between VSV-ZEBOV and -REBOV GPs. We then constructed VSV pseudo-typed with chimeric GPs by exchanging residues at 502–527 between ZEBOV and REBOV GPs and examined their infectivity in MGL/CD301-K562 cells. As shown in Fig. 2B, the infectivity was reversed, indicating that the residue 502–527 determined the difference in infectivity between VSV-ZEBOV and -REBOV GPs.

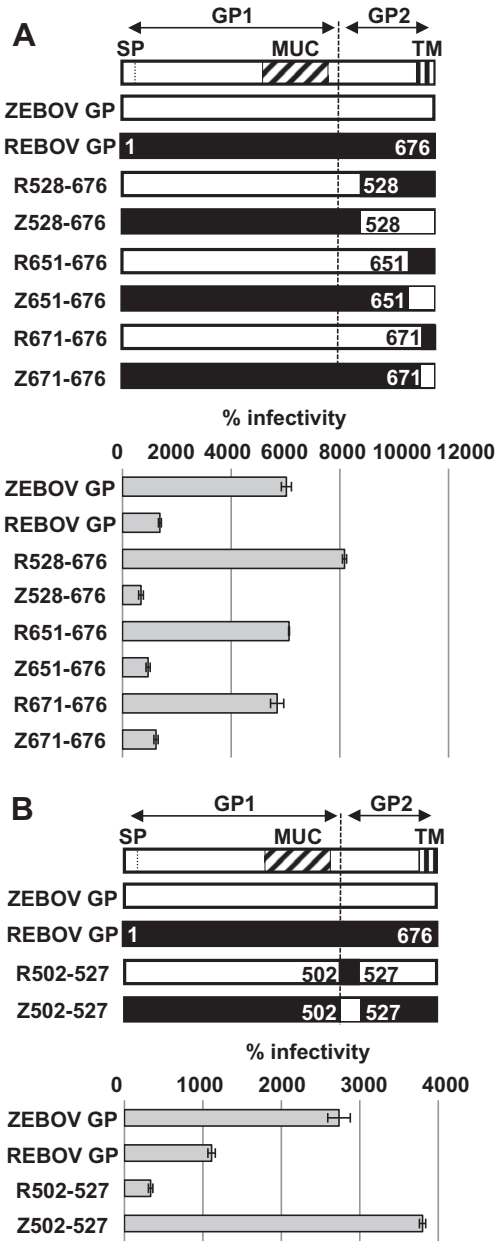

Fig. 2. The effect of each region of GP2 on the different infectivity between ZEBOV and REBOV. VSV pseudo-typed with chimeric cDNA was constructed and MGL/CD301-K562 cells (1×10^5 cells) were infected with these viruses. GPs were swapped at the residues 528–676, 651–676, or 671–676 in panel A. Residues at 502–527 were swapped in panel B. The infected cells were counted using a flow cytometer. The titers were standardized to infected K562-mock cells with the same amount of viruses (i.e., both virus stocks were diluted to give $0.4–1 \times 10^3$ infectious units/ml in K562-mock cells) [4]. The ratio of infectivity of MGLCD301-K562 cells to infectivity of K562-mock cells was determined for each pseudo-typed virus. All experiments were performed three times, and representative results are shown.

3.3. The importance of histidine 516 for the infectivity of EBOV to cells expressing MGL

The residues at 502–527 of GP consist of part of epitope (amino acid residues 505, 507–509, 511 and 514) of a neutralizing antibody, KZ52, derived from a human survivor of the 1995 Kikwit outbreak [18], and of β19 sheet, (residues 513–519) which stabilizes the internal fusion loop of EBOV GP by forming antiparallel β-structure with β20 sheet (residues 544–550). Out of these, six

