Characterization of envelope glycoproteins of unisolated bat-derived viruses

（コウモリ由来ウイルスの
表面糖タンパク質の性状解析）

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Contents

Abbreviations

ADE	antibody-dependent enhancement
BatIV	bat-derived influenza virus
BDBV	Bundibugyo virus
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
EBOV	Ebola virus
FCS	fetal calf serum
GFP	green fluorescent protein
GP	glycoprotein
HA	hemagglutinin
HEK	human embryonic kidney
hMGL	human macrophage galactose-type C-type lectin
IAV	influenza A virus
IU	infectious unit
LLOV	Lloviu virus
MARV	Marburg virus
MDCK	Madin-Darby canine kidney
MEM	minimal essential medium
MLR	mucin-like region
NA	neuraminidase
NP	nucleoprotein
OD	optical density
PBS	phosphate-buffered saline
RESTV	Reston virus
SEM	scanning electron microscopy
SUDV	Sudan virus
TAFV	Taï forest virus
TEM	transmission electron microscopy
VLP	virus-like particle
VP	viral protein
VSV	vesicular stomatitis virus

Preface

Zoonotic pathogens, which infect both humans and other animals, are considered to be the major sources of emerging and re-emerging infectious diseases in humans [1,2], and most of them are viral infections, which has been increasingly reported in the past few decades. Recently, bats (order *Chiroptera*), which include almost one quarter of all known mammalian species and are one of the most diverse and geographically dispersed animals, have been identified or suspected as reservoirs of zoonotic viruses (e.g. lyssavirus, henipavirus, severe acute respiratory syndrome coronavirus, and filovirus) [3-6], and thus particular attention has been paid to viruses harbored in bats. Currently, it is also possible to detect novel bat-derived viral genomes without isolation of infectious viruses or identification of virions by modern molecular techniques, such as next-generation sequencing [7,8]. However, zoonotic potential of unisolated bat-derived viruses have not been evaluated yet. Due to the expanding area of human activity, increasingly overlapping the habitats of bats, zoonoses caused by bat-derived pathogens will continue to emerge. Thus, it is important to understand their biological property, such as the viral life cycle and pathogenicity, in order to predict and prevent the emergence of zoonoses.

In this study, I focused on two novel unisolated viruses derived from bats, Lloviu virus (LLOV) and bat-derived influenza viruses (BatIVs). LLOV belongs to the family *Filoviridae* [9]. Filoviruses, represented by Ebola and Marburg viruses, are known to cause severe hemorrhagic fever in humans and/or nonhuman primates with high mortality rate. While sporadic outbreaks of Ebola and Marburg virus diseases have been reported in African countries, the biggest Ebola virus disease outbreak occurred in West African countries from December 2013. There were 28,637 cases of Ebola virus disease and 11,314 deaths reported at 22 November 2015 (Ebola Situation Reports, WHO, http://apps.who.int/ebola/ebola-situation-reports). BatIVs have been proposed to be related to influenza A viruses (IAVs) based on the phylogenetic analysis of their nucleotide sequences [10,11].

IAVs cause respiratory infection in mammalian and avian species including humans, pigs, horses, and poultry. Thus, influenza has been considered to be one of the most important zoonoses. It is noted that both LLOV and BatIVs are related to previously known zoonotic viruses, underscoring the need to assess their zoonotic potential.

In order to gain information on the biological property LLOV and BatIVs, I characterized their glycoproteins, which play important roles in viral replication cycles, particularly virus entry into host cells. The viral envelope glycoprotein (GP) is the only spike protein of filoviruses, and responsible for both receptor binding and fusion of the virus envelope with the host cell membrane. Filovirus GP undergoes proteolytic cleavage by host proteases such as furin, resulting in the two subunits, GP1 and GP2. GP1 contains a putative receptor binding region and mucin-like region (MLR) that has a number of potential N- and O-linked glycosylation sites. IAVs have two envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA is cleaved into HA1 and HA2 subunits by trypsin-like proteases of host cells. HA1 is responsible for virus binding to sialic acid receptors on the cell surface, and HA2 mediates membrane fusion, thereby delivering the viral genomic RNA into the cytoplasm of target cells. NAs have sialidase activity that enables mature virus particles to be released from infected cells after budding.

I employed a replication-incompetent vesicular stomatitis virus (VSV) pseudotype system which enabled us to directly analyze the glycoprotein functions. In chapter I, I show that LLOV GP is serologically distinct from the other known filovirus GPs and that LLOV GP mediates cellular entry in a manner similar to the other filovirus GPs while showing preferential tropism for some bat cells. In chapter II, I demonstrate that VSVs pseudotyped with the glycoproteins of BatIVs have preferential tropism for some particular bats cells and that BatIV HAs may recognize some glycoproteins as receptors but not sialoglycans which are typical receptors of IAVs.

Chapter I:
Characterization of the envelope glycoprotein of a novel filovirus, Lloviu virus

Introduction

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% [12]. 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. Among ebolaviruses, a difference in pathogenicity was suggested. EBOV is thought to be the most pathogenic, killing up to approximately 90% of patients, whereas RESTV has never caused lethal infection in humans [13] and is less pathogenic in experimentally infected nonhuman primates than EBOV [14].

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), whose name was derived from the cave in which it was first found [9]. LLOV is phylogenetically distinct from other filoviruses and thus proposed to belong to the new genus *Cuevavirus*, species *Lloviu cuevavirus*, in the family *Filoviridae* [9]. 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 [15].

The envelope GP is responsible for both receptor binding and fusion of the virus envelope with the host cell membrane [16,17]. 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 [17,18]. 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 [19,20]. 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 [21-23]. It was also shown that MLR contains epitopes for antibody-dependent enhancement (ADE) of filovirus infection *in vitro* [24,25].

