# Characterization of the Envelope Glycoprotein of a Novel Filovirus, Lloviu Virus

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Lloviu virus (LLOV), a novel filovirus detected in bats, is phylogenetically distinct from viruses in the genera *Ebolavirus* and *Marburgvirus* in the family *Filoviridae*. While filoviruses are known to cause severe hemorrhagic fever in humans and/or nonhuman primates, LLOV is biologically uncharacterized, since infectious LLOV has never been isolated. To examine the properties of LLOV, we characterized its envelope glycoprotein (GP), which likely plays a key role in viral tropism and pathogenicity. We first found that LLOV GP principally has the same primary structure as the other filovirus GPs. Similar to the other filoviruses, virus-like particles (VLPs) produced by transient expression of LLOV GP, matrix protein, and nucleoprotein in 293T cells had densely arrayed GP spikes on a filamentous particle. Mouse antiserum to LLOV VLP was barely cross-reactive to viruses of the other genera, indicating that LLOV is serologically distinct from the other known filoviruses. For functional study of LLOV GP, we utilized a vesicular stomatitis virus (VSV) pseudotype system and found that LLOV GP requires low endosomal pH and cathepsin L, and that human C-type lectins act as attachment factors for LLOV entry into cells. Interestingly, LLOV GP-pseudotyped VSV infected particular bat cell lines more efficiently than viruses bearing other filovirus GPs. These results suggest that LLOV GP mediates cellular entry in a manner similar to that of the other filoviruses while showing preferential tropism for some bat cells.

Filoviruses are nonsegmented, negative-stranded RNA viruses grouped into two genera, *Marburgvirus* and *Ebolavirus*. These filoviruses are known to cause severe hemorrhagic fever in human and/or nonhuman primates, with case mortality rates of up to 90% (1). There is one known species of *Marburgvirus*, *Marburg marburgvirus*, consisting of two viruses, Marburg virus (MARV) and Ravn virus. On the other hand, five distinct species are known in the genus *Ebolavirus*, *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï forest ebolavirus*, *Bundibugyo ebolavirus*, and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus (SUDV), Taï forest virus (TAFV), Bundibugyo virus (BDBV), and Reston virus (RESTV), respectively. A difference in pathogenicity was suggested to exist among ebolaviruses. EBOV is thought to be the most pathogenic, killing up to approximately 90% of patients, whereas RESTV has never caused a lethal infection in humans (2) and is less pathogenic in experimentally infected nonhuman primates than EBOV (3).

Recently, a filovirus-like RNA genome was detected in the lungs, livers, rectal swabs, and/or spleens of bat (*Miniopterus schreibersii*) carcasses found in Cueva del Lloviu, Asturias, Spain. This novel filovirus was designated Lloviu virus (LLOV); its name was derived from the cave in which it was first found (4). LLOV is phylogenetically distinct from other filoviruses; thus, it is proposed to belong to the new genus *Cuevavirus*, species *Lloviu cuevavirus*, in the family *Filoviridae* (4). However, the biological properties of this novel virus are uncharacterized, since infectious LLOV has not been isolated yet.

Filovirus particles consist of at least seven structural proteins, including the nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and polymerase (L) genes (5). The envelope GP is responsible for both receptor binding and fusion of the virus envelope with the host cell membrane (6, 7). GP undergoes proteolytic cleavage by host proteases such as furin, resulting in the two subunits, $GP_1$ and $GP_2$, which are linked by a disulfide bond (7, 8). GP is highly glycosylated with large amounts of N- and O-linked glycans, most of which are located in its middle one-third, designated the mucin-like region (MLR), which plays an important role in attachment to the preferred target cells (9, 10). Although MLR is found in all known filovirus GPs, its highly variable amino acid sequences and sugar chain structures suggest different GP properties among filovirus species. Membrane-anchored cellular C-type lectins have been found to facilitate filovirus infection *in vitro* through binding to glycans on the MLR (11–13). It was also shown that MLR contains epitopes for antibody-dependent enhancement (ADE) of filovirus infection *in vitro* (14, 15).

To provide information for estimation of the infectivity and potential pathogenicity of LLOV, this study focused on GP, which likely plays a major role in the replication cycle and the pathogenicity of filoviruses (10, 16). In this study, we investigated the morphology of virus-like particles consisting of LLOV GP, VP40, and NP, compared the antigenicity of GP among filoviruses, and analyzed the ability of GP to mediate virus entry into cells. Here, we show that LLOV GP has the potential to mediate viral entry

into cells of various animal species, including primates, in a manner similar to that of the other filoviruses while showing preferential tropism for particular bat cells.

## MATERIALS AND METHODS

**Cells.** Human embryonic kidney 293 (HEK293), HEK293T, and African green monkey kidney Vero E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fatal calf serum (FCS) and penicillin-streptomycin. Bat cell lines ZFB11-97 and SuBK12-08 were established by transfecting an expression plasmid encoding the Simian virus 40 large T antigen (pCXN2-Flag-SV40LT; kindly provided by H. Sawa and Y. Orba, Hokkaido University Research Center for Zoonosis Control) into primary kidney cells of bats captured in Zambia. The transfected cells were selected by culturing in the presence of G418 (200 μg/ml). ZFB11-97, SuBK12-08, and Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (MEM) with 10% FCS, L-glutamine, and penicillin-streptomycin. SK-L cells (17) were cultured in MEM with 10% FCS, L-glutamine, penicillin-streptomycin, and 0.3% tryptose phosphate broth (GIBCO). Bat cell lines BKT1, FBKT1, YubFKT1, IndFSPT1, and DemKT1 were established as described previously (18). Bat species were identified by morphology, habitat, and BLAST searches using the sequences of their cytochrome *b* genes (nucleotide positions 1 to 400). BKT1, FBKT1, YubFKT1, IndFSPT1, DemKT1, human chronic myelogenous leukemia (K562), and K562 clones expressing human macrophage galactose-type C-type lectin (hMGL) or dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (19, 20) were grown in RPMI 1640 medium with 10% FSC, L-glutamine, and penicillin-streptomycin.

**Construction of plasmids expressing GP, NP, and VP40.** Coding regions of the GP, NP, and VP40 genes of LLOV were synthesized in pBS II SK vector (FASMAC) based on the nucleotide sequence of LLOV (GenBank accession number JF828358). The NP and VP40 genes were synthesized according to the coding regions reported in the database. Since the ebolavirus envelope GP is expressed through transcriptional editing (21, 22), the coding region of the GP gene was synthesized with an additional adenosine at the putative editing site. After digestion by restriction enzymes, each gene was cloned into mammalian expression vector pCAGGS (23). The expression plasmids for EBOV (strain Mayinga), SUDV (strain Boniface), TAFV (strain Cote d'Ivoire), BDBV (strain Bundibugyo), RESTV (strain Pennsylvania), and MARV (strains Angola and Musoke) were constructed as described previously (24).