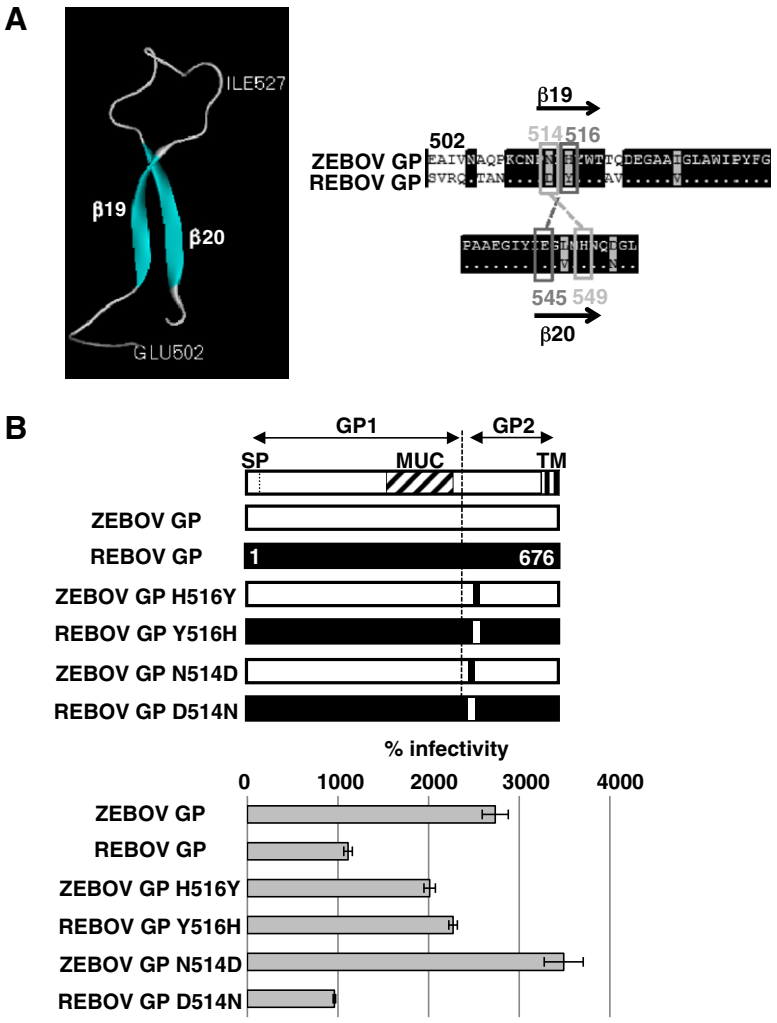

Fig. 3. The effect of residues 514 and 516 of GP2 on the different infectivity of ZEBOV and REBOV. (A) X-ray crystal structure of GP2, including β 19 and β 20 sheets. (B) VSV pseudo-typed viruses were constructed with chimeric GPs swapped at the residues 514 and 516. MGL/CD301-K562 cells (1×10^5 cells) were infected with these viruses. The infected cells were counted using a flow cytometer. The titers were standardized to infected K562-mock cells with the same amount of viruses (i.e., both virus stocks were diluted to give 0.4–1×10^3 infectious units/ml in K562-mock cells) [4]. The ratio of infectivity of MGL/CD301-K562 cells to infectivity of K562-mock cells was determined for each pseudo-typed virus. All experiments were performed three times, and representative results are shown.

amino acids, residues at 505, 507–509, 514, and 516, were different between ZEBOV and REBOV GPs. Judging from the crystal structure of ZEBOV GP [14], asparagine at 514 and histidine at 516 of GP in β19 sheet were predicted to interact with histidine at 549 and glutamic acid at 545, respectively, by hydrogen bonding at low pH (Fig. 3A). Aspartic acid at 514 of REBOV GP and histidine at 516 of ZEBOV GP might more easily form hydrogen bonds with counterparts compared to asparagine at 514 of ZEBOV GP and tyrosine at 516 of REBOV GP, thus affecting their ability to mediate entry into C-type lectin-expressing cells.