To provide information for estimation of the infectivity and potential pathogenicity of LLOV, this study focused on GP, which likely plays major role in the replication cycle and the pathogenicity of filoviruses [20,26]. In this study, we investigated the morphology of virus-like particles (VLPs) 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 the other filoviruses while showing preferential tropism for some particular bat cells.

Materials and methods

Cells. Human embryonic kidney (HEK) 293, HEK293T, and African green monkey kidney Vero E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and penicillin-streptomycin. Bat cell lines ZFBK11-97 and SuBK12-08 were established by transfecting an expression plasmid encoding the Simian virus 40 large T antigen (pCXN2-Flag-SV40LT kindly provided by Drs. 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). ZFBK11-97, 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 [27] were cultured in MEM with 10% FCS, L-glutamine, and penicillin-streptomycin, and 0.3% tryptose phosphate broth (GIBCO). Bat cell lines BKT1, FBKT1, YubFKT1, IndFSPT1, and DemKT1, were established as described previously [28]. Bat species were identified by morphology, habitat, and BLAST searches using the sequences of their *cytochrome b* genes (nucleotide positions 1-400). 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 intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) [29,30] 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 [31,32], 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 [33]. 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 [34].

Purification of virus-like particles (VLPs). HEK293T cells were transfected with plasmids encoding GP, VP40, and NP of LLOV, EBOV, SUDV, TAFV, BDBV, RESTV or MARV (strain Angola) using TransIT LT-1 regent (Mirus) according to the manufacturer's instructions. Forty-eight hours later, VLPs were purified from culture supernatants by ultracentrifugation at 28,000×*g* at 4°C for 1.5 hours with a 25% sucrose cushion. 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 hours 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 5-20% SuperSep (Wako), separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with 1:2000-diluted mouse antisera to filovirus VLPs (see below), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch). The bound antibodies were visualized with Immobilon Western (Millipore).

Mouse antisera and enzyme-linked immunosorbent assay (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 ELISA was performed as described previously [35]. 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 (OD) at 450 nm was measured.

Electron microscopy. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out as described previously [36,37]. Purified VLPs fixed with 0.25% glutalaldehyde were adsorbed to a 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 [25], 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 80kV. For SEM, cells transfected with plasmids expressing LLOV GP, VP40, and NP were fixed with 2.5% glutalaldehyde 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, substituted 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.

Vesicular stomatitis virus (VSV) pseudotyped with filovirus GPs. Using VSV containing the green fluorescent protein (GFP) gene instead of the receptor-binding VSV G protein gene (VSVΔG*-G) [16], 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 [16]. The genome copy number of each pseudotyped VSV preparation was quantified by real-time RT-PCR. Real-time RT-PCR was performed using One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa Bio) and a CFX96 Real Time System (BIO RAD) with primers (GFP498-F: CAAGATCCGCCACAACATCG and 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 [25]. K562 clones expressing hMGL and DC-SIGN grown on 96-well plates were infected with VSV pseudotyped with filovirus GPs (50-150 IU determined in K562 cells), and infectivities were determined by counting the number of GFP-positive cells using flow cytometry as described previously [29,30]. For the assays of ADE of infection, 10-fold serially diluted mouse antisera were mixed with equal volumes of pseudotyped VSVs (50-150 IU determined in K562 cells). After 1-hour incubation at room temperature, the mixture was inoculated into K562 cells grown on 96-well plates. At 20 hours post-inoculation, 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 fluorescent microscope, and IUs per 10^6 genome copies were calculated.

Inhibitor treatments. Vero E6 cells were pretreated with ammonium chloride (Wako), monensin

(Sigma), 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 200–2000 IUs/10^6 cells. At 20 hours post-inoculation, GFP-positive cells were counted with the IN Cell Analyzer 2000 (GE Healthcare).

Results

Characterization of the primary structure of LLOV GP. Though the envelope GPs of ebolaviruses are expressed through transcriptional editing [31,32], 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 [9]. 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 910-916. Based on sequence comparison with the EBOV genome, which has the editing site at positions 880-816, we assumed that this stretch of 7 adenosines could be 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 at position 505-508 recognized by host ubiquitous proteases such as furin) and MLR, similarly 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 120-130 kD) and its cleavage product, the GP_1 subunit (approximately 100 kD), by western blotting (Fig. 1C). These results suggested that LLOV GP principally might share biological 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 VLPs (Fig. 2D and H). Numerous spikes were observed on the VLP surface and immuno-TEM with an anti-LLOV GP monoclonal antibody confirmed the presence