**Purification of VLPs.** HEK293T cells were transfected with plasmids encoding GP, VP40, and NP of LLOV, EBOV, SUDV, TAFV, BDBV, RESTV, and MARV (strain Angola) using TransIT LT-1 reagent (Mirus) according to the manufacturer's instructions. Forty-eight hours later, VLPs were purified from culture supernatants by ultracentrifugation at $28,000 \times g$ at 4°C for 1.5 h with a 25% sucrose cushion. Virus-like particle (VLP) pellets were resuspended in phosphate-buffered saline (PBS).

**SDS-PAGE and Western blotting.** HEK293T cells were transfected with plasmids encoding filovirus GPs and lysed 48 h after transfection with a lysis buffer (10 mM Tris HCl [pH 7.8], 0.15 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and protease inhibitor mixture) (Roche). Cell lysates were mixed with SDS-PAGE sample buffer with or without 5% 2-mercaptoethanol. After electrophoresis on 5 to 20% SuperSep (Wako), separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with mouse antisera (diluted 1:2,000) to filovirus VLPs (see below), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson Immuno-Research). The bound antibodies were visualized with Immobilon Western (Millipore).

**Mouse antisera and ELISA.** Five-week-old female BALB/c mice were immunized twice intraperitoneally with purified VLPs (100 μg/mouse) at 3-week intervals. Antisera were collected 7 days after the second immunization. Animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocol was approved by the Hokkaido University

Animal Care and Use Committee. The GP-based enzyme-linked immunosorbent assay (ELISA) was performed as described previously (25). Serum samples were serially diluted with PBS containing 0.05% Tween 20, 0.5% bovine serum albumin, and 2% FCS. Bound antibodies were visualized by adding peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) and 3,3′,5,5′-tetramethylbenzidine (Sigma). The reaction was stopped by adding 1 N phosphate acid to the mixture, and the optical density at 450 nm ($OD_{450}$) was measured.

**Electron microscopy.** Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out as described previously (26, 27). Purified VLPs fixed with 0.25% glutaraldehyde were adsorbed to collodion-carbon-coated copper grids and negatively stained with 2% phosphotungstic acid solution (pH 5.8). For immuno-TEM, we used an anti-LLOV GP monoclonal antibody (LGP14-2), produced in this study as described previously (15), and an immunogold-conjugated goat anti-mouse IgG (H + L) polyclonal antibody (BB International). Samples were examined with an H-7650 electron microscope (Hitachi) at 80 kV. For SEM, cells transfected with plasmids expressing LLOV GP, VP40, and NP were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide in the same buffer. The fixed samples were dehydrated with a series of ethanol gradients, replaced with t-butanol, and dried in an ES-2030 freeze dryer (Hitachi). Dried specimens were coated with platinum by using mild spatter E-1046 (Hitachi). The samples were observed with an S-4700 electron microscope (Hitachi) at 15 kV.

**VSV pseudotyped with filovirus GPs.** Using vesicular stomatitis virus (VSV) containing the green fluorescent protein (GFP) gene instead of the receptor-binding VSV G protein gene (VSVΔG*-G) (6), pseudotyped viruses with GPs of EBOV, RESTV, MARV strains Angola and Musoke, and LLOV (VSVΔG*-Zaire, VSVΔG*-Reston, VSVΔG*-Angola, VSVΔG*-Musoke, and VSVΔG*-Lloviu, respectively) were generated, and the infectious units (IUs) of stock viruses were determined in Vero E6 cells according to a previous study (6). The genome copy number of each pseudotyped VSV preparation was quantified by real-time reverse transcription-PCR (RT-PCR). Real-time RT-PCR was performed using a One Step SYBR PrimeScript RT-PCR kit II (TaKaRa Bio) and a CFX96 real-time system (Bio-Rad) with primers (GFP498-F, CAAGATCCGCCACA ACATCG; GFP-617R, GACTGGGTGCTCAGGTAGTG) to detect the GFP gene in the VSV genome.

**Virus titration.** To determine infectivities of VSVs pseudotyped with filovirus GPs, appropriately diluted virus stocks were pretreated with an anti-VSV G monoclonal antibody, VSV-G(N)1-9, to abolish the background infectivity of parental VSVΔG*-G (15). K562 clones expressing hMGL and DC-SIGN grown on 96-well plates were infected with VSV pseudotyped with filovirus GPs (50 to 150 IU; determined in K562), and infectivities were determined by counting the number of GFP-positive cells using flow cytometry as described previously (19, 20). For the assays of antibody-dependent enhancement (ADE) of infection, 10-fold serially diluted mouse antisera were mixed with equal volumes of pseudotyped VSVs (50 to 150 IU; determined in K562). After a 1-h incubation at room temperature, the mixture was inoculated into K562 cells grown on 96-well plates. At 20 h postinoculation, GFP-positive cells were counted with an IN Cell Analyzer 2000 (GE Healthcare). To determine the infectivities in adherent cells of different animal origins, cell monolayers grown on 96-well plates were infected with VSVs pseudotyped with filovirus GPs. Twenty hours later, the virus infectivity in each cell line was determined by counting the number of GFP-expressing cells under a fluorescence microscope, and IUs per $10^6$ genome copies were calculated.

**Inhibitor treatments.** Vero E6 cells were pretreated with ammonium chloride (Wako), monensin (Sigma), or cathepsin B or L inhibitors (CA-074Me and FY-dmk, respectively; Calbiochem) for 30 min at 37°C. Treated cells were then infected with VSVΔG*-Zaire, VSVΔG*-Angola, VSVΔG*-Lloviu, and VSVΔG*-G appropriately diluted to yield 200 to 2,000 IUs/$10^6$ cells. At 20 h postinoculation, GFP-positive cells were counted with the IN Cell Analyzer 2000 (GE Healthcare).