To test this hypothesis, we constructed pseudo-typed viruses carrying GPs whose amino acid residues at positions 505–509, 514, and 516 were replaced mutually, and examined the infectivity of these viruses to MGL/CD301-K562 cells. The infectivity of pseudo-typed virus with ZEBOV GP which had the REBOV GP region of residues at positions 505–509 was almost the same as that of the virus with ZEBOV wild-type GP (data not shown). Similarly, exchanging this region of REBOV GP to that of ZEBOV GP showed limited effect on the infectivity of the virus. Substitutions of the residue at position 514 did not affect the infectivity (Fig. 3B). By contrast, the infectivities of pseudo-typed viruses bearing ZEBOV GP H516Y or REBOV GP Y516H were significantly lower or higher, respectively, than those of the viruses with the wild-type GPs (Fig. 3B). These results strongly suggest that a histidine residue at position 516 of ZEBOV GP contributes to the efficient entry into MGL/CD301-K562 cells.

4. Discussion

Epidemiological data showed that REBOV has never caused severe hemorrhagic fever in humans and was less virulent in nonhuman primates compared with the other EBOV species (i.e., ZEBOV, SEBOV, and CEBOV). However, the reason why REBOV was less pathogenic in comparison to the other EBOVs in humans remains unknown. In the present study, we demonstrated that a histidine residue at position 516 of ZEBOV GP2 is a critical determinant for different infectivity between ZEBOV and REBOV in MGL/CD301-K562 cells.

Previous studies already implicated the mechanism of GP processing with lower pathogenesis of REBOV as stated below. The full-length GP is cleaved by furin at the trans-Golgi apparatus into subunits, GP1 and GP2. It was previously shown that the furin-cleavage efficiency of REBOV GP was lower than ZEBOV and SEBOV

GPs [19]. However, there were reports showing that furin-cleavage is not essential for ZEBOV replication *in vitro* and *in vivo* [20–22]. Adenoviral gene transfer of ZEBOV GP was reported to cause cell damage on explanted cynomolgus monkey carotid arteries and human saphenous veins, whereas REBOV GP was toxic only in cynomolgus monkey carotid arteries [23]. A mucin-like domain of ZEBOV GP was found to be critical for the cytotoxic effect [23]. We previously showed that MGL/CD301 functioned as an attachment factor enhancing EBOV infection *in vitro* by interaction with a mucin-like domain and that infectivity of pseudo-typed viruses bearing ZEBOV GP or SEBOV GP was significantly higher than that of viruses with REBOV GP [10]. In addition, we showed that the binding capacity of MGL/CD301 contributes to different infectivity between VSV-ZEBOV and -REBOV. These findings strongly suggested that the failure of REBOV to interact with MGL/CD301, which is predominantly mediated by the GP1, should be considered as a basis for lower pathogenesis of REBOV.

The role of GP2 in the pathogenesis of EBOV was also previously explored, but to a limited extent. GP2 mediates fusion of viral and host membranes at low pH [2,4,5,19]. In the present study, we showed significantly reduced infectivity of pseudo-typed viruses in MGL/CD301-expressing cells by replacing ZEBOV GP2 to REBOV GP2 in comparison to wild-type ZEBOV GP (Fig. 1). This is the first report to show that GP2 determines the different infectivity mediated by MGL/CD301 between ZEBOV and REBOV.

In MGL/CD301-K562 cells, infectivity of VSV-ZEBOV GP was significantly reduced by replacing histidine to tyrosine at position 516 (Fig. 3). One possibility is that the formation of the salt bridge between histidine at 516 and glutamic acid at 545 makes the fusion motif more stable at a low pH. Assessing the X-ray crystal structure of ZEBOV GP, it can be seen from the distance between the two amino acids that they are close enough to interact with each other. It has been suggested that a low pH level is required for activity of endosomal proteases like cathepsin B and cathepsin L [12]. Another possibility is that histidine at 516 is involved in the efficiency of GP1 cleavage by cathepsin L.

In conclusion, we demonstrate that the difference in the primary structure of GP2 of ZEBOV and REBOV plays an essential role in their ability to infect cells expressing MGL/CD301, which is a marker for dendritic cells [24,25]. Previous study suggested that the efficiency of C-type lectin-mediated entry of Marburg virus, another member of filovirus family, was controlled not only by binding affinity between C-type lectins and GP1 but also by some mechanisms mediated by GP2 [26]. Although further investigations are required to prove our hypotheses, this study provides new insights for better understanding of the mechanisms of the C-type lectin-mediated entry of Ebola virus, which may have a possible link to its pathogenicity.

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