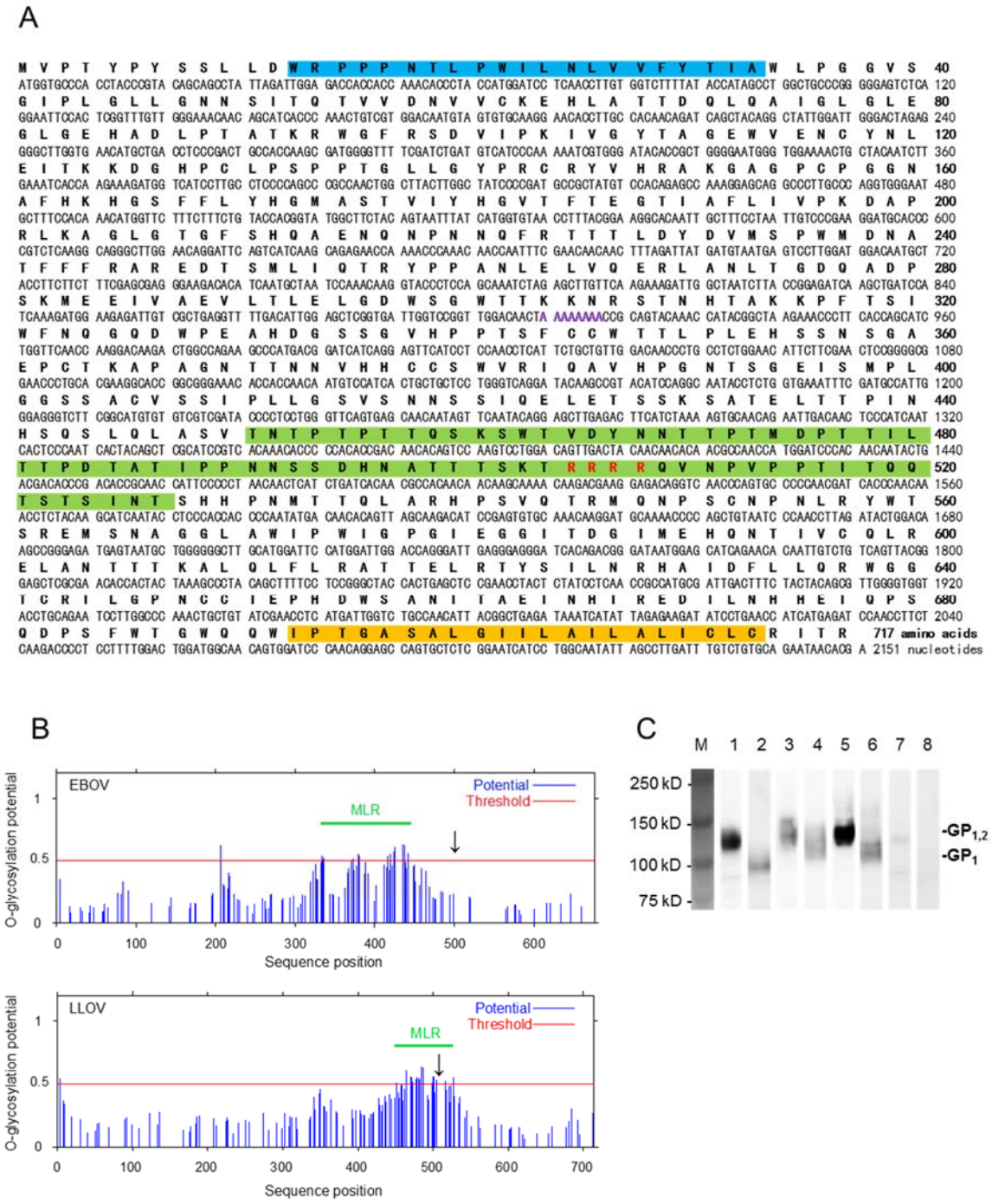

Fig. 1 Primary structure of LLOV envelope GP. Nucleotide and deduced amino acid sequences of LLOV GP (A). Deduced amino acid sequences are shown in bold letters. Blue, green, and yellow lines represent the signal peptide, MLR, and transmembrane domain predicted by GENETYX Ver.10, NetOGlyc, and TopPred 0.01, respectively. Purple and red letters represent the putative editing and cleavage sites, respectively. Comparison of EBOV and LLOV MLRs (B). Potential O-glycosylation sites were predicted by NetOGlyc, and MLRs were defined as the regions between the first and last amino acid residues showing the score over the threshold (0.5). Arrows indicate the cleavage sites. Western blotting of filovirus GPs (C). 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 non-reducing (lanes 1, 3, 5, and 7) or reducing (lanes 2, 4, 6, and 8) conditions.