## RESULTS

**Characterization of the primary structure of LLOV GP.** Although the envelope GPs of ebolaviruses are expressed through transcriptional editing (21, 22), similar characteristics were not shown for LLOV GP in a previous study, and the reported LLOV GP gene did not have an open reading frame of the single-transmembrane GP (4). Thus, we first analyzed the nucleotide and deduced amino acid sequences of LLOV GP and found that the nucleotide sequence of LLOV GP had 7 adenosines at positions 910 to 916. Based on sequence comparison to the EBOV genome, which has an editing site at positions 880 to 886, we assumed that this stretch of 7 adenosines is the editing site of the LLOV GP gene. Accordingly, an open reading frame of the full-length transmembrane GP gene was produced by adding an adenosine at this putative editing site (Fig. 1A). We found that LLOV envelope GP had a potential cleavage site (i.e., RRRR; recognized by host ubiquitous proteases, such as furin) and MLR, similar to the other filoviruses. The predicted MLR of LLOV GP differed from that of EBOV GP in length and location (i.e., LLOV MLR was located over the cleavage site and was a little shorter than EBOV MLR) (Fig. 1A and B). We confirmed the approximate molecular size of LLOV GP (GP$_{1,2}$, approximately 120 to 130 kDa) and its cleavage product, the GP$_1$ subunit (approximately 100 kDa), by Western blotting (Fig. 1C). These results suggested that LLOV GP shares many characteristics with the other filovirus GPs.

**Morphology of VLPs consisting of LLOV GP, VP40, and NP.** Filoviruses are characterized by their filamentous forms. However, LLOV particles have never been verified morphologically. To determine the possible shape of LLOV particles, we investigated the morphology of VLPs consisting of LLOV GP, VP40, and NP. TEM analyses revealed that LLOV VLPs were filamentous (Fig. 2A and E), like EBOV (Fig. 2C and G) and MARV (Fig. 2D and H) VLPs. Numerous spikes were observed on the VLP surface, and immuno-TEM with an anti-LLOV GP monoclonal antibody confirmed the presence of LLOV GPs on the surface (Fig. 2B and F). Similar to the other filoviruses (28), VLPs with a uniform diameter of approximately 70 nm and various lengths were observed. This was consistent with a previous study showing that the diameters of VLPs were narrower than those reported for actual EBOV particles (80-nm diameter) (1, 29). By SEM, numerous filamentous structures were observed on the surfaces of cells transfected with plasmids expressing LLOV GP, VP40, and NP (Fig. 3), which has similarity to EBOV budding (27). These results suggested that LLOV shared morphological characteristics with the other known filoviruses.

**Antigenic comparison among filovirus GPs.** While LLOV is shown to be phylogenically distinct from the other filovirus species, serological information is lacking. The amino acid sequence of LLOV GP has 35 and 28% similarity to those of EBOV and MARV, respectively. To compare the antigenic relationships among filovirus GPs, we produced antisera to each GP by immunizing mice with VLPs and performed GP-based ELISA (Fig. 4). We found that anti-LLOV GP sera showed exclusive reactivity to the LLOV GP antigen (Fig. 4A). Similarly, anti-MARV GP sera only reacted with the MARV GP antigen (Fig. 4G). Antisera to EBOV, SUDV, TAFV, BDBV, and RESTV GPs showed slight cross-reactivity with ebolavirus antigens at the lowest dilution of the sera but not with the LLOV and MARV GP antigens (Fig. 4B to

F). These results indicated that LLOV was distinct not only phylogenically but also serologically from the other known filoviruses.

**Functional study of LLOV GP with chemical inhibitors.** For the functional study of LLOV GP, we produced VSV pseudotyped with LLOV GP, which could infect Vero E6 cells, and confirmed that the full-length GP produced with 8 adenosines at the putative editing sites was fully functional as a single transmembrane GP. It has been shown that endosomal acidification and proteolytic processing with the cellular cysteine proteases cathepsin L and/or B are required for filovirus GP-mediated entry in vitro (6, 7, 30). To investigate the requirement of these factors for the LLOV GP function, we examined the infectivities of pseudotyped VSV in Vero E6 cells pretreated with chemical inhibitors (Fig. 5). Pretreatment of cells with ammonium chloride and monensin markedly reduced the infectivity of VSVΔG*-Lloviu, as was the case with VSVΔG*-Zaire, VSVΔG*-Angola, and VSVΔG*-G, suggesting that LLOV GP requires a low pH for cellular entry. We found that pretreatments with a cathepsin L inhibitor at concentrations of 2.5 and 10 μM significantly reduced the infectivities of VSVΔG*-Lloviu, -Zaire, and -Angola. However, treatments at 0.625 μM did not show any inhibitory effects on the VSVΔG*-Lloviu infectivity; rather, it enhanced the infectivity. Interestingly, when cells were treated with a cathepsin B inhibitor, the infectivity of VSVΔG*-Zaire was reduced significantly in a dose-dependent manner, whereas much less inhibition was observed in the VSVΔG*-Lloviu and -Angola infectivities. These cathepsin inhibitors did not affect the infectivity of VSVΔG*-G.

**Human C-type lectin-mediated entry of pseudotyped VSVs.** C-type lectins expressed on the host cell surface are thought to serve as an attachment factor for filovirus GP, and C-type lectin-mediated entry is believed to be one of the important factors responsible for filovirus tropism and pathogenicity (11, 19, 20). Thus, we investigated the potential of LLOV GP to use the human C-type lectins hMGL and DC-SIGN, both of which are known to enhance filovirus infectivity (Fig. 6). VSVΔG*-Lloviu infected DC-SIGN-expressing cells more efficiently than hMGL-expressing cells, which was similar to VSVΔG*-Zaire and VSVΔG*-Reston. VSVΔG*-Lloviu infected hMGL-expressing cells more efficiently than VSVΔG*-Musoke (P < 0.05) but less so than VSVΔG*-Angola (P < 0.05); however, there were no significant differences among VSVΔG*-Lloviu, VSVΔG*-Zaire, and VSVΔG*-Reston. On the other hand, VSVΔG*-Lloviu infected DC-SIGN-expressing cells more efficiently than VSVΔG*-Angola (P < 0.05) and VSVΔG*-Musoke (P < 0.01) and less efficiently than VSVΔG*-Zaire (P < 0.05), but there was no significant difference between VSVΔG*-Lloviu and VSVΔG*-Reston.

