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Enzyme-Linked Immunosorbent Assay for Detection of Filovirus Species-Specific Antibodies[▽]

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Several enzyme-linked immunosorbent assays (ELISAs) for the detection of filovirus-specific antibodies have been developed. However, diagnostic methods to distinguish antibodies specific to the respective species of filoviruses, which provide the basis for serological classification, are not readily available. We established an ELISA using His-tagged secreted forms of the transmembrane glycoproteins (GPs) of five different Ebola virus (EBOV) species and one Marburg virus (MARV) strain as antigens for the detection of filovirus species-specific antibodies. The GP-based ELISA was evaluated by testing antisera collected from mice immunized with virus-like particles as well as from humans and nonhuman primates infected with EBOV or MARV. In our ELISA, little cross-reactivity of IgG antibodies was observed in most of the mouse antisera. Although sera and plasma from some patients and monkeys showed notable cross-reactivity with the GPs from multiple filovirus species, the highest reactions of IgG were uniformly detected against the GP antigen homologous to the virus species that infected individuals. We further confirmed that MARV-specific IgM antibodies were specifically detected in specimens collected from patients during the acute phase of infection. These results demonstrate the usefulness of our ELISA for diagnostics as well as ecological and serosurvey studies.

Ebola virus (EBOV) and Marburg virus (MARV) belong to the family *Filoviridae* and cause severe hemorrhagic fever in primates (20). While MARV consists of a single species, *Lake Victoria marburgvirus*, four distinct EBOV species are known: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Côte d'Ivoire ebolavirus* (CIEBOV), and *Reston ebolavirus* (REBOV). The phylogenetically distinct *Bundibugyo ebolavirus* (BEBOV) was recently identified in Uganda and was proposed to be a new species of EBOV (Fig. 1) (31).

EBOV and MARV are filamentous, enveloped, single-stranded, negative-sense RNA viruses. The virus genome encodes seven structural proteins, nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), minor matrix protein (VP24), and RNA-dependent RNA polymerase (L). EBOV also expresses at least one secreted nonstructural glycoprotein (sGP) (20). GP is responsible for receptor binding and fusion of the viral envelope with host cell membranes (11, 22, 35) and has an important role in the pathogenesis of filovirus infection (3, 23, 36). GP is the main target of neutralizing antibodies, and most of the known ZEBOV-specific monoclonal antibodies (MAbs) show little cross-reactivity to other filovirus species (24, 27, 34).

Serological diagnostic methods based on enzyme-linked immunosorbent assays (ELISAs) using the recombinant EBOV and MARV NP antigens have been developed to detect filovirus-specific antibodies (5, 17). Using a ZEBOV NP antigen, NP-specific antibodies were broadly detected in animals infected with ZEBOV, SEBOV, CIEBOV, or REBOV (17), indicating strong cross-reactivity among EBOV species. It is predicted, however, that the antibody response to GP is more species specific due to the larger genetic variability with this protein, which is supposed to be the main target of the host humoral immune response. Therefore, in this study we developed a filovirus species-specific ELISA using recombinant GP antigens to serologically distinguish filovirus species.

MATERIALS AND METHODS

Plasmids. Viral RNA extracted from the supernatant of Vero E6 cells infected with ZEBOV, SEBOV, CIEBOV, BEBOV, REBOV, or MARV strain Angola was used for the cloning of the respective GP cDNAs lacking the transmembrane domain and cytoplasmic tail. The cDNAs of truncated EBOV and MARV GPs with a C-terminal histidine (His) tag (His-EBOV-GP and His-MARV-GP, respectively) were cloned into a pATX vector. Finally, the cDNA fragments of His-EBOV-GP and His-MARV-GP were inserted into the mammalian expression vector pCAGGS/MCS, which contains the chicken β-actin promoter (13). All clones were confirmed by sequencing prior to expression.

MAbs. Hybridoma cells producing EBOV GP-specific MAb ZGP42/3.7 (IgG1) (24, 26), which recognizes a linear epitope on GP comprising the sequence GEWAFWENKKN, and MARV GP-specific MAb AGP127-8 (IgG1) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) and RPMI medium (Sigma), respectively, supplemented with fetal calf serum (FCS) and antibiotics. Mouse ascites were obtained by a standard procedure, and MAbs were purified from ascites fluid using protein A-agarose columns (Bio-Rad). The S139/1 monoclonal antibody (IgG2a), which binds to the hemagglutinin of influenza A viruses (37), was used as a negative control.

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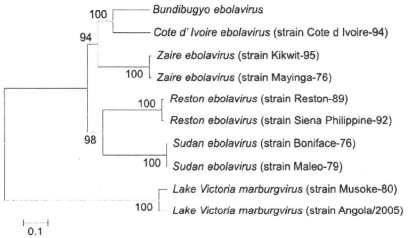

FIG. 1. Phylogenetic analysis of filovirus GP amino acid sequences. The phylogenetic tree was constructed by using the neighbor-joining method. For the construction of this tree, we used 10 GP amino acid sequences, each comprising a whole GP amino acid sequence. Numbers at branch points indicate bootstrap values (1,000 replicates).

Sera and plasma. Five-week-old female BALB/c mice were immunized twice intraperitoneally with 100 μg virus-like particles (VLPs) (14, 21) in 3-week intervals, and the serum samples were collected 7 to 10 days after the second immunization. Convalescent-phase plasma samples were collected from cynomolgus macaques vaccinated and/or infected with EBOV as described previously (27). ZEBOV convalescent-phase human plasma (patients 2 to 7) and serum (patients 1 and 8) samples were obtained 51 to 135 days after the onset of ZEBOV infection during the 1995 outbreak in Kikwit, Democratic Republic of the Congo (25). SEBOV convalescent-phase patient serum samples (patients 9 and 10) were collected about 2 months after onset during the Ebola hemorrhagic fever outbreaks in Uganda in 2000 associated with SEBOV (2). These EBOV-infected human samples were kindly provided by T. G. Ksiazek (Centers for Disease Control and Prevention). MARV-infected human blood samples (patients 11 to 21) were collected within a few days after the onset of symptoms from admitted patients from the 2004-2005 outbreak in Angola (29). Blood collections during outbreak investigations were approved under the special response protocol established between the World Health Organization and national authorities.

Expression and purification of His-EBOV-GP and His-MARV-GP. Human epithelial kidney 293T cells cultured in high-glucose DMEM containing 10% FCS and antibiotics were transfected with pCAGGS vectors expressing His-EBOV-GP (pCHis-ZEBOV-GP, pCHis-SEBOV-GP, pCHis-CIEBOV-GP, pCHis-BEBOV-GP, or pCHis-REBOV-GP) or His-MARV-GP (pCHis-MARV-GP) using TransIT LT1 (Mirus). Forty-eight hours after transfection, the supernatants were collected, and the recombinant GPs were purified by using the Ni-nitrilotriacetic acid (NTA) purification system (Invitrogen) according to the manufacturer's instructions. The majority of contaminant protein was removed with wash buffer containing 15 mM imidazole. Finally, bound proteins were collected with elution buffer containing 250 mM imidazole. To monitor inevitable nonspecific reactions (i.e., nonspecific antibodies) to FCS-derived impurities in each GP preparation, control antigens (FCS-derived proteins nonspecifically bound to the Ni beads) were prepared by using the Ni-NTA column under the same conditions. The eluted protein was concentrated by using Amicon Ultra 4 spin columns (Millipore) and dialyzed against phosphate-buffered saline (PBS) at 4°C overnight. Purified His-EBOV-GP and His-MARV-GP were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. Western blotting was performed by using ZGP42/3.7, AGP127-8, and anti-His MAbs (Covance).

Antigens prepared from cell lysates and VLPs. Membrane lysates of 293T cells transfected with pCAGGS expressing full-length GP were prepared by using the Mem-PER eukaryotic membrane protein extraction reagent kit (Pierce) according to the manufacturer's instructions. To generate VLPs, 293T cells were transfected with plasmids expressing major viral structural proteins, GP, NP, and VP40 (10, 33). After 48 h, supernatants were overlaid on 25% sucrose and ultracentrifuged at 28,000 × *g* at 4°C for 1.5 h. The VLPs were recovered from the pellet and disrupted with 0.05% Triton X-100 in the presence of 30 mM potassium chloride for the use of ELISA antigens. The GP amounts in the membrane lysates and VLPs were quantified by Western blotting using MAb ZGP42/3.7 or AGP127-8, and the GP concentrations of each preparation were calculated based on the standard band intensities provided by known concentrations of His-GP. Membrane lysates or supernatants of 293T cells transfected with empty pCAGGS vectors were used to prepare control antigens for ELISA using cell lysates or VLPs, respectively.

ELISA. ELISA plates (Nunc Maxisorp) were coated with the GP antigens (100 ng of GP/50 μl/well) or control antigens in PBS at 4°C overnight and then washed with PBS containing 0.05% Tween 20 (PBST). Unspecific binding of the antibodies was avoided by blocking with 3% skim milk (150 μl/well) for 2 h at room temperature. Monkey plasma samples were preincubated with 2% FCS to absorb antibodies to FCS components, since they were exposed to FCS by the injection of the vaccines or viruses diluted in DMEM containing FCS. After washing three times with PBST, 50 μl of appropriately diluted serum or plasma samples or the GP-specific MAb in PBST containing 1% skim milk was added and incubated for 1 h at room temperature. After washing three times with PBST, the bound antibodies were detected by using the following secondary antibodies conjugated with horseradish peroxidase diluted in 1% skim milk in PBST: goat anti-mouse IgG (Jackson ImmunoResearch), goat anti-monkey IgG (Rockland), goat anti-human IgG (Jackson ImmunoResearch), or donkey anti-human IgM (Jackson ImmunoResearch). After incubation for 1 h at room temperature and three PBST washes, 50 μl of 3,3′,5,5′-tetramethylbenzidine (Sigma) was added to each well, and the mixture was incubated for 15 min at room temperature. The reaction was stopped by adding 1 N sulfuric acid to the mixture, and the optical density (OD) at 450 nm was measured.

Phylogenetic analysis. Phylogenetic analysis was based on whole amino acid sequences of filovirus GPs. The sequences were analyzed by using GENETYX (Genetyx Corp., Japan) for Windows software, version 7. A phylogenetic tree was constructed by using the neighbor-joining bootstrap method (1,000 replicates) in MEGA 4.0 software (28). Amino acid sequences of ZEBOV strain Mayinga-76, ZEBOV strain Kikwit-95, SEBOV strain Boniface-76, SEBOV strain Maleo-79, CIEBOV strain Côte d'Ivoire-94, BEBOV, REBOV strain Reston-89, REBOV strain Siena Philippine-92, MARV strain Musoke-80, and MARV strain Angola/2005 used in phylogenetic analyses were obtained from GenBank under accession numbers Q05320, P87666, Q66814, Q66798, Q66810, ACI28624, Q66799, Q89853, P35253, and Q1PD50, respectively.

Statistical analyses. OD values higher than 3 standard deviations above the averages of negative-control samples at a 1:100 dilution were considered positive. To test the specificity of each reaction, ELISA data (i.e., OD values) were analyzed by using one-way analysis of variance (ANOVA). The differences between OD values were compared by using the two-sided *t* test with the Bonferroni-Holm correction for multiple comparisons (4). All statistical analyses were performed with the computer program R (version 2.2.8).