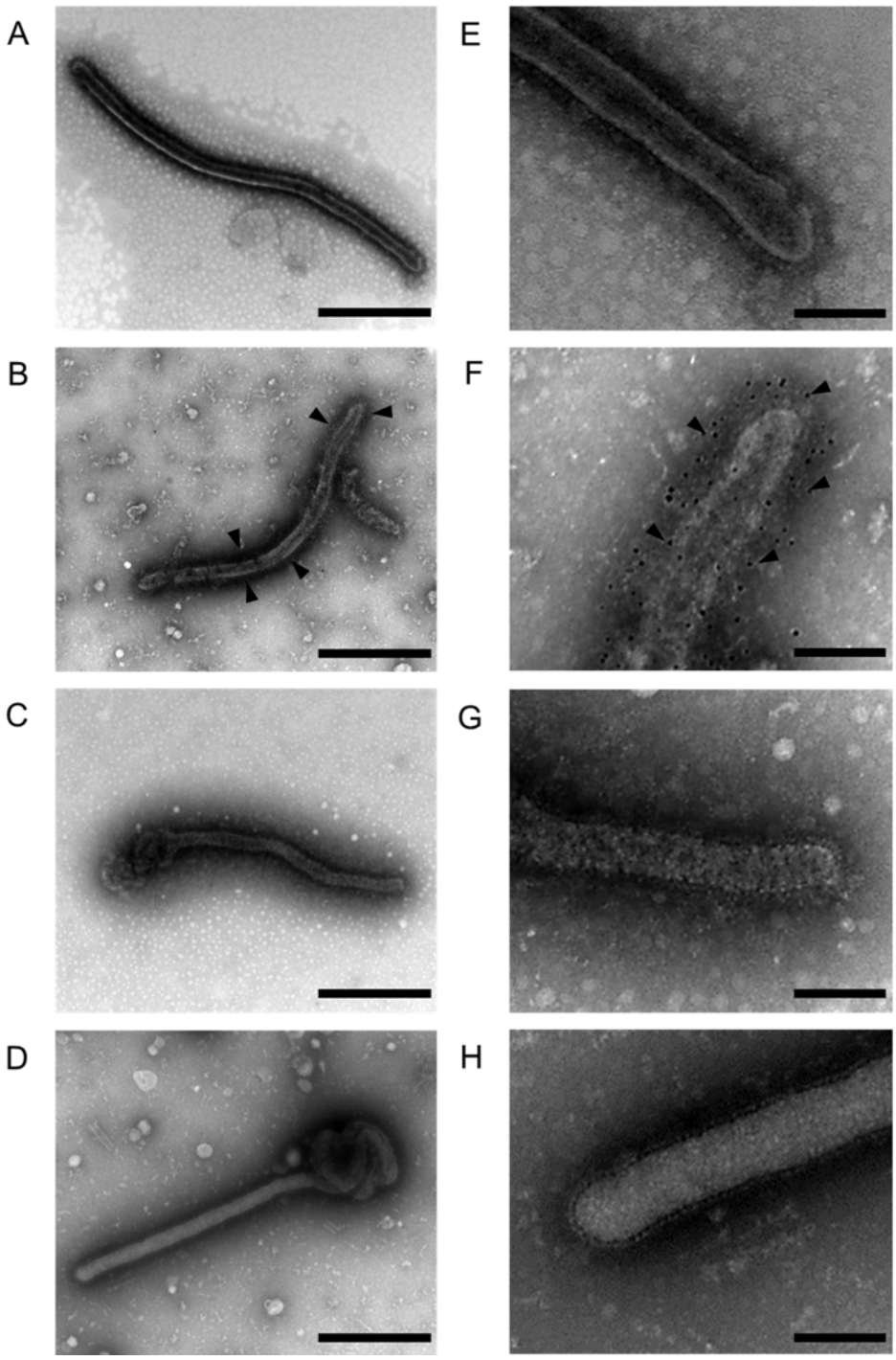

Fig. 2 TEM of filovirus VLPs. Purified VLPs produced from 293T cells transfected with plasmids expressing LLOV (A, B, E, and F), EBOV (Zaire) (C and G), and MARV (Angola) (D and H) proteins were fixed and stained as described in Materials and Methods. For immuno-TEM (B and F), an anti-LLOV GP monoclonal antibody was used. Scale bars represent 500 nm (A to D) and 200 nm (E to H). Arrowheads indicate gold particles.

of LLOV GPs on the surface (Fig. 2B and F). Similarly to the other filoviruses [38], VLPs with a uniform diameter of approximately 70 nm and varied lengths were observed. This was consistent with a previous study showing that the diameters of VLPs were narrower than reported for actual EBOV particles (80-nm diameter) [12,39]. 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 a similarity to EBOV budding [37]. 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 with 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 functional study of LLOV GP, we produced VSV pseudotyped with LLOV GP, which could infect Vero E6 cells, and thus 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

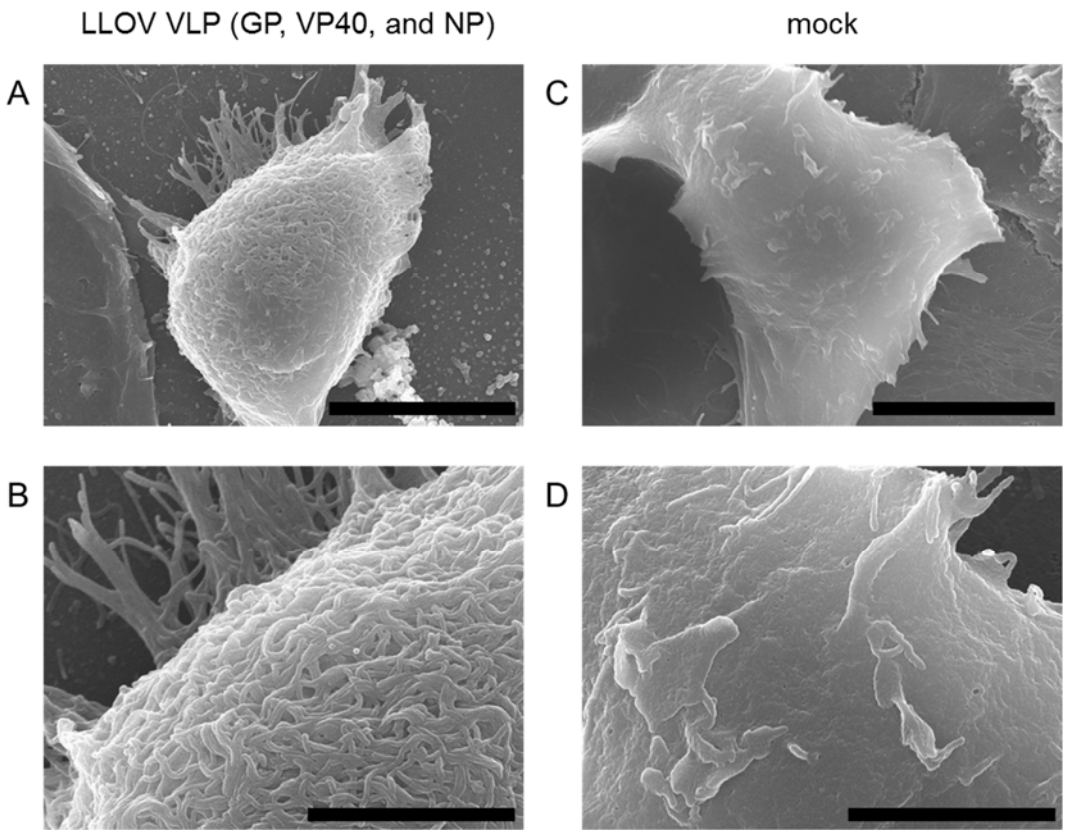

Fig. 3 SEM of LLOV VLPs. HEK293T cells transfected with pCAGGS expressing LLOV GP, VP40, and NP (A and B) or pCAGGS alone (C and D) were fixed at 48 hours after transfection. Samples were observed with an S-4700 scanning electron microscope (Hitachi). Scale bars represent 5 μm (A and C) and 2 μm (B and D).