**Difference in ADE activity between anti-EBOV and anti-LLOVGP antisera.** Antibody-dependent enhancement of infection is also a known in vitro phenomenon observed for comparatively highly lethal filoviruses (e.g., Zaire and Angola) (15, 31, 32). To investigate the potential of LLOV GP to induce ADE antibodies, K562 cells were infected with VSVΔG*-Lloviu or VSVΔG*-Zaire in the presence of mouse antisera specific to the respective viruses (Fig. 7). We confirmed the ADE activity of the anti-Zaire serum as indicated by markedly enhanced infectivities of VSVΔG*-Zaire at serum dilutions of 1:10 and 1:100 (Fig. 7A). In contrast, only minimal ADE activity was seen in the anti-LLOV serum, although similar amounts of specific IgG antibodies were detected in both antisera by ELISA (Fig. 4). Consist with the ab-

## A

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M V P T Y P Y S S L L D W R P P P N T L P W I L N L V V F Y T I A W L P G G V S   40
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G I P L G L L G N N S I T Q T V V D N V V C K E H L A T T D Q L Q A I G L G L E   80
GGAATTCCAC TCGGTTTGTT GGGAAACAAC AGCATCACCC AAACTGTCGT GGACAATGTA GTGTGCAAGG AACACCTTGC CACAACAGAT CAGCTACAGG CTATTGGATT GGGACTAGAG 240
G L G E H A D L P T A T K R W G F R S D V I P K I V G Y T A G E W V E N C Y N L   120
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E I T K D G H P C L P S P P T G L L G Y P R C R Y V H R A K G A G P C P G G N   160
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R L K A G L G T G F S H Q A E N Q N P N N Q F R T T T L D Y D V M S P W M D N A   240
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T F F F R A R E D T S M L I Q T R Y P P A N L E L V Q E R L A N L T G D Q A D P   280
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S K M E I V A E V L T L E L G D W S G W T T K K N R S T N H T A K K P F T S I   320
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E P C T K A P A G N T T N N V H H C C S W V R I Q A V H P G N T S G E I S M P L   400
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E L A N T T T K A L Q L F L R A T T E L R T Y S I L N R H A I D F L L Q R W G G   640
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T C R I L G P N C C I E P H D W S A N I T A E I N H I R E D I L N H H E I Q P S   680
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Q D P S F W T G W Q Q W I P T G A S A L G I I L A I L A L I C L C R I T R   717 amino acids
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## B
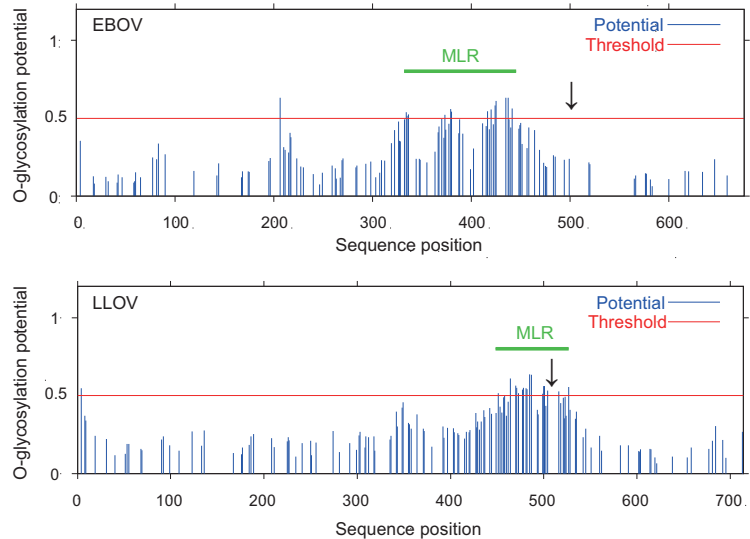


## C
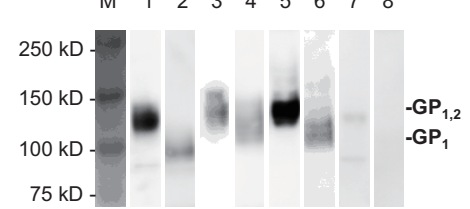


FIG 1 Primary structure of LLOV envelope GP. (A) Nucleotide and deduced amino acid sequences of LLOV GP. Deduced amino acid sequences are shown in boldface letters. Blue, green, and yellow lines represent the signal peptide, MLR, and transmembrane domain predicted by GENETYX Ver.10, NetOGlyc, and TopPred 0.01, respectively. Purple and red letters represent the putative editing and cleavage sites, respectively. (B) Comparison of EBOV and LLOV MLRs. Potential O-glycosylation sites were predicted by NetOGlyc, and MLRs were defined as the regions between the first and last amino acid residues showing a score above the threshold (0.5). Arrows indicate the cleavage sites. (C) Western blotting of filovirus GPs. Proteins in the lysate of HEK293T cells transfected with the plasmid expressing LLOV GP (lanes 1 and 2), EBOV GP (lanes 3 and 4), MARV GP (lanes 5 and 6), or empty vector (lanes 7 and 8) were separated by SDS-PAGE under nonreducing (lanes 1, 3, 5, and 7) or reducing (lanes 2, 4, 6, and 8) conditions.

sence of cross-reactive IgG in ELISA, little cross-ADE activity was observed in the ADE assay.

**Cellular tropism of LLOV GP.** To estimate the GP-dependent tropism that is likely reflected by the prevalence of LLOV recep-