RESULTS

Expression and purification of recombinant EBOV and MARV GPs. The expression and secretion of His-EBOV-GP and His-MARV-GP in the supernatants of 293T cells transfected with a plasmid encoding His-GP were confirmed by immunoblotting using anti-GP and anti-His MAbs (data not shown). These recombinant GPs were purified as described in Materials and Methods. All purified His-GPs were detected by SDS-PAGE and immunoblotting using anti-GP and anti-His MAbs as prominent protein bands of the predicted size of the transmembrane anchor-minus EBOV and MARV GPs (Fig. 2). These purified GPs were used as antigens for the ELISA described in the following experiments.

Sensitivity of the GP-based ELISA. The sensitivity of the purified GP-based ELISA was tested by using anti-EBOV-GP MAb ZGP42/3.7 and anti-MARV-GP MAb AGP127-8. Serial 10-fold dilutions of the antibodies (10^{-5} to 10^{2} μg/ml) were prepared, and the reactivity to each GP antigen was examined (Fig. 3a to c). The negative-control MAb, S139/1, did not bind to any His-GPs in the ELISA. At concentrations ranging from 0.1 μg/ml to 100 μg/ml, ZGP42/3.7 reacted with all His-EBOV-GPs but not His-MARV-GP, whereas AGP127-8 reacted specifically with His-MARV-GP but not any of the His-EBOV-GPs. The detection limit for specific antibodies using this assay

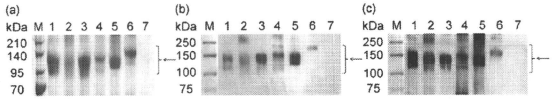

FIG. 2. Identification and characterization of purified His-GPs. (a) His-EBOV-GP and His-MARV-GP were analyzed by 8% SDS-PAGE and stained with Coomassie brilliant blue. (b and c) Immunoblotting of purified His-GPs was performed by using MAbs to EBOV (ZGP42/3.7) and MARV GPs (AGP127-8) (b) and His tags (c). Arrows indicate the locations of the His-GPs. The protein bands represent His-ZEBOV-GP (lane 1), His-SEBOV-GP (lane 2), His-CIEBOV-GP (lane 3), His-BEBOV-GP (lane 4), His-REBOV-GP (lane 5), and His-MARV-GP (lane 6). Lane 7 shows FCS-derived proteins used as a control antigen (see Materials and Methods).

was approximately 0.01 to 0.1 μg/ml. On the other hand, ELISA using membrane lysates of GP-transfected cells or VLPs under similar conditions with the GP-based ELISA showed lower sensitivity, except for the Angola serum and VLP combination (Fig. 3d to i). This is most likely due to the interference by the residual detergent and/or irrelevant proteins in the lysates and VLP antigen preparations.

Specificity of the GP-based ELISA. Next, the species specificity of the ELISA was assessed by testing the antisera of mice immunized with VLP containing the respective EBOV and MARV GPs. We found that species-specific IgG antibodies were clearly detected in these mouse antisera (Fig. 4a to f). All the anti-EBOV IgG antibodies in the sera showed low reactivity to heterologous EBOV GPs, and no cross-reactivity to MARV GP was found (Fig. 4a to e). Similarly, anti-MARV VLP serum antibodies reacted to MARV GP but not to EBOV GPs (Fig. 4f). These results indicated that this purified GP-based ELISA sufficiently detected filovirus species-specific antibodies. On the other hand, the VLP-based ELISA was less sensitive and detected more appreciable cross-reactive antibodies in some of the mouse sera, likely specific to NP and VP40, than the purified GP-based ELISA (Fig. 4g to i).

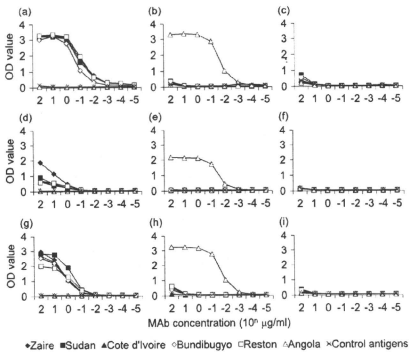

FIG. 3. Sensitivity of ELISAs. His-GPs (a, b, and c), GP-expressing cell lysates (d, e, and f), and VLP (g, h, and i) were used as antigens. The GP amounts were standardized by Western blotting as described in Materials and Methods. Serial 10-fold dilutions of MAbs to EBOV (a, d, and g) and MARV (b, e, and h) were prepared and tested. S139/1 (specific to influenza virus hemagglutinin) was used as a negative-control antibody (c, f, and i).

Analysis of clinical samples in the GP-based ELISA. To further confirm the specificity of our ELISA, we used convalescent-phase plasma samples obtained from monkeys experimentally infected with ZEBOV or SEBOV (Fig. 5). The cutoff OD values (i.e., the mean plus 3 standard deviations of the five negative serum samples) were 0.23, 0.22, 0.29, 0.22, 0.17, 0.20, and 0.13 for His-ZEBOV-GP, His-SEBOV-GP, His-CIEBOV-GP, His-BEBOV-GP, His-REBOV-GP, His-MARV-GP, and control antigens, respectively. According to these thresholds, all infected monkey serum samples tested were EBOV antibody positive. We detected IgG antibodies in the ZEBOV-infected monkey plasma with higher reactivity against His-ZEBOV-GP than against any heterologous GP antigens. Although IgG antibodies in the SEBOV-infected monkey plasma showed binding to all His-EBOV-GPs, the highest reactivity was observed with the homologous antigen His-SEBOV-GP. Neither of these plasma antibodies reacted with MARV GP.

We then examined IgG antibody levels in serum or plasma derived from ZEBOV-, SEBOV-, and MARV-infected patients (Fig. 6a). The cutoff OD values obtained from the five negative-control sera for IgG antibodies were 0.20, 0.17, 0.24, 0.18, 0.14, 0.27, and 0.23 for His-ZEBOV-GP, His-SEBOV-GP, His-CIEBOV-GP, His-BEBOV-GP, His-REBOV-GP, His-MARV-GP, and control antigens, respectively. For most of the samples tested, IgG antibodies to homologous GP antigens were detected with the highest reactivity (Fig. 6a). All of the samples derived from ZEBOV-infected patients cross-reacted with His-CIEBOV-GP and His-BEBOV-GP antigens, whereas only one of the SEBOV-infected human samples (sample 9) showed cross-reactivity with His-MARV-GP. Overall, the level of cross-reactivity was consistent with the phylogenetic relationship among EBOV species (Fig. 1). On the other hand, for most of the samples from patients infected with MARV Angola, IgG antibodies to His-MARV-GP were specifically detected, except for specimen 17, which showed no IgG response to any GP. Interestingly, IgG antibodies detected in specimen 11 showed remarkable cross-reactivity with the heterologous antigens His-CIEBOV-GP and His-BEBOV-GP.

We next evaluated whether GP-specific IgM antibodies could be detected in the patient serum or plasma samples using the GP-based ELISA (Fig. 6b). The cutoff values for IgM ELISA were 0.23, 0.32, 0.31, 0.28, 0.30, 0.22, and 0.36 for His-ZEBOV-GP, His-SEBOV-GP, His-CIEBOV-GP, His-BEBOV-GP, His-REBOV-GP, His-MARV-GP, and control

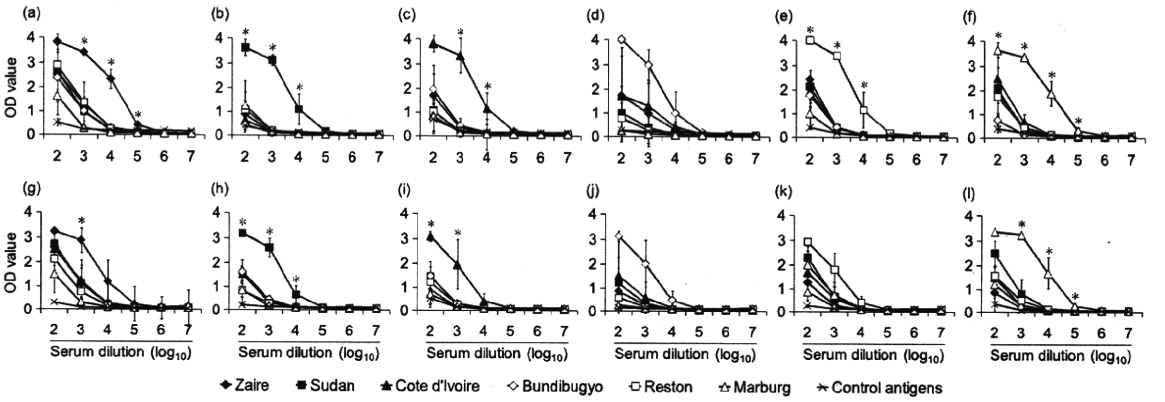

FIG. 4. IgG antibodies detected in mouse antisera. Serial 10-fold dilutions of anti-ZEBOV (a and g), anti-SEBOV (b and h), anti-CIEBOV (c and i), anti-BEBOV (d and j), anti-REBOV (e and k), and anti-MARV (f and l) sera obtained from mice immunized with EBOV and MARV VLPs were tested for IgG antibodies reacting with His-GPs (a, b, c, d, e, and f) and VLPs (g, h, i, j, k, and l). Averages and standard deviations for three mice of each group are shown. Asterisks indicate statistically significant differences in OD values between the homologous antigen and all other antigens ($P < 0.05$).

antigens, respectively. ZEBOV- or SEBOV-specific IgM antibodies were detected only in patients 2, 6, 9, and 10. In contrast, MARV-specific IgM antibodies were detected in 8 out of the 11 specimens derived from MARV Angola-infected patients. No obvious IgM cross-reactivity to heterologous GP antigens was found in these samples.

DISCUSSION

In this study, we established a GP-based ELISA to detect filovirus species-specific antibodies. To date, lysates from Vero E6 cells infected with live EBOV and MARV or recombinant EBOV and MARV NPs have been used as antigens in ELISAs for the detection of filovirus-specific antibodies (5, 7, 17). Since the NPs of EBOV and MARV contain similar amino acid sequences (18), common antibody epitopes seem to be present (12). Indeed, cross-reactivity among all EBOV species was to be expected (16, 17). Therefore, NP antigens may be useful for the detection of genus-specific antibodies but not for the detection of species-specific humoral responses (7, 16, 17).

The heterogeneity of EBOV and MARV GPs has been demonstrated at the genetic level through sequence analyses (17, 19). An ELISA using recombinant ZEBOV GP expressed in a baculovirus-insect cell expression system was reported previously (16), but it is known that the protein glycosylation pathways in insect cells differ from those in mammalian cells (6). This may significantly affect the antigenic properties of filovirus GPs, since large amounts of both N- and O-linked carbohydrate chains are present in GP molecules. To overcome this difficulty, we used mammalian 293T cells for the expression of GP antigens and verified the sensitivity and specificity of GP-based ELISAs. Our results were consistent with a previous study suggesting that anti-EBOV GP antibodies were highly species specific and showed little cross-reactivity to GPs of other EBOV species (27). These findings indicated that most antibodies induced against filovirus GPs recognized

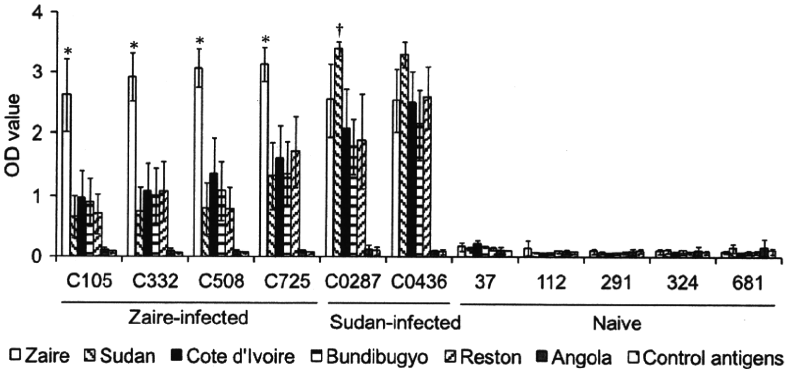

FIG. 5. IgG antibodies detected in experimentally infected monkey plasma by ELISA using His-GPs. Monkeys C105, C332, C508, and C725 were infected with ZEBOV, whereas monkeys C0287 and C0436 were infected with SEBOV. Infected monkey sera were diluted at 1:1,000. Naïve monkey sera were diluted at 1:100. Each bar represents the average and standard deviation of data from three independent experiments. Asterisks indicate statistically significant differences in OD values between the Zaire antigen and all other antigens ($P < 0.05$). The dagger shows statistically different reactions between His-SEBOV-GP and all the other antigens ($P < 0.05$) except His-ZEBOV-GP.