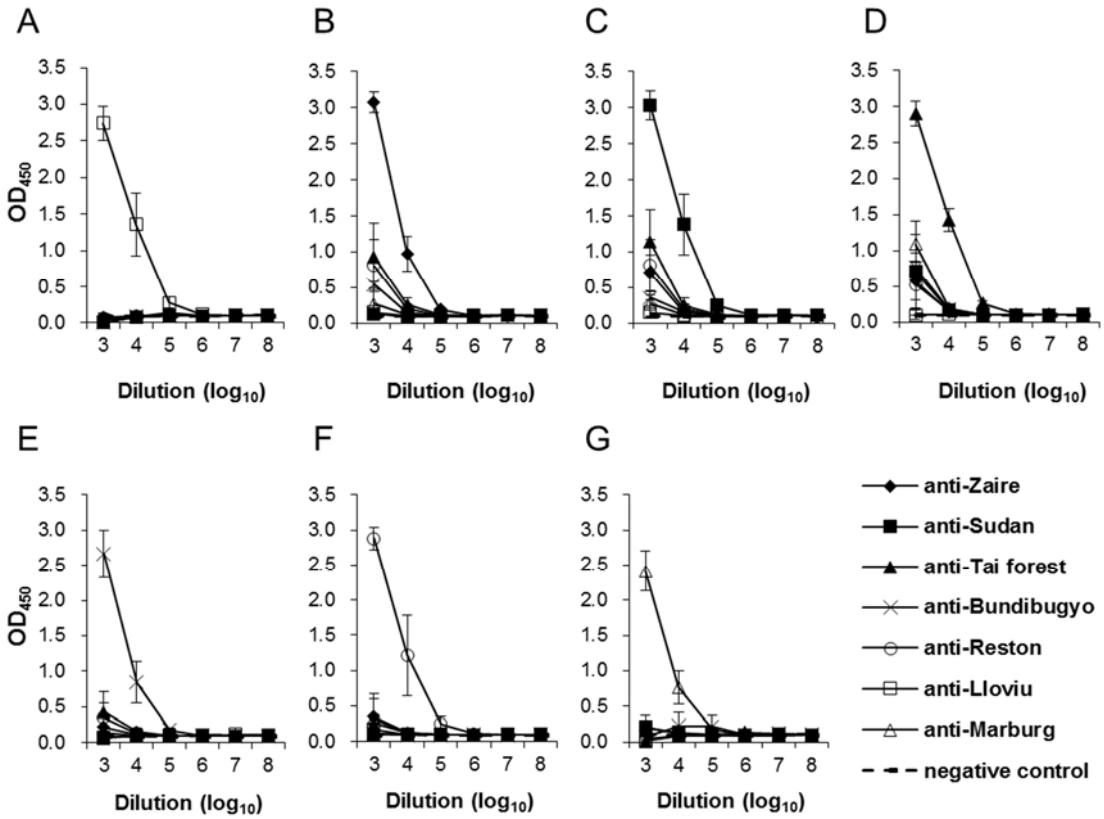

Fig. 4 Cross-reactivities of anti-GP sera among filoviruses in ELISA. Ten-fold serial dilutions of mouse antisera to EBOV (anti-Zaire), SUDV (anti-Sudan), TAFV (anti-Tai forest), BDBV (anti-Bundibugyo), RESTV (anti-Reston), LLOV (anti-Lloviu), and MARV (anti-Marburg) were tested for IgG reactivities 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.

in vitro [16,17,40]. 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, and rather 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 were observed in the VSVΔG*-Lloviu, -Zaire, 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 [21,29,30]. 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 than VSVΔG*-Angola ($P < 0.05$), but 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 ($p <$