tors, we infected various cell lines of different animal origins (Table 1) with pseudotyped VSV, and their infectivities were compared (Fig. 8A). VSVΔG*-Lloviu infected cells that were derived from the human, African green monkey, pig, dog, and bat in a
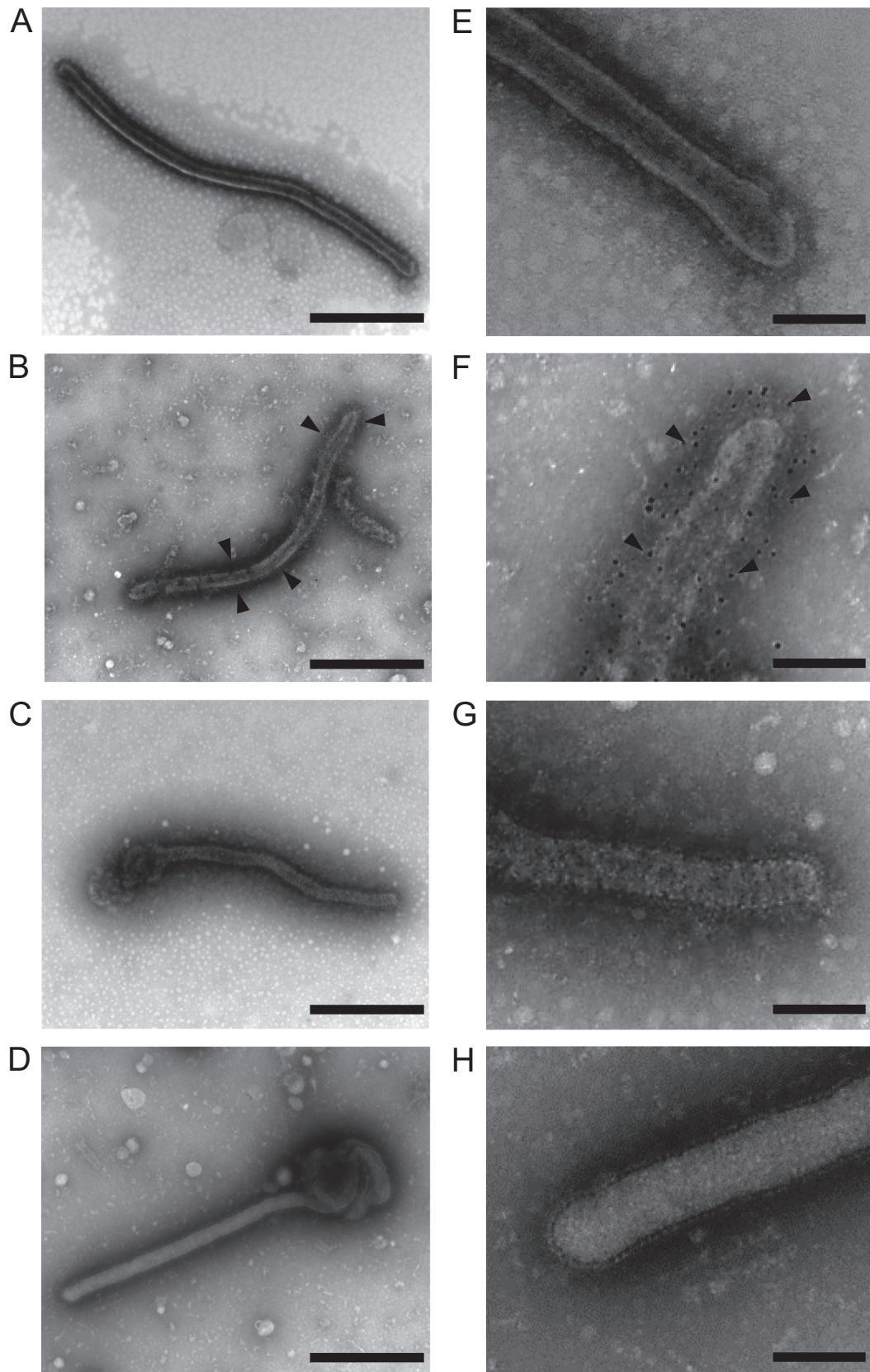
**FIG 2** TEM of filovirus VLPs. Purified VLPs produced from 293T cells transfected with plasmids expressing LLOV (A, B, E, and F), EBOV (Zaire) (C and G), and MARV (Angola) (D and H) proteins were fixed and stained as described in Materials and Methods. For immuno-TEM (B and F), an anti-LLOV GP monoclonal antibody was used. Scale bars represent 500 nm (A to D) and 200 nm (E to H). Arrowheads indicate gold particles.
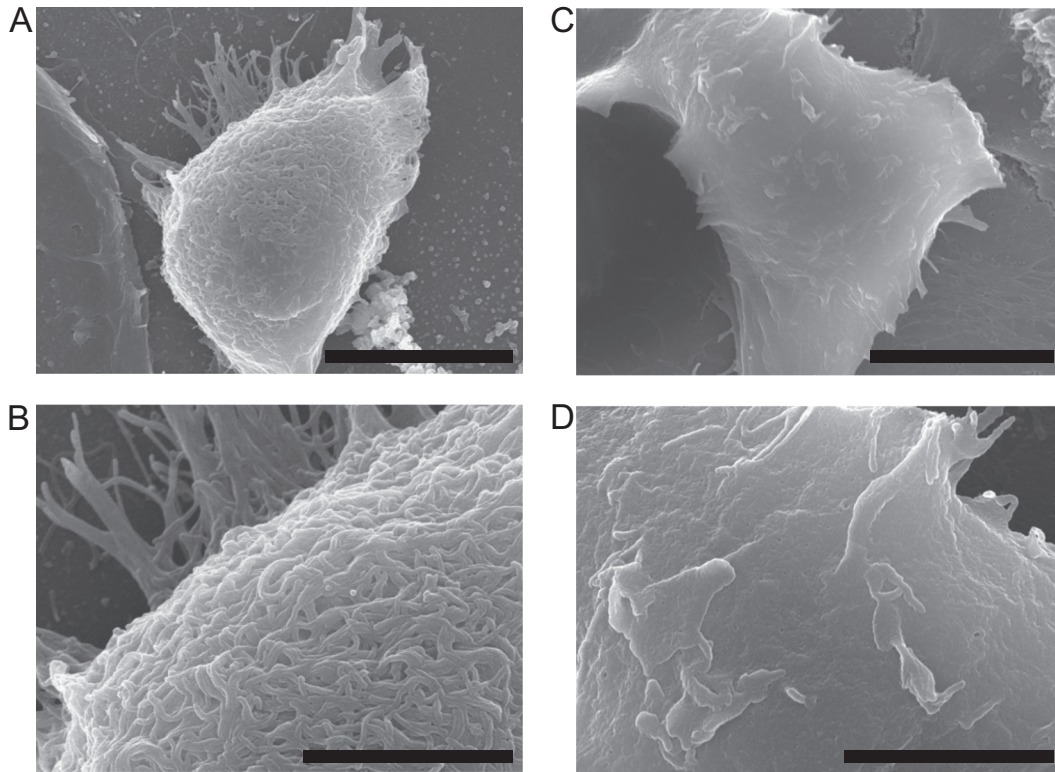
**FIG 3** SEM of LLOV VLPs. HEK293T cells transfected with pCAGGS expressing LLOV GP, VP40, and NP (A and B) or pCAGGS alone (C and D) were fixed at 48 h after transfection. Samples were observed with an S-4700 scanning electron microscope (Hitachi). Scale bars represent 5 μm (A and C) and 2 μm (B and D).

manner similar to that of VSVΔG*-Zaire and VSVΔG*-Reston (Fig. 8A). VSVΔG*-Angola and VSVΔG*-Musoke had higher IUs in these cells than VSVΔG*-Lloviu, VSVΔG*-Zaire, and VSVΔG*-Reston, except in a cell line from the Yaeyama flying fox