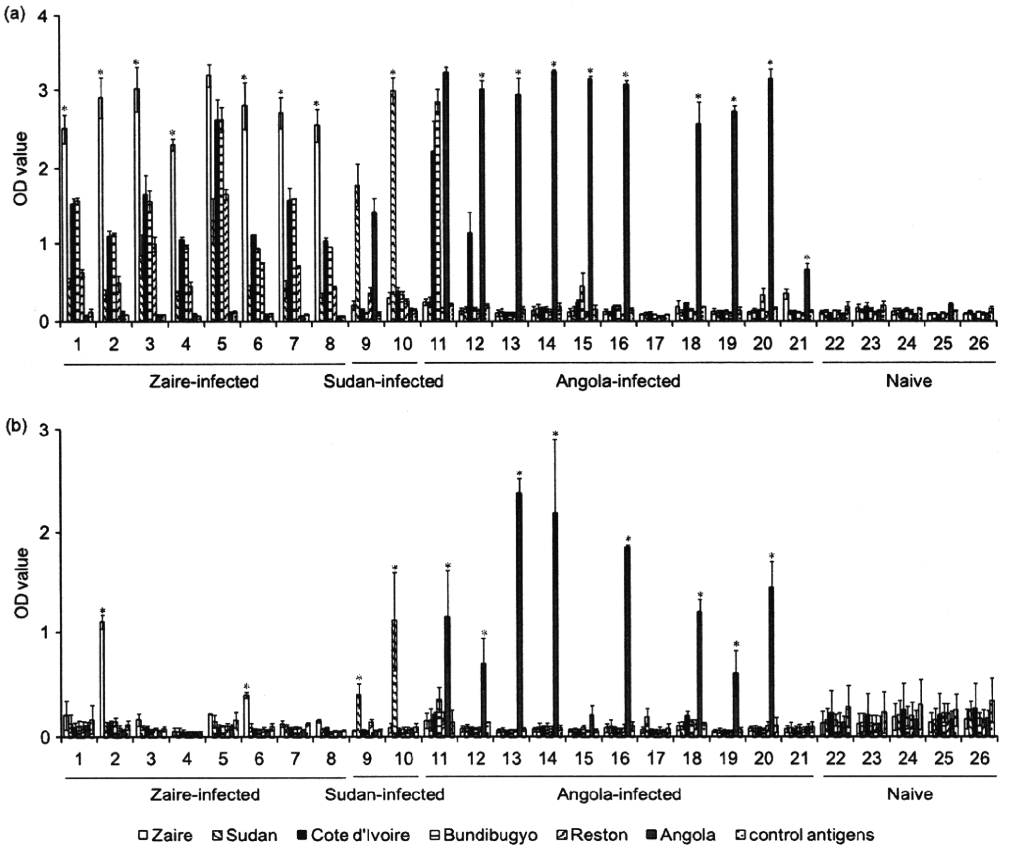

FIG. 6. IgG and IgM antibodies detected in human samples. OD values for specific IgG (a) and IgM (b) antibodies in the patient sera are shown. Sera from 21 individuals were analyzed at 1:1,000 dilutions. Naïve human sera (1:100 dilution) were used as a negative control. Each bar represents the average and standard deviation of data from independent experiments. Asterisks indicate statistically significant differences in OD values between the homologous antigen and all other antigens ($P < 0.05$).

epitopes in the variable regions of the protein. Expectedly, the serological classification mirrors the phylogenetic relationship of the different GPs (Fig. 1). Interestingly, serological characterization of anti-BEBOV antibodies clearly supports the molecular investigations (31) suggesting that BEBOV represents a new species within the EBOV genus.

IgG antibodies in some of the serum and plasma samples collected from infected monkeys and humans showed appreciable cross-reactivity to heterologous antigens, whereas antibodies in the mouse sera produced by immunization with VLPs specifically reacted to the homologous antigens. This result led us to the conjecture that VLP immunization and live-virus infection induce a distinct antibody repertoire or that the antibody repertoire of mice differs from that of primates. Interestingly, the plasma of patient 11 infected with MARV Angola contained IgG, but not IgM, antibodies cross-reactive to His-CIEBOV-GP and His-BEBOV-GP. It might be possible that prior to infection with MARV Angola, this patient was infected with CIEBOV, BEBOV, or another unknown filovirus whose GP has epitopes shared among CIEBOV and BEBOV. In the plasma of patient 17, neither IgG nor IgM antibodies were readily detected. An explanation for this observation might be differences of immunological conditions in individuals, or alternatively, the blood samples have been collected before a detectable antibody response was induced.

Notably, our GP-based ELISA detected MARV Angola-specific IgM antibodies in most of the plasma samples collected during the acute or subacute phase of infection, although it was reported previously that the detection of antibodies is of only limited use for acute-case diagnosis due to a lack of a detectable antibody response (8). The present study suggests that if proper antigen and sensitive assays are available, IgM antibodies can be useful for the diagnosis of acute EBOV and MARV infections and support the use of antigen capture ELISA and reverse transcription-PCR, the most commonly used technologies.

Despite the more recent discovery of REBOV in domestic pigs in the Philippines (1) and the discovery of fruit bat species as potential reservoirs for EBOV and MARV (9, 15, 30, 32), the search for the reservoirs and potential amplifying hosts remains ongoing. Advanced diagnostic technologies are welcome here, and our new GP-based species-specific antibody detection ELISA may be a useful tool for future ecological and seroepidemiological studies in areas of Central Africa and parts of Asia where the disease is endemic.

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C-type lectins do not act as functional receptors for filovirus entry into cells

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ABSTRACT

Cellular C-type lectins have been reported to facilitate filovirus infection by binding to glycans on filovirus glycoprotein (GP). However, it is not clearly known whether interaction between C-type lectins and GP mediates all the steps of virus entry (i.e., attachment, internalization, and membrane fusion). In this study, we generated vesicular stomatitis viruses pseudotyped with mutant GPs that have impaired structures of the putative receptor binding regions and thus reduced ability to infect the monkey kidney cells that are routinely used for virus propagation. We found that infectivities of viruses with the mutant GPs dropped in C-type lectin-expressing cells, parallel with those in the monkey kidney cells, whereas binding activities of these GPs to the C-type lectins were not correlated with the reduced infectivities. These results suggest that C-type lectin-mediated entry of filoviruses requires other cellular molecule(s) that may be involved in virion internalization or membrane fusion.

1. Introduction

Ebola virus (EBOV) and Marburg virus (MARV) are enveloped negative-strand RNA viruses that constitute the family *Filoviridae*. Filovirus infection causes severe hemorrhagic fever in humans and non-human primates and mortality rates have ranged up to 90%. *Zaire ebolavirus* (ZEBOV) has caused multiple large outbreaks with the highest mortality rates (~90%) among EBOV species. Among MARVs, strain Angola (MARV-A) caused the largest outbreak in 2004–05 in Angola, with the highest mortality rate (90%) [1].

It has been shown that the filovirus entry into host cells depends on endosomal acidification [2,3] and proteolysis of the glycoprotein (GP) by endosomal cysteine proteases like cathepsin B and/or L [4]. Filovirus GP is the only spike protein on the surface of the virion, and therefore GP is responsible for both receptor binding and membrane fusion. GP is comprised of two molecules, GP1 and GP2, which are linked by a disulfide bond. GP1 contains a putative receptor binding region (RBR) [5,6] and a mucin-like region (MLR) that has a number of potential N- and O-linked glycosylation sites [7,8]. GP2 has a transmembrane domain, cytoplasminc tail and an internal fusion loop [1].

GP1, in particular MLR, is highly glycosylated by both N- and O-glycans, and these glycans are thought to be recognized by cellular C-type lectins such as liver-specific C-type lectin asialoglycoprotein receptor (ASGP-R) [9,10], dendritic cell- and liver/lymph node-specific ICAM-3-grabbing nonintegrin (DC-SIGN and L-SIGN) [10–18], human macrophage galactose-type C-type lectin (hMGL) [18,19], and liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) [12,17]. Though these C-type lectins show different specificities, depending on the structures of target glycans, all have been reported to promote filovirus entry. Hepatocytes, dendritic cells, monocytes and macrophages are thought to be the preferred target cells of filoviruses, and infection of these cells is important for hemorrhagic manifestation and immune disorders [20–23]. Thus, increased infection of these cells might be directly involved in the pathogenesis of filovirus infection [18,24].

Though the C-type lectins have been reported to enhance filovirus infection, DC-SIGN and L-SIGN did not confer susceptibility for EBOV to non-susceptible cells, i.e. CD4+T-cells [11] and Ramons B cells [14]. In readily susceptible cells, it was reported that the internalization of DC-SIGN and L-SIGN themselves was not essential for trafficking EBOV into endosomal compartments [14]. These studies suggest that C-type lectins promote the filovirus entry by enhancing the virion attachment on the cell surface but not by enhancing the virion internalization. However, it has not been clarified yet whether C-type lectins independently act as a functional receptor mediating all the steps of viral entry including attachment, internalization, and membrane fusion. In the present study, to confirm the role of the C-type lectins in filovirus entry, we generated mutant GPs whose RBRs were impaired, and examined their abilities