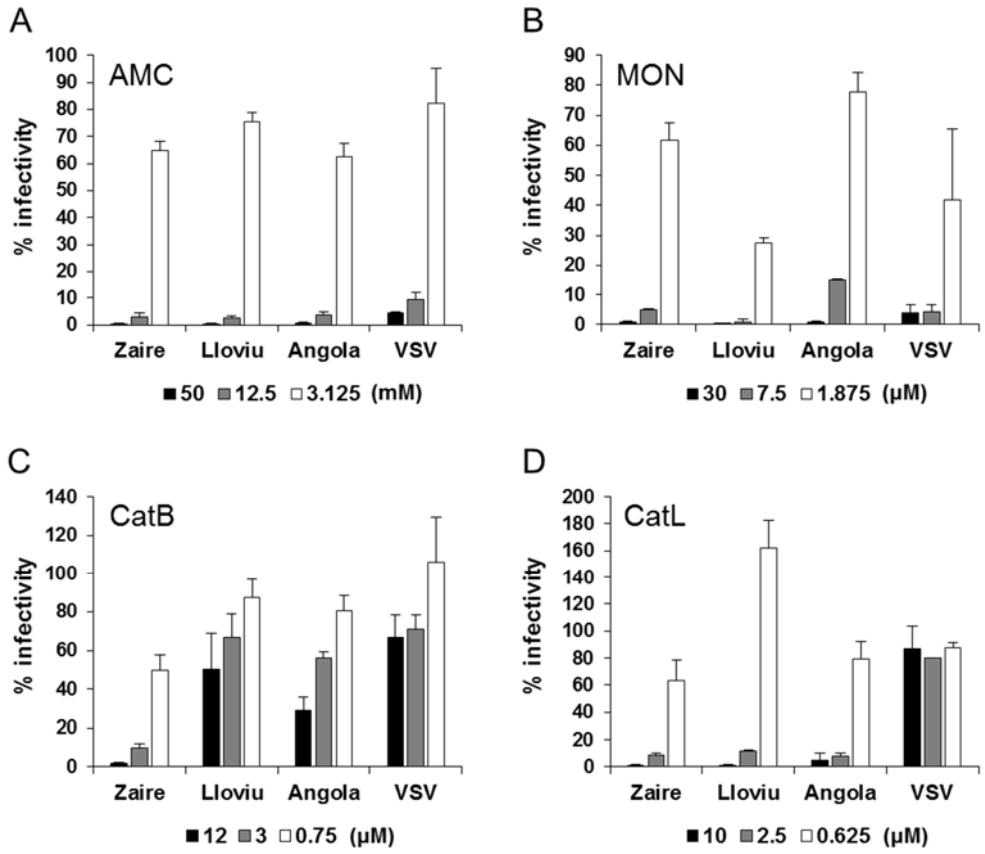

Fig. 5 Effects of chemical inhibitors on infectivities of pseudotyped VSVs. Vero E6 cells were pretreated with ammonium chloride (AMC; A), monensin (MON; B), and cathepsin B and L inhibitors (Cat B; C, and Cat L; D) for 30 min at 37°C. The treated cells were then infected with VSVΔG*-Zaire (Zaire), VSVΔG*-Angola (Angola), VSVΔG*-Lloviu (Lloviu), and VSVΔG*-G (VSV) appropriately diluted to yield 200–2000 IUs/10^6 cells. At 20 hours post-inoculation, 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.

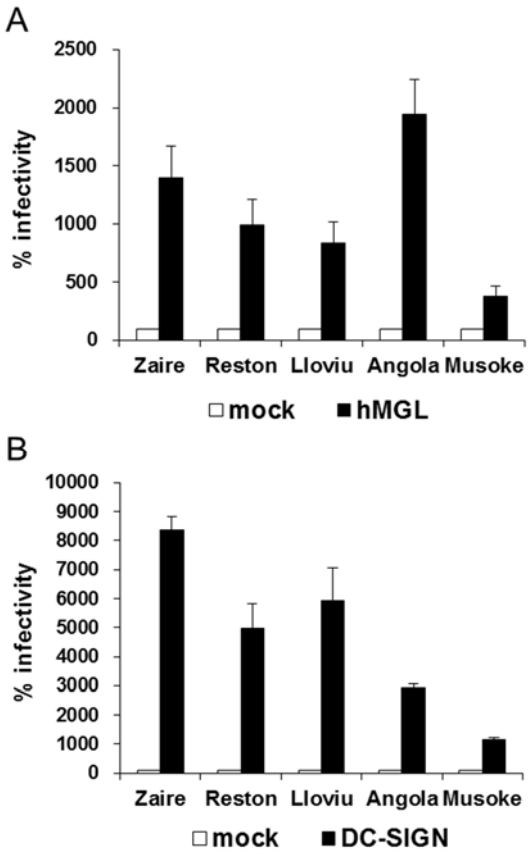

Fig. 6 Infectivities of pseudotyped VSVs in C-type lectin-expressing cells. K562 cells expressing hMGL or DC-SIGN were infected with VSVΔG*-Zaire, VSVΔG*-Reston, VSVΔG*-Lloviu, VSVΔG*-Angola, and VSVΔG*-Musoke. The infectivity of each pseudotyped VSV on K562 clones was determined by counting the number of GFP-positive cells using flow cytometry, and the percentages of infectivity in K562-hMGL and -DC-SIGN were determined by setting the number of the infected K562 to 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).

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) [25,41,42]. 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 the serum dilutions of 1:10 and 1:100 (Fig 7A). By 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. 4A and B). Consist with the absence 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 receptors, we infected various cell lines of different animal origins (Table 1) with pseudotyped VSVs 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 similar manner to 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 [43-47], we focused on these bat cells and relative infectivities were determined (Fig. 8B). Interestingly, VSVΔG*-Lloviu infected IndFSPT1 and SuBK12-08 more efficiently than the other viruses tested.

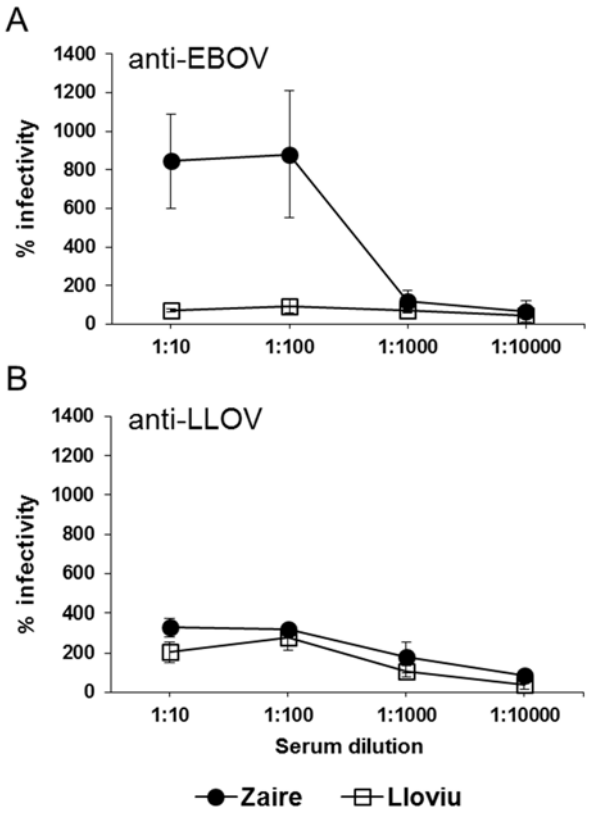

Fig. 7 ADE activities of antisera to EBOV and LLOV GPs. Ten-fold serially diluted mouse anti-EBOV (A) and anti-LLOV (B) sera ware mixed with equal volumes of the VSVΔG*-Zaire and VSVΔG*-Lloviu. Relative infectivity was determined by setting the number of infected K562 cells without antisera to 100%.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