(*Pteropus dasymallus yayeyamae*; FBKT1) (Fig. 8A). Since some species of bats are suspected to be natural reservoirs of filoviruses (33–37), we focused on these bat cells and relative infectivities were determined (Fig. 8B). Interestingly, VSVΔG*-Lloviu in-
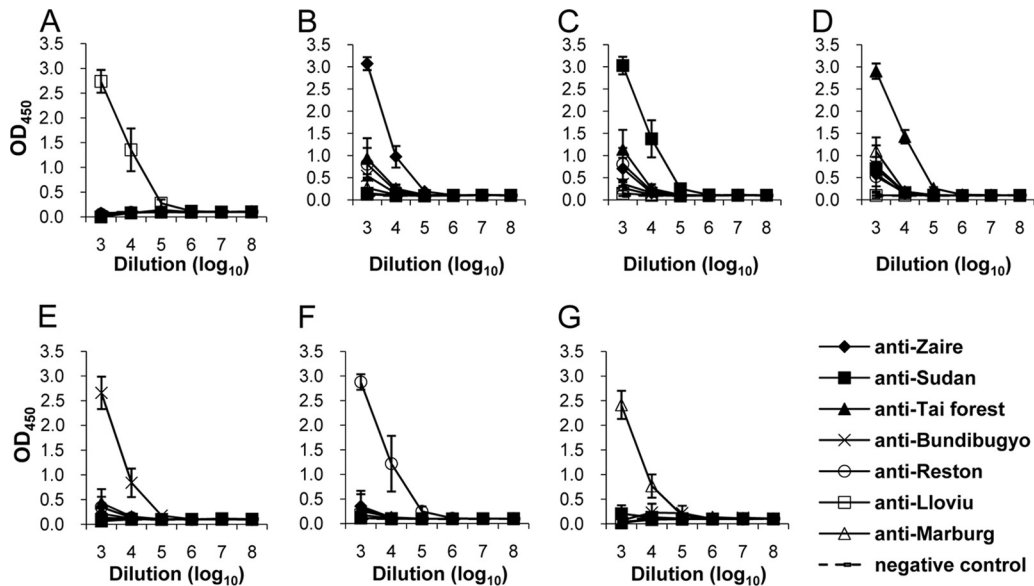


**FIG 4** Cross-reactivities of anti-GP sera among filoviruses in ELISA. Ten-fold serial dilutions of mouse antisera to EBOV (anti-Zaire), SUDV (anti-Sudan), TAFV (anti-Tai forest), BDBV (anti-Bundibugyo), RESTV (anti-Reston), LLOV (anti-Lloviu), and MARV (anti-Marburg) were tested for IgG reactivities to LLOV (A), EBOV (B), SUDV (C), TAFV (D), BDBV (E), RESTV (F), and MARV (G) GP antigens. Three mice were used for each virus, and averages and standard deviations are shown.
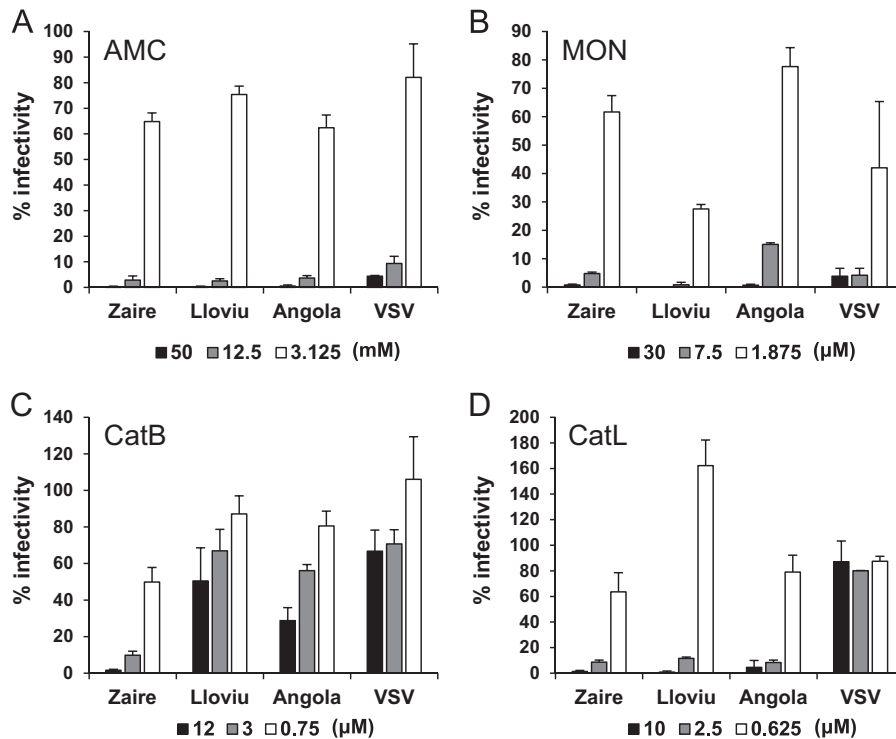
**FIG 5** Effects of chemical inhibitors on infectivities of pseudotyped VSVs. Vero E6 cells were pretreated with ammonium chloride (AMC) (A), monensin (MON) (B), and cathepsin B (CatB) (C) and L (CatL) (D) inhibitors for 30 min at 37°C. The treated cells were then infected with VSVΔG*-Zaire (Zaire), VSVΔG*-Angola (Angola), VSVΔG*-Lloviu (Lloviu), and VSVΔG*-G (VSV) appropriately diluted to yield 200 to 2,000 IUs/$10^6$ cells. At 20 h postinoculation, GFP-positive cells were counted using an IN Cell Analyzer 2000 (GE Healthcare). The percentages of infectivity were determined by setting the number of the untreated cells to 100%. Each experiment was performed three times, and averages and standard deviations are shown.

fected IndFSPT1 and SuBK12-08 more efficiently than the other viruses tested.

## DISCUSSION

This study provides fundamental information on the properties of LLOV GP. The RNA editing that is required to produce the full-length transmembrane GP is a common characteristic of viruses belonging to the genus *Ebolavirus* (21, 22). The presence of the editing site in the LLOV GP gene supports the notion that this virus is more related to ebolaviruses than marburgviruses. Phylogenic analyses also suggested that LLOV has the same ancestor as ebolaviruses (4). On the other hand, LLOV GP has MLR and a furin cleavage site, both of which are common features shared by previously known filoviruses (38, 39). By TEM and SEM of VLPs, we further found morphological similarity between LLOV and other filoviruses, which have numerous GP spikes located on the filamentous VLP surface. Viral entry assays with chemical inhibitors also suggest that LLOV GP, as well as GPs of the other filoviruses, requires low endosomal pH and proteolytic processing for virus entry into cells. Taken together, these results indicate that the structure and function of LLOV GP are primarily similar to those of the other filovirus GPs.