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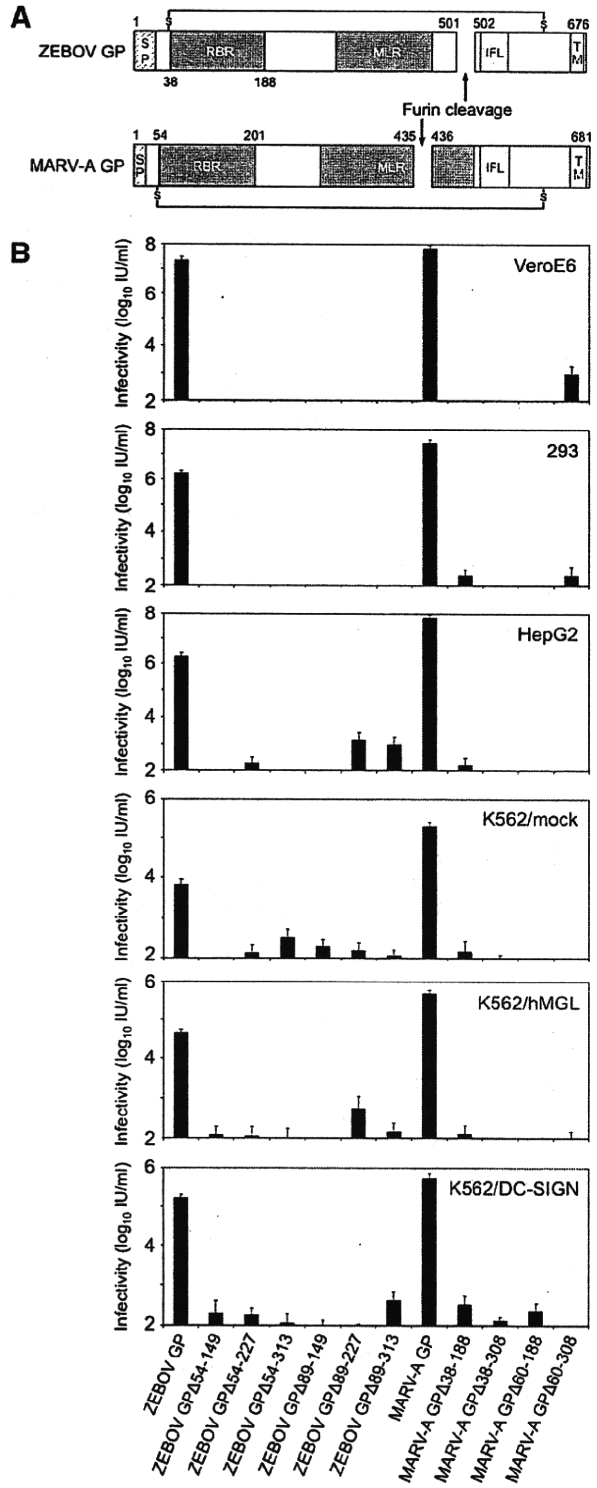

Fig. 1. Infectivity of VSVΔG* pseudotyped with GPΔRBR. Functional domains and putative regions of ZEBOV GP and A-MARV GP are represented in schematic forms (A) (SP; signal peptide, RBR; receptor binding region, MLR; mucin-like region, IFL; internal fusion loop, and TM; transmembrane domain). Infectivities of the viruses in Vero E6, 293, HepG2, K562/mock, K562/hMGL, and K562/DC-SIGN were determined by counting GFP-positive cells and the infectious units (IUs) are indicated on the vertical lines (B). All experiments were done at least three times and averages and standard deviations are shown.

to infect C-type lectin-expressing cells without the interaction between RBR and its unknown putative counterpart(s).

2. Materials and methods

2.1. Cells

293T, Vero E6, and HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. HepG2 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. K562 cell clones expressing hMGL (K562/hMGL), DC-SIGN (K562/DC-SIGN), and mock transfected (K562/mock) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics.

2.2. Viruses

Construction of mutant GPs was done as previously described [18]. The modified GP genes were then ligated into pCAGGS and used to express GPs on 293T cells. Vesicular stomatitis virus expressing green fluorescent protein (GFP) (VSVΔG*) pseudotyped with GP was generated in 293T cells as previously described [2,18].

Table 1
Characteristics of entry deficient mutant GPs.

	Protein expression[a]	Virion incorporation[b]	Reference
ZEBOV GP	++++	++++	
ZEBOV GPΔ54-149	++	+	
ZEBOV GPΔ54-227	++++	+++	
ZEBOV GPΔ54-313	ND	ND	
ZEBOV GPΔ89-149	++	+	
ZEBOV GPΔ89-227	++++	++++	
ZEBOV GPΔ89-313	ND	ND	
D55A	++++	++++	[27]
L57A	++++	++++	[27]
L57I	++++	++++	[27]
L57F	++++	++++	[27]
L57K	++++	++++	[27]
L63A	++++	+++	[27]
R64E	++++	++++	[27]
F88A	++++	++	[27,28]
K95A	++++	+++	[27]
R134A	++++	++	[29]
K140A	++++	+++	[29]
G143A	++++	+++	[29]
I170A	++++	+++	[27]
MARV-A GP	++++	++++	
MARV-A GPΔ38-188	+	++++	
MARV-A GPΔ38-308	ND	ND	
MARV-A GPΔ60-188	+	++++	
MARV-A GPΔ60-308	ND	ND	
L41A	++++	++++	
K79A	++++	++++	
K118A	++++	++++	
G127A	++++	++++	
Y146A	ND	ND	

++++: >75% of wild-type GP.
+++: 50–75% of wild-type GP.
++: 25–50% of wild-type GP.
+: <25% of wild-type GP.
ND: GP specific bands not detected.
[a] Intensities of GP specific bands in the lysate of 293T cells.
[b] The ratio between intensities of GP and the VSV M specific band in the supernatant of 293T cells.

2.3. Western blot analysis

Anti-ZEBOV GP monoclonal antibody (MAb) 42/3.7 recognizing a linear epitope (amino acid positions 286–296) of ZEBOV GP [25], anti-MARV-A GP MAb 127-8 recognizing a linear epitope (amino acid positions 410–430) of MARV-A GP [25], and anti-VSV matrix protein (M) MAb 192/1 [18] were used for detection of the proteins. Peroxidase-conjugated AffiPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch) and Immobilon Western (Millipore) were used for visualization of the protein bands. Intensities of specific bands were measured with ImageJ [26].

2.4. Lectin-binding assay

VSVΔG* pseudotyped with GPs was purified by ultracentrifugation through a 25% sucrose cushion and diluted in phosphate-buffered saline (PBS). The GP amounts in the VLPs were quantified by Western blotting using MAb ZGP42/3.7 or AGP127-8, and standardized based on the band intensities. Enzyme-linked immunosorbent assay (ELISA) plates were coated with the diluted viruses (2 mg/ml) and then blocked with 3% bovine serum albumin in PBS. After each well was washed with Dulbecco's Tris-buffered saline (dTBS), biotinylated soluble recombinant hMGL (hMGL ECD) or DC-SIGN (DC-SIGN ECD) [18] in dTBS was added. To detect C-type lectins bound to the viruses, horseradish peroxidase (HRP)-streptavidin (Jackson ImmunoResearch) and 3,3,5,5-tetramethylbenzidine (Sigma) were used.

3. Results and discussion

We first constructed RBR-deletion mutant GPs of ZEBOV GP (Δ54-149, Δ54-227, Δ54-313, Δ89-149, Δ89-227, and Δ89-313) and MARV-A GP (Δ38-188, Δ38-308, Δ60-188, and Δ60-308) (Fig. 1A), and viruses pseudotyped with these mutant GPs were generated. Lysates of GP-expressing 293T cells and culture supernatants containing pseudotyped viruses were examined by SDS-PAGE and Western blot analysis to verify the expression and the virion incorporation of the GPs (Table 1). Though MAbs 42/3.7 and 127-8 failed to react with ZEBOV GPΔ54-313, ZEBOV GPΔ89-313, MARV-A GPΔ38-308, and MARV-A GPΔ60-308, the other mutant GPs were detected by these antibodies. Although ZEBOV GPΔ54-149, ZEBOV GPΔ89-149, MARV-A GPΔ38-188, and MARV-A GPΔ60-188 showed significantly lower band intensities than wild-type GP, the expression on 293T cells and incorporation into the virion of these mutant GPs were verified. We then tested the infectivity of VSVΔG* pseudotyped with GPs in the various cell lines (Fig. 1B). The infectivity of VSVΔG* bearing the RBR-deletion mutant GPs was undetectable or significantly lower than VSVΔG* bearing wild-type GPs in all the cells tested, including the C-type lectin-expressing cells. These results indicated that GPs lacking

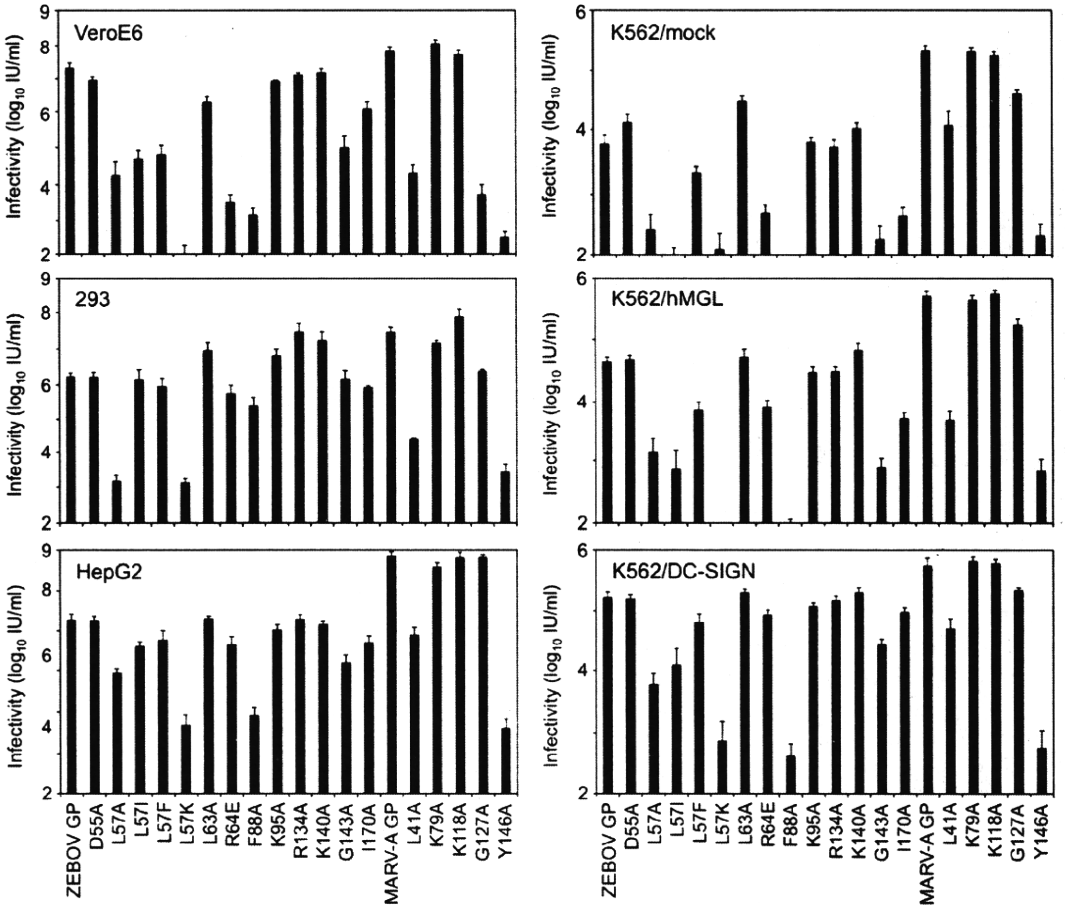

Fig. 2. Infectivity of VSVΔG* pseudotyped with mutant GPs having single amino acid substitutions. The infectious units determined for each virus in Vero E6, 293, HepG2, K562/mock, K562/hMGL, and K562/DC-SIGN are indicated on the vertical lines. All experiments were done at least three times and averages and standard deviations are shown.

RBR did not confer the sufficient infectivity to VSVΔG*, even when the C-type lectins existed on the target cell surface.