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
ZFBK11-97	Gambian epauletted fruit bat[f]	*Epomophorus gambianus*	Kidney
SuBK12-08	Schreiber's bat[g]	*Miniopterus schreibersii*	Kidney

[a] Nucleotide sequence identity is 98% (manuscript in preparation).
[b] Previously described [28].
[c] Nucleotide sequence identity of *cytochrome b* genes is 99%.
[d] Nucleotide sequence identity of *cytochrome b* genes is 98%.
[e] Nucleotide sequence identity of *cytochrome b* genes is 100%.
[f] Nucleotide sequence identity of *cytochrome b* genes is 89%.
[g] Nucleotide sequence identity of *cytochrome b* genes is 98%.

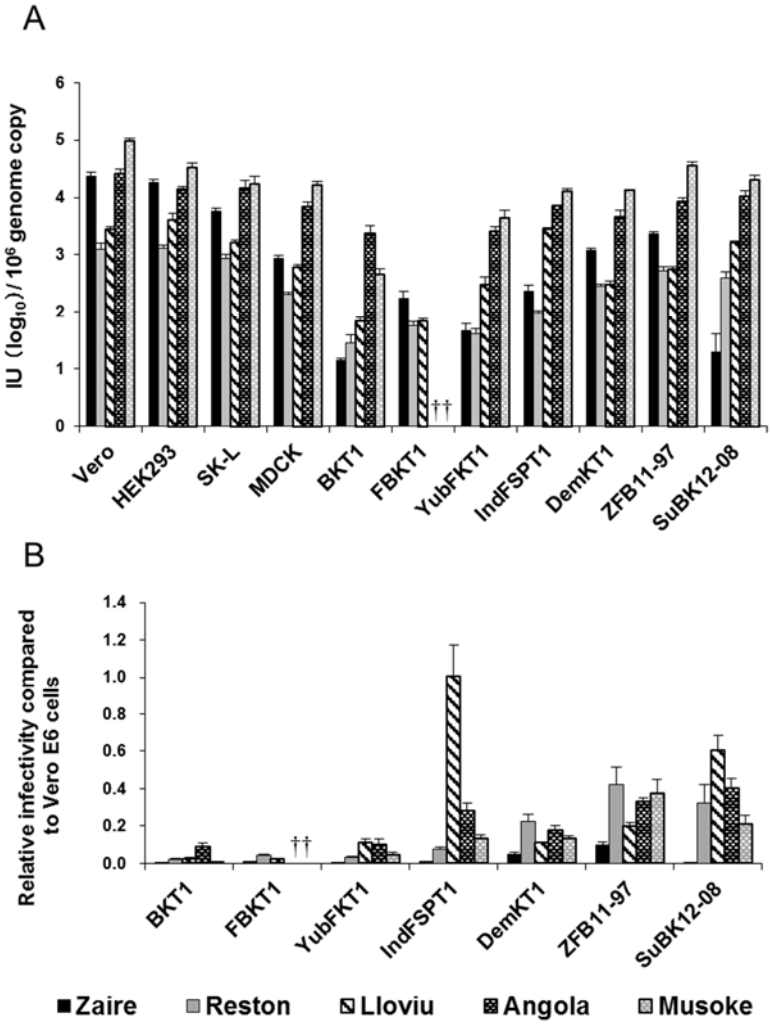

Fig. 8 Infectivities of pseudotyped VSVs in mammalian cell lines. VSVΔG*-Zaire, VSVΔG*-Reston, VSVΔG*-Lloviu, VSVΔG*-Angola, and VSVΔG*-Musoke were inoculated into several mammalian cell lines. Infectious units (IUs) of each virus in different cell lines were determined by counting the number of GFP-expressing cells and each IU was then standardized based on 10^6 copies of the VSV genome determined by real-time RT-PCR (A). Relative infectivities in bat cell lines are given by setting each IU in Vero E6 to 1.0 (i.e., [IU in bat cells]/[IU in Vero E6 cells] (B). 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 (†).

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* [31,32]. 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 might have the same ancestor as ebolaviruses [9]. On the other hand, LLOV GP has MLR and a furin cleavage site, both of which are common features shared by previously known filoviruses [48,49]. 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 cathepsin inhibitor, FY-dmk, was suggested to inhibit not only cathepsin L but also cathepsin B and likely other endosomal cysteine proteases [50]. FY-dmk at the lowest concentration tested in this study (0.625 μM) did not reduce infectivities of neither VSVΔG*-Lloviu, VSVΔG*-Zaire, nor VSVΔG*-Angola, suggesting that cathepsin L is not essential for LLOV infection similarly to the other filoviruses [50,51]. It was also shown that Cathepsin B and Cathepsin L activities are not required for EBOV replication in a mouse model [51]. Thus, further studies are needed to clarify the *in vivo* importance of the GP cleavage by cathpsins 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 [21,29,52-54]. 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 [23,29,30,55]. 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) [56-58]. Like EBOV and RESTV GPs, LLOV GP showed greater preference for DC-SIGN than hMGL compared with MARV GPs, suggesting that LLOV GP, particularly its MLR, might have a carbohydrate structure comparable to that of ebolaviruses. Our data suggest that LLOV GP has a tropism to cells expressing C-type lectins and might potentially 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 [21,23,52]. 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 residues (e.g., the amino acid at position 547 in MARV GP) contributing to the efficiency of ADE- and C-type lectin-mediated entry [25,30]. 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) [21,23,52].