However, the requirement of cathepsin L might be controversial, since a high concentration (>1 μM) of FY-dmk was suggested to inhibit not only cathepsin L but also cathepsin B and likely other endosomal cysteine proteases (40). FY-dmk at the lowest concentration tested in this study (0.625 μM) did not reduce infectivities of VSVΔG*-Lloviu, VSVΔG*-Zaire, or VSVΔG*-Angola, suggesting that cathepsin L is not essential for LLOV infection, similar to the other filoviruses (40, 41). It was also shown that cathepsin B and cathepsin L activities are not required for EBOV replication in a mouse model (41). Thus, further studies are needed to clarify the *in vivo* importance of the GP cleavage by cathepsins and some other host proteases for filovirus infection.

Hepatocytes, dendritic cells, monocytes, and macrophages, all of which express cell surface C-type lectins, are thought to be the preferred target cells of filoviruses, and increased infection of these cells might be directly involved in the pathogenesis of filovirus infection (11, 19, 42–44). We demonstrated that LLOV GP utilized human C-type lectins, hMGL and DC-SIGN, most likely as attachment factors, as reported with the other filovirus GPs (13, 19, 20, 45). These C-type lectins have different glycan specificities (i.e., hMGL and DC-SIGN preferentially react with O- and high-mannose-type N-glycans, respectively) (46–48). Like EBOV and RESTV GPs, LLOV GP showed greater preference for DC-SIGN than hMGL, unlike MARV GPs, suggesting that LLOV GP, particularly its MLR, has a carbohydrate structure comparable to that of ebolaviruses. Our data suggest that LLOV GP has a tropism to cells expressing C-type lectins and infect human immune cells, such as dendritic cells and macrophages.

Like C-type lectins, ADE antibodies mostly recognize epitopes on MLR of filovirus GPs, leading to enhanced infectivity (11, 13, 42). Thus, MLR is thought to play important roles for these two attachment functions of GP. In addition to the primary structure of MLR (i.e., the presence of different epitopes and sugar chains among filoviruses), the GP2 region seems to have key amino acid
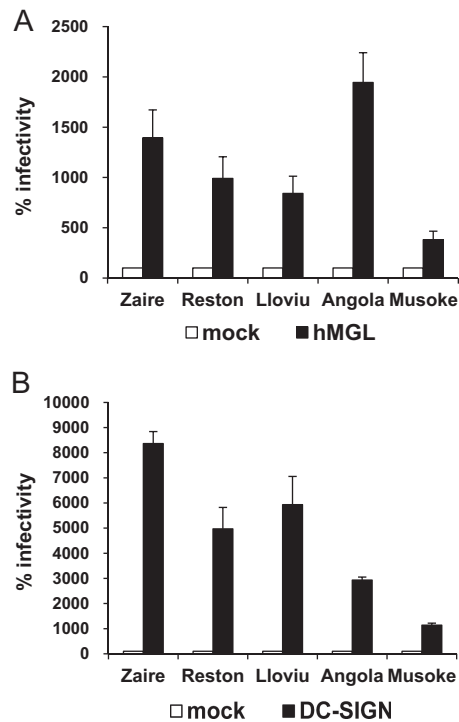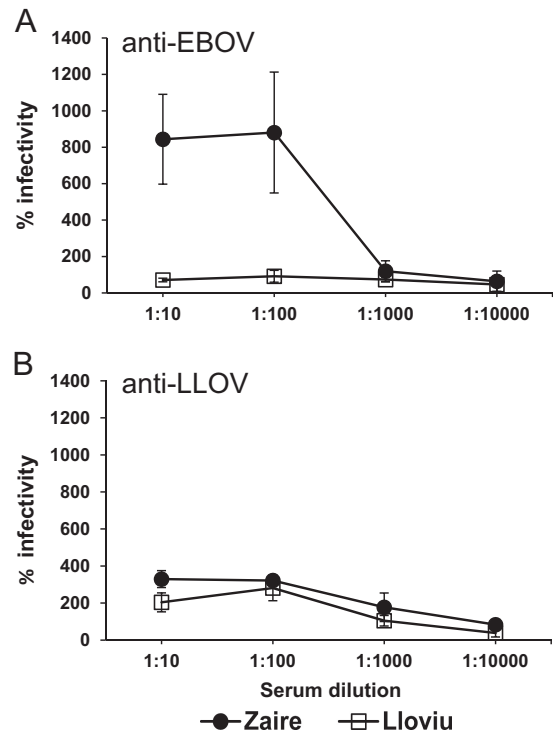
FIG 6 Infectivities of pseudotyped VSVs in C-type lectin-expressing cells. K562 cells expressing hMGL or DC-SIGN were infected with VSVΔG*-Zaire, VSVΔG*-Reston, VSVΔG*-Lloviu, VSVΔG*-Angola, and VSVΔG*-Musoke. The infectivity of each pseudotyped VSV on K562 clones was determined by counting the number of GFP-positive cells using flow cytometry, and the percentages of infectivity in K562-hMGL and K562-DC-SIGN were determined by setting the number of the infected K562 cells as 100%. Each experiment was performed three times, and averages and standard deviations are shown. Statistical significance of the differences was determined by Student's t test (see P values in the text).



FIG 7 ADE activities of antisera to EBOV and LLOV GPs. Ten-fold serially diluted mouse anti-EBOV (A) and anti-LLOV (B) sera were mixed with equal volumes of VSVΔG*-Zaire and VSVΔG*-Lloviu. Relative infectivity was determined by setting the number of infected K562 cells without antisera to 100%. Each experiment was performed three times, and averages and standard deviations are shown.

residues (e.g., the amino acid at position 547 in MARV GP) contributing to the efficiency of ADE- and C-type lectin-mediated entry (15, 20). Although detailed functional mapping and structural analysis are still needed, our data indicate that the overall properties of LLOV GP for ADE- and C-type lectin-mediated entry are comparable to those of RESTV GP rather than highly virulent EBOV and MARV (strain Angola) (11, 13, 42).

VSVΔG*-Lloviu infected all cell lines tested, as well as VSVΔG*-Zaire and VSVΔG*-Reston (Fig. 5A). Recently, RESTV was detected in pigs in the Philippines and China, and more recent studies have revealed that EBOV causes respiratory disease in pigs (49–53), suggesting a role of this animal in filovirus ecology. In this study, VSVΔG*-Lloviu infected pig cells (SK-L cells) in a manner similar to that of VSVΔG*-Zaire and VSVΔG*-Reston. Although GP is not the only determinant controlling filovirus pathogenicity, our data suggest that LLOV at least meets the minimum requirements to infect pig cells.