However, deletion of entire RBR polypeptides might cause not only a defect of binding ability to the putative functional receptor but also defects in other essential functions such as membrane fusion. Therefore we constructed mutant ZEBOV GPs with single amino acid substitutions in RBR, which were reported to impact the receptor binding capacity, leading to reduced infectivity [27–29]. Based on the amino acid sequence alignment between ZEBOV and MARV-A GPs, MARV-A mutant GPs that had corresponding mutations were also constructed (Table 1). The expression and virion incorporation of each mutant GP were compared with those of wild-type GPs by Western blot analysis (Table 1). Consistent with previous studies [27–29], all the mutant GPs were expressed and incorporated into the virion except MARV-A GP Y146A. The infectivity of the VSVΔG* pseudotyped with mutant GPs was tested in the same cell lines used in Fig. 1 (Fig. 2). As expected, almost all mutant ZEBOV GPs conferred lower infectivity to VSVΔG* in Vero E6, 293, and K562/mock cells than wild-type ZEBOV GP. Similarly, mutations in MARV-A GP (L41A and G127A) significantly reduced the infectivities of the viruses. In the C-type lectin-expressing cells (HepG2, K562/hMGL, and K562/DC-SIGN), the infectivities of the viruses bearing the mutant GPs were also lower than those of the viruses with wild-type GPs, and were likely reduced parallel to the infectivities in Vero E6, 293, and K562/mock cells. These results suggested that the reduced infectivity caused by the mutations in RBR could not be complemented by the interaction between the glycans on GP and C-type lectins.

In a lectin-binding assay using pseudotyped viruses and soluble recombinant hMGL (hMGL ECD) and DC-SIGN (DC-SIGN ECD), we further confirmed that the binding capacities of GPs to these lectins were not significantly reduced by the mutations that gave the lowest infectivities to VSVΔG* in K562/hMGL and K562/DC-SIGN (i.e., F88A and L41A of ZEBOV and MARV-A GPs, respectively) (Fig. 3). This finding indicated that there was no remarkable correlation between GP binding capacity to C-type lectins and reduced infectivity of the viruses with the mutant GPs, and suggested a limited contribution of the interaction between C-type lectin and GP to the subsequent steps in filovirus entry.

In the present study, we demonstrated that the structure of RBR was essential for the entry of filoviruses even when C-type lectins existed on the cell surface, suggesting that the C-type lectins were not independently able to mediate filovirus entry into cells. Therefore, we conclude that C-type lectin-mediated entry of filoviruses requires other cellular molecule(s) that may be critical for virion internalization and/or membrane fusion. Identification of the unknown ubiquitous receptor(s) or coreceptor(s) is essential for further understanding of the molecular mechanisms of filovirus cellular entry and may provide information on the link to the tropism and pathogenesis of filovirus infection.

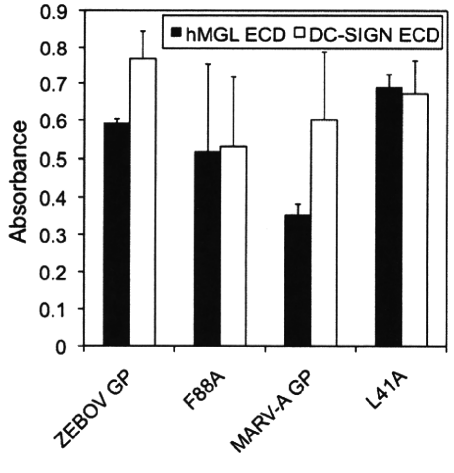

Fig. 3. Binding capacity of the C-type lectins to VSVΔG* pseudotyped with MARV GPs. ELISA plates were coated with purified VSVΔG* bearing mutant GPs. Biotinylated recombinant soluble hMGL ECD (2.5 mg/ml) and DC-SIGN ECD (2.5 mg/ml) were incubated with the viruses and visualized as described in Materials and Methods. All experiments were done in triplicate, and average results and standard deviations are shown.

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Different Potential of C-Type Lectin-Mediated Entry between Marburg Virus Strains[▽]

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The glycoproteins (GPs) of filoviruses are responsible for virus entry into cells. It is known that GP interacts with cellular C-type lectins for virus attachment to cells. Since primary target cells of filoviruses express C-type lectins, C-type lectin-mediated entry is thought to be a possible determinant of virus tropism and pathogenesis. We compared the efficiency of C-type lectin-mediated entry between Marburg virus strains Angola and Musoke by using a vesicular stomatitis virus (VSV) pseudotype system. VSV pseudotyped with Angola GP (VSV-Angola) infected K562 cells expressing the C-type lectin, human macrophage galactose-type C-type lectin (hMGL), or dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) more efficiently than VSV pseudotyped with Musoke GP (VSV-Musoke). Unexpectedly, the binding affinity of the C-type lectins to the carbohydrates on GPs did not correlate with the different efficiency of C-type lectin-mediated entry. Site-directed mutagenesis identified the amino acid at position 547, which switched the efficiency of C-type lectin-mediated entry. In a three-dimensional model of GP, this amino acid was in close proximity to the putative site of cathepsin processing. Interestingly, the cathepsin inhibitors reduced the infectivity of VSV-Angola less efficiently than that of VSV-Musoke in C-type lectin-expressing K562 cells, whereas only a limited difference was found in control cells. The amino acid at position 547 was critical for the different effects of the inhibitors on the virus infectivities. These results suggest that the efficiency of C-type lectin-mediated entry of filoviruses is controlled not only by binding affinity between C-type lectins and GP but also by mechanisms underlying endosomal entry, such as proteolytic processing by the cathepsins.

Marburg virus (MARV) and Ebola virus (EBOV), which belong to the family *Filoviridae*, have produced sporadic outbreaks of hemorrhagic fever in Africa. After the initial outbreak of MARV infection in 1967 in Europe, which resulted in 7 deaths among 32 confirmed patients (41), there were three small, isolated outbreaks of MARV infection in Africa between 1975 and 1987. During one of the outbreaks in Kenya in 1980, one of the two patients died (42), and experimental studies showed that this Kenyan MARV Musoke strain (Musoke) killed monkeys within 12 days after infection (6). On the other hand, throughout a recent outbreak of MARV infection in Angola, 84% of the 422 patients died (29). This MARV Angola strain (Angola) produced fatal disease in monkeys within 8 days after inoculation and was thought to be more pathogenic than the Musoke strain (5, 17). Among EBOVs, a difference in pathogenicity was also suggested. Zaire EBOV is thought to be the most pathogenic EBOV, killing approximately up to 90% of patients, whereas Reston EBOV has never caused lethal infection in humans (31) and is less pathogenic in experimentally infected nonhuman primates than Zaire EBOV (16). However, the factors that influence the different pathogenicity among filoviruses remain unclear.

The envelope glycoprotein (GP) of filoviruses is the only spike protein and is responsible for both receptor binding and membrane fusion. GP is comprised of two molecules, GP1 and GP2, which are linked by a disulfide bond. GP1 contains the receptor-binding domain, which is responsible for the viral attachment to cell surface molecules (9, 25). GP2 has the heptad repeat regions required for assembling GP as a trimer and the internal fusion loop, which is thought to interact with the cellular membrane (50). Although the trigger to promote the conformational change leading to membrane fusion is not fully understood, it was recently suggested that endosomal proteolysis of EBOV GP by cysteine proteases such as cathepsins B and L plays an important role in inducing membrane fusion (4).

Both MARV and EBOV GPs are heavily glycosylated and contain both N- and O-linked carbohydrate chains with different terminal sialylation patterns that seem to depend on the virus strains and cell lines used for virus propagation (12, 18,

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39, 48). The middle one-third of the GP molecule particularly varies among filoviruses and includes a mucin-like region (MLR) that contains a number of potential N- and O-linked glycosylation sites (32, 52). It is thought that carbohydrate chains on GP are recognized by cellular C-type lectins, such as the liver-specific C-type lectin asialoglycoprotein receptor (ASGP-R) (3), dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), liver/lymph node-SIGN (L-SIGN) (1, 2, 20, 30, 33, 40), human macrophage galactose-type C-type lectin (hMGL) (46), and liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) (8, 19, 20, 34). While these C-type lectins show different specificities, depending on the structures of target glycans, all have been reported to promote filovirus entry. Hepatocytes, dendritic cells (DCs), monocytes, and macrophages are thought to be the preferred target cells of filoviruses, and infection of these cells is important for hemorrhagic manifestation and immune disorders (7, 13, 15, 36). Thus, increased infection of these cells might be directly involved in the pathogenesis of filoviruses (16).

In the present study, using the vesicular stomatitis virus (VSV) pseudotype system (VSV that contains the green fluorescent protein [GFP] gene rather than the receptor-binding GP gene [VSVΔG*]) described previously (45), we compared the properties of Angola and Musoke GPs and found a significant difference in the ability to utilize hMGL and DC-SIGN for their entry. Importantly, GP binding affinity for the C-type lectins was not the primary factor contributing to the difference. We identified a single amino acid involved in the different efficiency of C-type lectin-mediated entry between Angola and Musoke. Three-dimensional analysis suggested that this amino acid might affect the processing of GP by endosomal cysteine proteases and/or flexibility of the GP internal fusion loop. Here, mechanisms underlying the different efficiencies for C-type lectin-mediated entry of filoviruses are discussed.

MATERIALS AND METHODS

Viruses and cells. VSVΔG* expressing GFP pseudotyped with MARV GPs was generated as previously described (45). The viruses were treated with neutralizing monoclonal antibody I1 to VSV G protein before use (28). The virus titer was determined by counting the number of cells expressing GFP using fluorescence microscopy or flow cytometry.

Vero E6 and HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. Human chronic myelogenous leukemia (K562) cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics. K562 clones expressing hMGL (K562/hMGL) were generated as previously described (46). cDNA encoding DC-SIGN was isolated from a placenta cDNA library (Invitrogen) and then cloned into a mammalian cell expression vector, pcDNA3.1(+) (Invitrogen). K562 cells were transfected with the plasmid using Attractene transfection reagent (Qiagen). After selection with Geneticin (G418 sulfate; Calbiochem), DC-SIGN-positive cells (K562/DC-SIGN) were enriched with immunomagnetic beads by using monoclonal antibody CD209 (Beckman Coulter). K562 cells transfected with the empty vector pcDNA3.1(+) and selected by Geneticin were used as control cells (K562/mock).

Expression of soluble recombinant hMGL and DC-SIGN. Soluble hMGL was purified by affinity chromatography on a column of galactose-Sepharose 4B as described previously (44). The expression plasmid pET-15b encoding the extracellular domain (ECD) of DC-SIGN was similarly constructed. The plasmid was subsequently used to transform *Escherichia coli* BL21/DE3 pLysS. The recombinant DC-SIGN ECD was prepared from inclusion bodies in *E. coli*. The recombinant DC-SIGN ECD was bound to mannose-Sepharose 4B and eluted with 10 mM EDTA. Subsequently, biotinylation of these soluble proteins was performed using EZ-Link sulfo-NHS-LC-biotin (Pierce).

Lectin-ELISA analysis. VSVΔG* pseudotyped with GPs was purified by ultracentrifugation through a 25% sucrose cushion and diluted to give a titer of 5×10^5 infectious units (IU)/ml in phosphate-buffered saline (PBS). Enzyme-linked immunosorbent assay (ELISA) plates were coated with the viruses and then blocked with 3% bovine serum albumin in PBS. After each well was washed with Dulbecco's Tris-buffered saline (dTBS), biotinylated hMGL or DC-SIGN in dTBS was added. To detect C-type lectins bound to the viruses, horseradish peroxidase (HRP)-streptavidin (Jackson ImmunoResearch) and 3,3′,5,5′-tetramethylbenzidine (Sigma) were used.

Mutagenesis. To construct the mutant GPs, MARV GP cDNAs were cloned into the pATX vector, kindly provided by H. Ebihara (Laboratory of Virology, Department of Health and Human Services, Rocky Mountain Laboratories, Division of Intramural Research, NIAID, NIH). By using the primers containing the sequences of the desired regions and the class IIS restriction enzyme, the BsmBI site, the MLR-deletion mutant, and chimeric GP constructs were generated. Mutant GPs with a single substitution (A/H504T, A/G547V, A/A596T, A/R618K, M/T504H, M/V547G, M/T596A, and M/K618R) were generated by using the primers containing the desired mutations and the BsmBI site. All the mutant GP genes were cloned into pCAGGS, the mammalian expression plasmid, and used for expression of the GPs in HEK293T cells.

Virus titration. The infectivity of VSVΔG* pseudotyped with GPs on K562 clones was determined by counting the number of GFP-positive cells using flow cytometry. To test C-type lectin-mediated entry, 10^5 cells of the K562 clones in 96-well plates were infected with the respective viruses, whose titers were standardized (i.e., all the viruses were diluted to give 1×10^5 to 5×10^5 IU/ml in Vero E6 cells that uniformly gave approximately 1×10^4 to 5×10^4 IU/ml in K562/mock cells), and the number of GFP-positive cells were counted. To investigate the effects of the cathepsin B and L inhibitors, CA-074Me and FY-dmk, respectively (Calbiochem), cells were treated with one of the inhibitors for 3 h before infection.

Binding assay. Approximately 10^6 infectious units (in Vero E6 cells) of purified VSVΔG* pseudotyped with GPs were incubated with 10^5 cells of K562/hMGL and K562/DC-SIGN for 1 h on ice. After being washed three times with PBS(+), cells were lysed to measure the amount of VSV matrix protein in the virions which bound on the cell surface.

SDS-PAGE and Western blotting. Cells or purified viruses were lysed with PBS containing 1% Triton X-100 and protease inhibitor cocktail Complete Mini (Roche), and the insoluble fraction was removed by centrifugation. Lysates were mixed with Laemmli sample buffer (Bio-Rad), electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 20% SuperSep (Wako), and blotted on a polyvinylidene difluoride (PVDF) membrane (Millipore). Non-specific binding to the membrane was blocked with 3% skim milk in PBS. A mixture of the sera obtained from mice immunized with Angola virus-like particles and Musoke virus-like particles for detecting MARV GPs or anti-VSV matrix protein monoclonal antibody (195-2) was incubated with the membrane. Peroxidase-conjugated AffiniPure goat anti-mouse IgG(H+L) (Jackson ImmunoResearch) and Immobilon Western (Millipore) were used for visualization. Intensities of specific bands were measured with ATTO CS Analyzer 2.1.

Molecular modeling. A three-dimensional model of Angola GP was generated by a homology modeling method using the crystal structure of EBOV GP (Protein Data Bank [PDB] code 3CSY) (27) as a template. The sequence alignment between MARV and EBOV GPs was based on that previously reported by Lee et al. (27). One hundred models of the first construction were generated using the automodel class in Modeller 9v6 (35), and the model with the lowest value of the Modeller objective function was selected. Next, to fill the gap of some potential loop conformation that the structural template of Zaire EBOV GP lacks (residues 174 to 197, 208 to 218, and 272 to 291 in the Angola GP numbering that correspond to residues 190 to 213, 224 to 225, and 279 to 298 in Zaire EBOV GP, respectively), two hundred models were generated by the loop model class (14). The best loop model was chosen by a combination of the Modeller objective function value and the discrete optimized protein energy (DOPE) statistical potential score (38). Then, the model, after addition of hydrogen atoms, was refined by energy minimization (EM) with the minimization protocols in the Discovery Studio 2.1 software package (Accelrys, San Diego, CA), using a CHARMM force field. Steepest descent, followed by conjugate gradient minimizations, was carried out until the root mean square (RMS) gradient was less than or equal to 0.01 kcal/mol/Å. The generalized Born implicit solvent model (43, 47) was used to model the effects of solvation. The model of Angola GP was finally evaluated by using PROCHECK (26), WHAT_CHECK (22), and Verify3D (10).

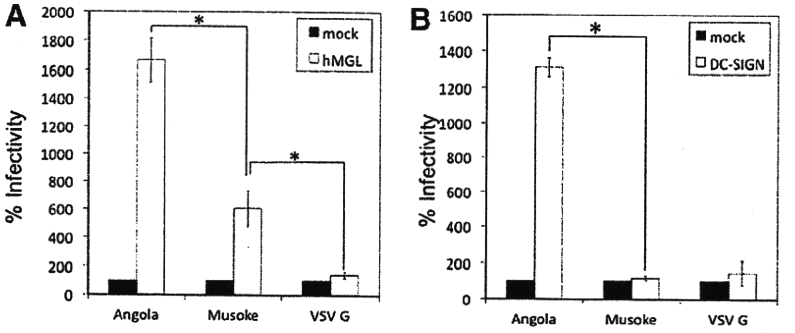

FIG. 1. Infectivity of VSVΔG* pseudotyped with MARV GPs in K562 cells expressing the C-type lectins. The infectivities of VSV-Angola and -Musoke were standardized using Vero E6 cells, as described in Materials and Methods, and approximately the same titers of viruses were used to infect K562/mock, K562/hMGL, and K562/DC-SIGN cells. The infected cells were counted using a flow cytometer, and the percentages of infectivity (i.e., relative infectivities) in K562/hMGL (A) and K562/DC-SIGN (B) cells were determined by setting the number of the infected K562/mock cells to 100% (46). All experiments were done in triplicate, and average results and standard deviations are shown. Statistical significance was determined by Student's t test. *, $P < 0.05$.

RESULTS

Efficiency of C-type lectin-mediated entry differs between MARV strains. We generated VSVΔG* bearing VSV G (VSV-VSV G), VSVΔG* bearing Angola GP (VSV-Angola), or VSVΔG* bearing Musoke GP (VSV-Musoke), and the infectivities of these viruses in K562/hMGL or K562/DC-SIGN cells were compared (Fig. 1). No significant enhancement of VSV-VSV G infectivity was seen in these C-type lectin-expressing cells. Consistent with a previous study (46), the viruses infected K562/hMGL cells more efficiently than they infected control K562/mock cells. In K562/DC-SIGN cells, the difference was observed only for VSV-Angola infectivity. It was noted that VSV-Angola showed significantly higher infectivity in these C-type lectin-expressing cells than VSV-Musoke, as was seen between Zaire and Reston EBOVs (46).

hMGL and DC-SIGN bind to MARV GPs in a different manner. To test the attachment of VSV-Angola and -Musoke to the surfaces of the cells expressing C-type lectins, a direct binding assay was performed (Fig. 2A). In both K562/hMGL and K562/DC-SIGN cells, only limited differences of the viruses attached on the cell surfaces were observed. For more quantitative analysis of the binding of MARV GPs to the C-type lectins, we next carried out a lectin-ELISA using soluble forms of hMGL and DC-SIGN and purified viruses (Fig. 2B and C). We found that both lectins bound to Angola and Musoke GPs in a dose-dependent manner and that hMGL had slightly higher ability to bind to Angola GPs than to bind to Musoke GPs, whereas DC-SIGN similarly bound to both GPs, confirming the different glycan specificities of these lectins (i.e., hMGL and DC-SIGN preferentially react with O-glycans and high-mannose-type N-glycans, respectively) (11, 21, 44).

MLRs and GP2 are important for efficient entry mediated by C-type lectins. MLRs of filoviruses have been shown to play an important role in interaction with the C-type lectins. To ascertain the contribution of the MLR of MARV GP to C-type lectin-mediated entry, we first constructed MLR-deletion mutants (AΔ and MΔ) (Fig. 3A) and examined the infectivities of VSVΔG* pseudotyped with these mutant GPs (VSV-AΔ and -MΔ) in Vero E6, K562/hMGL, and K562/DC-SIGN cells. VSV-AΔ and -MΔ showed no defects in their infectivities in Vero E6 cells, consistent with a previous study (32), indicating that glycosylation in the MLR and GP cleavage by furin are not essential to infect Vero E6 cells. In contrast, these viruses infected K562 cells expressing the C-type lectins much less efficiently than VSVΔG* pseudotyped with full-length wild-type GPs (Fig. 3B and C). These results indicate the major contribution of the MLR to C-type lectin-mediated entry of MARV.

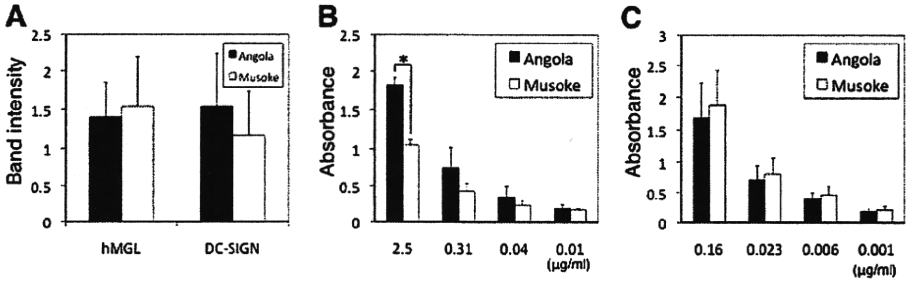

FIG. 2. Binding affinity of the C-type lectins to VSVΔG* pseudotyped with MARV GPs. (A) The amounts of VSV-Angola and -Musoke that attached on K562/hMGL or K562/DC-SIGN cells were shown as band intensities of VSV matrix protein. (B, C) A lectin-ELISA was performed, using purified VSV-Angola and -Musoke as antigens. Biotinylated recombinant soluble hMGL (B) and DC-SIGN ECD (C) were incubated at the indicated concentrations and visualized, as described in Materials and Methods. All experiments were done in triplicate, and average results and standard deviations are shown. Statistical significance was determined by Student's t test. *, $P < 0.05$.