Interestingly, neither VSVΔG*-Angola nor VSVΔG*-Musoke infected FBKT1 cells derived from the Yaeyama flying fox (*Pteropus dasymallus yayeyamae*), although the infectivities of these viruses in the other cell lines tested were uniformly higher than those of the other viruses (Fig. 5A). This finding suggests the existence of cellular receptors/coreceptors that interact with EBOV, RESTV, and LLOV GPs but not MARV GP. Although several cellular molecules were reported to be involved in filovirus entry

(e.g., T-cell immunoglobulin domain and mucin domain 1, Tyro 3 family, C-type lectin, or Niemann-Pick C1) (11–13, 42–47, 54–57), there is only limited information on these molecules in bats. It would be of interest to clarify whether these cellular molecules

TABLE 1 Origins of cell lines used in this study

| Cell line | Species | Zoological name | Organ |
|---|---|---|---|
| Vero E6 | African green monkey | *Chlorocebus* sp. | Kidney |
| HEK293 | Human | *Homo sapiens* | Kidney |
| SK-L | Pig | *Sus scrofa domesticus* | Kidney |
| MDCK | Dog | *Canis lupus familiaris* | Kidney |
| BKT1 | Greater horseshoe bat[a] | *Rhinolophus ferrumequinum* | Kidney |
| FBKT1 | Yaeyama flying fox[b] | *Pteropus dasymallus yayeyamae* | Kidney |
| YubFKT1 | Eastern bent-winged bat[c] | *Miniopterus fuliginosus* | Kidney |
| IndFSPT1 | Indian flying fox[d] | *Pteropus giganteus* | Spleen |
| DemKT1 | Leschenault's rousette[e] | *Rousettus leschenaulti* | Kidney |
| ZFB11-97 | Gambian epauletted fruit bat[f] | *Epomophorus gambianus* | Kidney |
| SuBK12-08 | Schreiber's bat[g] | *Miniopterus schreibersii* | Kidney |

[a] Nucleotide sequence identity is 98% (unpublished data).
[b] Previously described (18).
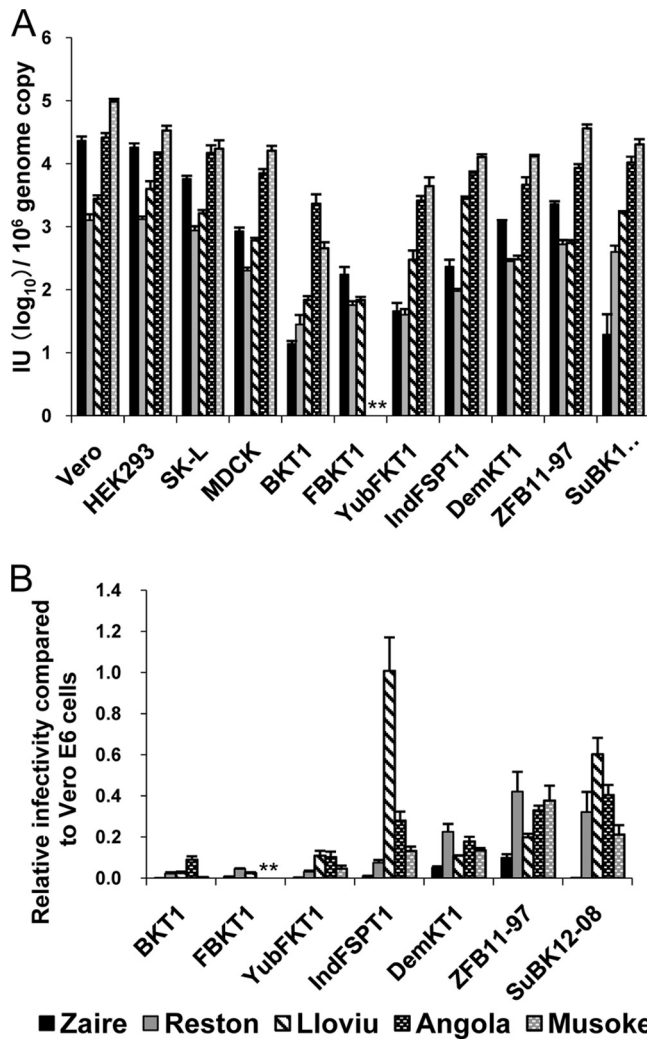[c] Nucleotide sequence identity is 99%.
[d] Nucleotide sequence identity is 98%.
[e] Nucleotide sequence identity is 100%.
[f] Nucleotide sequence identity is 89%.
[g] Nucleotide sequence identity is 98%.

FIG 8 Infectivities of pseudotyped VSVs in mammalian cell lines. VSVΔG*-Zaire, VSVΔG*-Reston, VSVΔG*-Lloviu, VSVΔG*-Angola, and VSVΔG*-Musoke were inoculated into several mammalian cell lines. (A) Infectious units (IUs) of each virus in different cell lines were determined by counting the number of GFP-expressing cells, and each IU value was standardized based on $10^6$ copies of the VSV genome as determined by real-time RT-PCR. (B) Relative infectivities in bat cell lines are given by setting each IU value in Vero E6 cells to 1.0 (i.e., [IU in bat cells]/[IU in Vero E6 cells]). Each experiment was performed three times, and averages and standard deviations are shown. Infectivities of VSVΔG*-Angola and VSVΔG*-Musoke were under the limit of detection (*).

play critical roles in tissue tropism and/or host range restriction of filoviruses. Alternatively, there might be a new receptor of LLOV in bats.

VSVΔG*-Lloviu infected SuBK12-08 and IndFSPT1 more efficiently than the other viruses (Fig. 5B). It should be noted that SuBK12-08 was derived from the same insectivorous bat species, Schreiber's bat (*Miniopterus schreibersii*), in which LLOV was first detected in Europe. However, since LLOV likely causes lethal infection of Schreiber's bat, this bat species may not serve as the natural host that can maintain this virus in nature. On the other hand, IndFSPT1 was derived from fruit bats. Considering that some species of fruit bats are suspected to be natural hosts of filoviruses (33, 58), strong tropism to this fruit bat species suggests that fruit bats also play some roles in the ecology of LLOV.

While LLOV seems to be highly pathogenic for some species of bats (e.g., Schreiber's bat), its ability to infect human and nonhuman primates and the pathogenic potential for these hosts can only be hypothesized, since infectious LLOV has never been isolated. In this study, we used a replication-incompetent VSV pseudotype system that enabled us to investigate the cellular tropism mediated by simple interaction between LLOV GP and its cellular ligands. Although a reverse genetics approach and *in vivo* experiments for infectious LLOV are needed to provide direct evidence of the viral pathogenicity and host specificity, our data suggest that the overall properties of LLOV GP are similar to those of the other filoviruses, and that LLOV has the potential, at least from the aspect of GP-receptor/coreceptor interaction, to infect many mammalian cells, including those of the human, monkey, and pig, with preferential tropism for some bat cells.

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