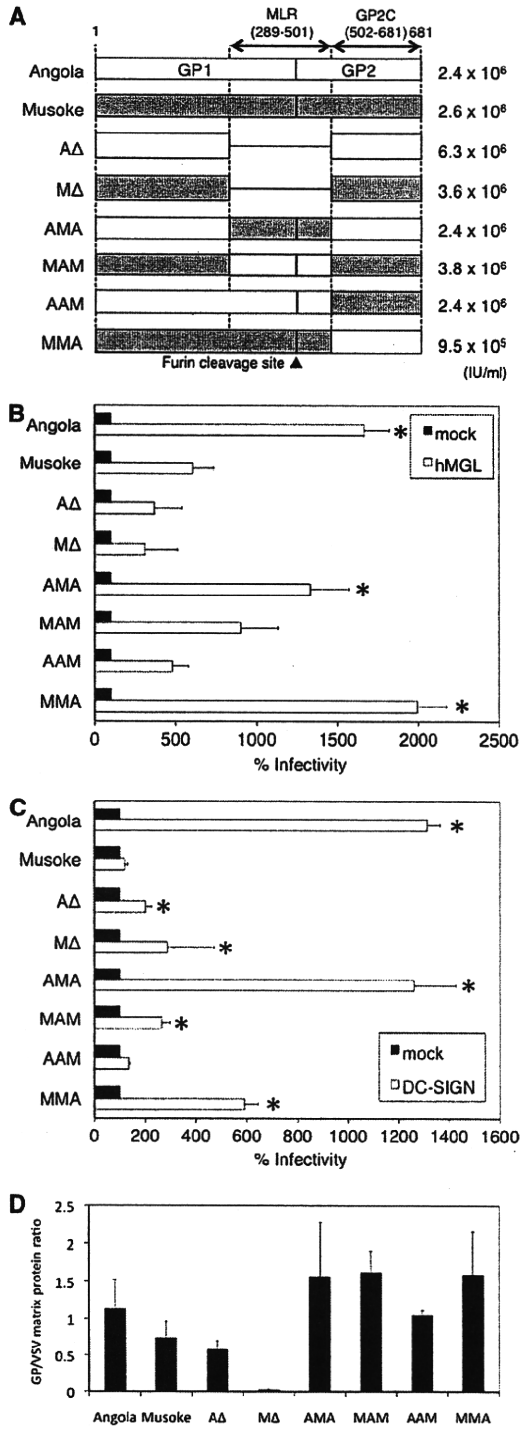

FIG. 3. Infectivity of VSVΔG* pseudotyped with the deletion or chimeric mutant GPs in C-type lectin-expressing cells. (A) The names of the MARV mutant GPs and the relevant amino acid positions are shown in the schematic. The number of infectious units determined for each virus in Vero E6 cells are shown on the right. (B, C) The relative infectivities of the viruses in K562/hMGL (B) and K562/DC-SIGN (C) cells were determined, as described in the legend of Fig. 1. All experiments were done in triplicate, and average results and standard deviations are shown. Statistical significance was determined (compared to the infectivity of VSV-Musoke in each K562/hMGL or K562/DC-SIGN cell) by Student's *t* test. *, $P < 0.05$. (D) The amounts of mutant GPs incorporated within the pseudotyped VSVΔG* were quantitated by using Western blotting of purified virions. Band intensities of MARV GPs and VSV matrix protein were determined, and their ratios are shown.

Next, we constructed chimeric GPs whose MLRs were swapped (AMA and MAM) (Fig. 3A), and the infectivities of VSVΔG* pseudotyped with these chimeric GPs (VSV-AMA and VSV-MAM) were tested (Fig. 3B and C). Unexpectedly, the relative infectivity of VSV-AMA in K562 cells expressing the C-type lectins was significantly higher than that of VSV-Musoke and similar to that of VSV-Angola. Replacement of the MLR of Musoke GP with that of Angola GP (MAM) showed only minimal effects on the enhancement of infectivity in the C-type lectin-expressing cells if compared with VSV-Musoke infectivity.

We finally replaced amino acid positions 502 to 681 of the GP2 regions (GP2C) of each (AAM and MMA) (Fig. 3A) and found that VSVΔG* pseudotyped with the chimeric Angola GP that had Musoke GP2C (VSV-AAM) infected both K562/hMGL and K562/DC-SIGN cells less efficiently than VSV-Angola (Fig. 3B and C), and the relative infectivities of VSV-AAM were similar to that of VSV-Musoke in both C-type lectin-expressing cell types. In contrast, the infectivities of VSV-MMA in both types of cells were significantly higher than that of VSV-Musoke or -AAM. These results indicate that GP2C is critical for the difference in the efficiency of C-type lectin-mediated entry between VSV-Angola and -Musoke. It was confirmed that all the chimeric GPs were similarly incorporated into virions (Fig. 3D). MΔ was not clearly detected by Western blotting. It was most likely due to the lack of the MLR containing many specific epitopes, resulting in the different reactivity of polyclonal serum to MΔ. We further confirmed that fully functional GPs were incorporated into VSV virions, since there is no significant difference in the infectivities in Vero E6 cells among these viruses.

Substitution of an amino acid at position 547 in the GP2 region influences the efficiency of C-type lectin-mediated entry. There are four different amino acids in GP2C between the Angola and Musoke GPs. To identify which amino acid(s) contributes to the different ability of C-type lectin-mediated entry between the Angola and Musoke strains, the following eight mutant GPs that contain single-amino-acid substitutions were constructed: four Angola-based mutant GPs (A/H504T, A/G547V, A/A596T, and A/R618K) and four Musoke-based mutant GPs (M/T504H, M/V547G, M/T596A, and M/K618R) (Fig. 4A). The infectivity levels of VSVΔG* pseudotyped with these mutant GPs in K562/hMGL or K562/DC-SIGN cells were compared (Fig. 4B and C). While the mutations at position 504, 596, or 618 did not affect the infectivity of the respective viruses in cells expressing the C-type lectins, the infectivities of VSVΔG* pseudotyped with mutant GPs with substitution at position 547 (VSV-A/G547V and -M/V547G) were clearly switched (i.e., the relative infectivities of VSV-Angola and -M/V547A in the C-type lectin-expressing cells were comparable and higher than those of VSV-Musoke and -A/G547V).

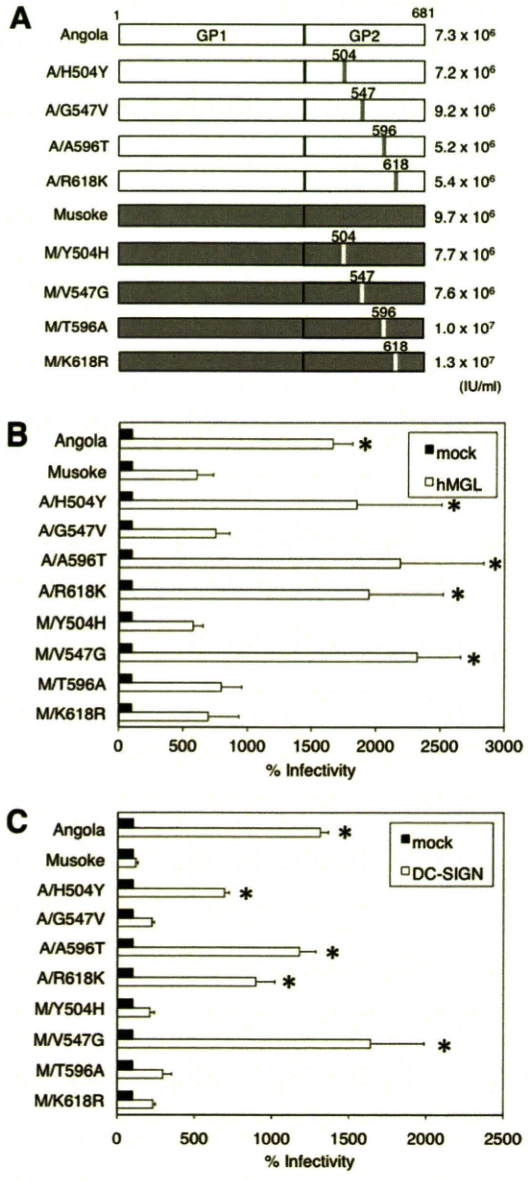

FIG. 4. Infectivity of VSVΔG* pseudotyped with the single-amino-acid mutant GPs in the C-type lectin-expressing cells. (A) The names of the MARV GP mutants and the positions of substituted amino acids are shown in the schematic. The number of infectious units determined for each virus in Vero E6 cells are shown on the right. (B, C) The relative infectivities of the viruses in K562/hMGL (B) and K562/DC-SIGN (C) cells were determined, as described in the legend of Fig. 1. All experiments were done in triplicate, and average results and standard deviations are shown. Statistical significance was determined (compared to the infectivity of VSV-Musoke in each K562/hMGL or K562/DC-SIGN cell) by Student's *t* test. *, $P < 0.05$.

Infectivities of VSV-Angola and -Musoke are reduced in different manners by cathepsin inhibitors in the C-type lectin-expressing cells. Because the GP binding affinity for C-type lectins (i.e., attachment) did not seem essential for the different ability levels of C-type lectin-mediated entry between the Angola and Musoke strains, we then focused on the following

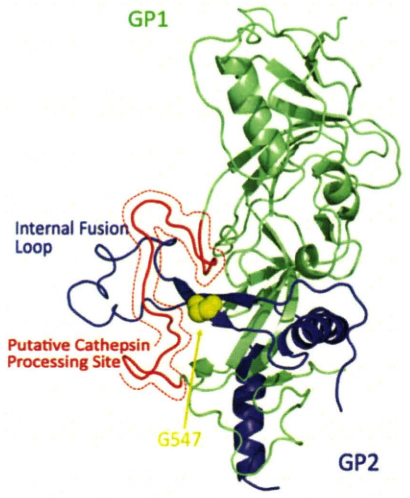

FIG. 5. Three-dimensional structure of the Angola GP monomer. The crystal structure of EBOV GP (PDB code 3CSY) was used as a template for homology modeling. GP1 (lime green) and GP2 (dark blue) are shown as ribbon models. Glycine at position 547 (G547, yellow) is shown as a space-filling model. The putative cathepsin cleavage site (amino acid residues 174 to 197 of Angola GP, corresponding to amino acid residues 190 to 213 of Zaire EBOV GP) is colored in red. This figure was prepared using PyMOL (DeLano Scientific LLC).

steps of viral entry. In EBOV and human coronavirus entry, it has been reported that proteolysis of GP by cellular endosomal cysteine proteases cathepsins B and/or L is necessary (4, 23, 24, 37). A three-dimensional model of Angola GP revealed that glycine at position 547 of Angola GP is presumed to form a β strand included in the internal fusion loop and is in close proximity to the putative cathepsin processing site (Fig. 5) (9, 27). Thus, we hypothesized that the amino acid change at position 547 affected the efficiency of cathepsin processing, which might lead to membrane fusion.

To test our hypothesis, VSVΔG* pseudotyped with wild-type or mutant (A/G547V and M/V547G) GPs was analyzed by comparing the infectivities of these viruses in Vero E6, control K562/mock, K562/hMGL, and K562/DC-SIGN cells pretreated with cathepsin inhibitors (Fig. 6). The infectivities of all the viruses were reduced by both of the inhibitors in a dose-dependent manner in all cells tested, suggesting that the proteolysis of GP by cathepsins B and/or L is also required for MARV entry. Interestingly, the infectivities of VSV-Angola and -M/V547G in K562 cells expressing the C-type lectins were less effectively reduced by these cathepsin inhibitors than those of VSV-Musoke and -A/G547V, whereas in Vero E6 and K562/mock cells, the differences in the infectivity at each concentration of the inhibitors were limited among the viruses. Paired Student's *t* test revealed significant differences ($P < 0.01$) in the following pairs: VSV-Angola and -Musoke, VSV-Angola and -A/G547C, and VSV-Musoke and -M/V547G (Fig. 6C, G, and H). And in Fig. 6D, the significant differences among these viruses were observed at a concentration of 10 μM by Student's *t* test. The infectivities of VSVΔG* pseudotyped with the other mutant GPs (i.e., VSV-A/H504T, -A/A596T, -A/

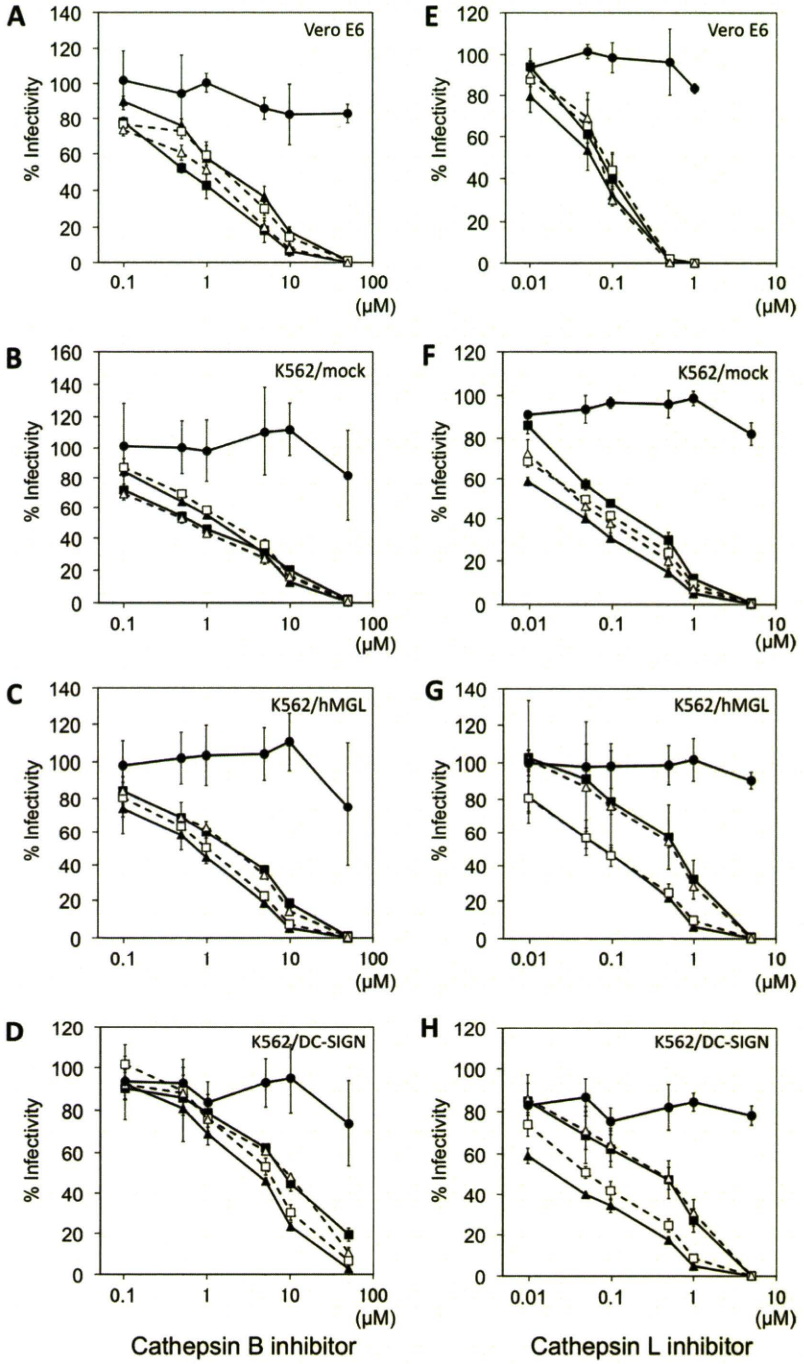

FIG. 6. Inhibition of virus infectivity by cathepsin inhibitors. Vero E6 (A and E), K562/mock (B and F), K562/hMGL (C and G), and K562/DC-SIGN (D and H) cells were pretreated with cathepsin B inhibitor (A, B, C, and D), cathepsin L inhibitor (E, F, G, and H), or dimethyl sulfoxide (DMSO) for 3 h and then infected with VSV-Angola (closed square), -Musoke (closed triangle), -A/G547V (open square), -M/V547G (open triangle) or -VSV G (closed circle). The number of infected cells, given in DMSO-treated cells, was used to set the 100% infectivity level for each cell type, and the relative infectivity was determined at each concentration of the inhibitors. All experiments were done in triplicate, and average results and standard deviations are shown.

R618K, -M/T504H, -M/T596A, and -M/K618R) were reduced by these cathepsin inhibitors similar to those of VSVΔG* pseudotyped with the respective wild-type GPs (data not shown).

DISCUSSION

It has been shown that cellular C-type lectins are utilized for the attachment of several viruses to host cells. hMGL, which

was originally founded as a macrophage-specific C-type lectin recognizing galactose/*N*-acetylgalactosamine, is expressed in monocyte-derived DCs and macrophages (44), and it was demonstrated that hMGL promoted the infection of EBOV and MARV (46). DC-SIGN, which recognizes high-mannose-type N-glycans and plays an important role in regulating the immune system (53), has also been shown to promote infection by several viruses (e.g., Ebola, Marburg, human immunodeficiency, hepatitis C, measles, dengue, and influenza viruses) (49, 53). A liver-specific C-type lectin, ASGP-R, which recognizes galactose in carbohydrate side chains, has been shown to be exploited for MARV infection of hepatocytes (3) and is thought to be one of the possible determinants of hepatotropism of MARV (17). Taken together, increased infection of these cells expressing C-type lectins and subsequent destruction of the host immune functions and coagulation system may be crucial for the pathogenesis of filoviruses. Our previous study (46) and the present study show that the different abilities used to utilize the C-type lectins among filoviruses to promote cellular entry might be correlated with the pathogenicities of the viruses. *In vivo* study may be needed to provide a direct link between the pathogenicity of MARV and its ability to use C-type lectins for entry into target cells.

Using deletion mutant GPs, we found that both hMGL and DC-SIGN principally recognized MLRs. Amino acid comparison between Angola and Musoke GPs indicates that the similarity of their MLRs is 86.4% (data not shown). The numbers of potential O-glycosylation sites vary between Angola and Musoke GPs (the number of these sites for Musoke GP is less than the number of these sites for Angola GP), whereas the potential N-glycosylation sites are relatively conserved, supporting our observation that hMGL, but not DC-SIGN, bound more efficiently to Angola GP than to Musoke GP in the lectin-ELISA. However, using chimeric mutant GPs, we showed that the structure of the MLR itself was not essential for the different levels of infectivity between VSV-Angola and -Musoke in K562 cells expressing these C-type lectins, suggesting that the capacity of the GP for binding to C-type lectins through the MLR (i.e., the glycosylation pattern of MLR) is not the only factor contributing to the efficiency of C-type lectin-mediated entry. Indeed, we identified that the amino acid at position 547 in GP2, but not in the MLR, was critical for the different efficiency levels of C-type lectin-mediated entry between VSV-Angola and -Musoke. It is of interest to confirm the importance of this amino acid in MARV infection by using a reverse genetics approach.

In our three-dimensional model, it seemed unlikely that glycine/valine at position 547 directly influenced the binding to the putative specific receptors, since substitution of this amino acid did not affect the infectivity of pseudotyped viruses in Vero E6 or control K562 cells (data not shown), and this amino acid was not located around the putative receptor-binding domain (9, 25). We showed that the effects of cathepsin inhibitors on the infectivity differ between Angola and Musoke only in the C-type lectin-expressing cells, and the single-amino-acid substitution at position 547 altered the effects of cathepsin inhibitors. However, no significant difference in the susceptibility to cathepsins was seen in direct digestion assays *in vitro* using soluble forms of the C-type lectins and purified virions (data not shown). These results suggest that glycine at position 547 increases sensitivity of GPs to endosomal cathepsins during C-type lectin-mediated entry, but our *in vitro* digestion assay did not provide actual conditions for interaction between GPs and C-type lectins in endosomes. It may be also possible that when the viruses are internalized to endosomes through the interaction with C-type lectins, (i) the amino acid at position 547 affects the flexibility of the fusion loop and/or the efficiency of conformational change, and (ii) glycine at position 547 weakens GP1-GP2 interaction, resulting in reduced cathepsin dependence in virus entry, as reported with EBOV GP (51). It is interesting to clarify how this amino acid contributes to the intramolecule interaction required for GP functions.

In summary, our data suggest that the efficiency of C-type lectin-mediated entry of filoviruses is determined not only by direct interaction between GP and C-type lectins but also by some mechanisms underlying endosomal entry, such as proteolytic processing, and likely by cathepsins or membrane fusion machinery. Although further investigations are required to prove our hypotheses, this study provides new insights into understanding the molecular basis of the C-type lectin-mediated entry of filoviruses, which may have a possible link to their pathogenicity.

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学内グラント 終了時報告書

平成20-21年度　学内グラント報告書

ペプチド結合リポソームを用いた，エボラウイルスに対するCTL誘導型ワクチンの開発

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緒言

エボラウイルスは，フィロウイルス科に属するRNAウイルスで，ヒトを含む霊長類に重篤な新興感染症であるエボラ出血熱を引き起こす．その致死率は時に90%を超え，最も危険なウイルスである[1]．1976年にスーダンとザイールで大流行して以来，中央・西アフリカで散発的に流行を繰り返しているが，米国においても1989年にサル検疫施設で発生し大問題となった．致死量が極めて少量で，血液や体液を介してヒトからヒトへ感染するため極めて脅威であり，Biosafety level 4 (BSL4) の封じ込めが必要である．自然宿主はコウモリの可能性が示唆されている[2]が，まだ特定されていない．我が国ではまだ発症例はないが，ペットのサルの輸入や旅行者の増加に伴い，日本に侵入する危険性は高まっており，一類感染症に指定されている．また，米国政府は，エボラウイルスをバイオテロに利用しうるカテゴリーAの生物兵器と位置づけている．しかしながら，効果的な予防・治療法は存在しない．以上の状況から，地球規模で，エボラウイルスに対するワクチンの開発は急務である．

エボラ出血熱の患者では，激しいリンパ球のアポトーシスがみられ，免疫機構がうまく働かない．しかし，生き延びたわずかの感染者では，ウイルス特異的細胞傷害性T細胞（CTL）やウイルスに対する抗体が存在する[3]．従って，エボラウイルス感染においても，一般的なウイルス感染症と同様に，体液性免疫及び細胞性免疫が有効にウイルスを排除すると考えられる．事実，マウスにウイルス中和抗体[4]やウイルス特異的CTL[5]を導入することによって，エボラウイルスへの抵抗性が示された．一方，サルを用いた実験では，細胞性免疫は有効にウイルスを排除した[6-8]が，中和抗体はウイルスをコントロールできなかった[9]．従って，CTLを中心とした細胞性免疫がエボラウイルスの排除に重要である可能性が高く，CTL誘導型ワクチンの開発が必要であると思われる．しかし，エボラ出血熱における細胞性免疫の研究はほとんど行われておらず，そのCTLエピトープはわずか3つしかわかっていない[10]．

エボラウイルスを扱うにはBSL4の封じ込めが必要であり，それが研究上の大きな障害である．我々は，組換えウイルスとMHCクラスIトランスジェニックマウスを用いることで，BSL3やBSL4実験施設が必要とされる危険なウイルスに対しても，BSL2実験室でCTLのエピトープを同定できる方法を開発した．さらに，エピトープに相当する短いペプチドをリポソーム表面に結合させてマウスに免疫することで，極めて強力にペプチド特異的CTLを誘導できることを示した[11]．我々は，これらの方法を使って，BSL3の封じ込めが必要な，重症急性呼吸器症候群（SARS）コロナウイルス[12,13]，及びH5N1亜型高病原性鳥インフルエンザウイルス[14]のHLA-A2拘束性CTLエピトープを多数同定した．そして，同定したエピトープを結合したペプチド結合リポソームを作製し，HLA-A2トランスジェニックマウスに免疫して，ウイルス特異的CTLの誘導を詳細に検討した[12-14]．現在，我々は，これらのデータを基にして，新型インフルエンザに対する新しいワクチンの開発・実用化をめざした研究を続けている（特許2件出願中）．

本研究では，同様の方法を用いて，エボラウイルスに対するCTLエピトープを多数同定し，そのエピトープを利用してペプチド結合リポソームを作製・解析して，エボラ出血熱に対するワクチン開発のための基礎

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