VSVΔG*-Lloviu infected all cell lines tested as well as VSVΔG*-Zaire and VSVΔG*-Reston (Fig. 8A). Recently, RESTV was detected in pigs in the Philippines and China, and more recent studies have revealed that EBOV causes respiratory disease in pigs [59-63], suggesting a potential role of this animal in filovirus ecology. In this study, VSVΔG*-Lloviu infected pig cells (SK-L cells) in a similar manner to 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. 8A). This finding suggests the existence of cellular receptors/coreceptors that interacts 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) [21-23,52-56,58,64-67], there is only limited information on these molecules in bats. It would be of interest to clarify whether these cellular molecules 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. 8B). 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 [43,68], strong tropism to this fruit bat species may suggest 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.

Summary

LLOV, a novel filovirus detected in bats, is phylogenetically distinct from viruses in the genus *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 GP, which likely plays a key role in viral tropism and pathogenicity. We first found that LLOV GP principally shares the primary structure with the other filovirus GPs. Similarly to the other filoviruses, VLPs produced by transient expression of LLOV GP, matrix protein, and nucleoprotein in HEK293T cells had densely arrayed GP spikes on a filamentous particle. Mouse antiserum to LLOV VLP was little cross-reactive to viruses of the other genera, indicating that LLOV is a serologically distinct from the other known filoviruses. For functional study of LLOV GP, we utilized a 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 the other filoviruses while showing preferential tropism for some bat cells.

Chapter II:
Characterization of the glycoproteins of bat-derived influenza viruses

Introduction

Influenza A viruses (IAVs), which belong to the family *Orthomyxoviridae*, have 8 segmented negative sense RNA genomes. IAV is one of the most important zoonotic pathogens, with high morbidity in humans, pigs, horses, and poultry. IAVs have two envelope glycoproteins, HA and NA, and are divided into subtypes based on antigenicity. IAVs of H1-16 HA and N1-9 NA subtypes have been isolated from water birds such as migratory ducks, the natural reservoir of IAVs [69-71].

HAs are expressed as trimers on the virion surface [72]. HA is initially synthesized as an inactive precursor HA0 and subsequently cleaved into HA1 and HA2 subunits by trypsin-like proteases of host cells [73]. The proteolytic cleavage of the HA molecule is essential for IAVs to acquire infectivity [74,75]. HA1 is responsible for virus binding to sialic acid receptors on the cell surface, and HA2 mediates membrane fusion under acidic conditions in endosomes, thereby delivering the viral genomic RNA into the cytoplasm of target cells [76,77]. NAs, expressed on the virion surface as tetramers, have sialidase activity that enables mature virus particles to be released from infected cells after budding [70,78].

Recently, IAV-like RNA genomes were detected in succession from 2 frugivorous bat species, little yellow-shouldered bats (*Sturnira lilium*) and flat-faced fruit bats (*Artibeus planirostris*) in Guatemala and Peru, respectively [10,11]. The nucleotide sequences of the HA and NA of these bat-derived influenza viruses (BatIVs) were divergent from all previously known IAVs and new subtypes, H17N10 and H18N11, have been proposed [10,11]. However, infectious viruses have not been isolated yet. Previous studies by others tried to rescue BatIVs using a reverse genetics approach, but failed to generate infectious BatIVs [79,80]. Thus, the information on the biological properties of BatIVs is mostly speculative and the possible functions of BatIV HAs and NAs are only hypothetical,

based on structural analyses [11,81-83].

In this study, we utilized VSV pseudotype system, enabling us to directly analyze the biological functions of the BatIV glycoproteins, which presumably play important roles in the replication cycle and pathogenicity. We found some bat cell lines susceptible to VSVs pseudotyped with BatIV HAs and NAs. Our data suggest that BatIVs do not use sialic acids as a viral receptor and may have a limited host range, at least considering receptor engagement.

Materials and Methods

Cells. HEK293, HEK293T, and Vero E6 cells were grown in DMEM with 10% FCS and penicillin-streptomycin. MDCK cells were grown in DMEM with 10% calf serum, L-glutamine, and penicillin-streptomycin. Bat cell lines BKT1, FBKT1, YubFKT1, IndFSPT1, DemKT1, ZFBK11-97, SuBK12-08, and ZFBS13-76A were established as described previously [28,84]. All bat cell lines were grown in RPMI-1640 medium with 10% FCS, L-glutamine, and penicillin-streptomycin.

Construction of plasmids expressing HAs and NAs. Coding regions of the HAs and NAs of BatIVs were synthesized in vector pUC19 or pUCFa, based on the nucleotide sequences of GenBank (accession numbers for H17 HA, N10 NA, H18 HA, and N11 NA: CY103892, CY103894, CY125945, and CY125947, respectively) (FASMAC). Each coding region of the viral proteins was amplified by PCR with primers including restriction sites, the kozak sequence, and the stop codon. After digestion by restriction enzymes, each gene was cloned into the mammalian expression vector pCAGGS [33]. H1 HA and N1 NA of A/WSN/1933 (H1N1) (WSN), and H3 HA and N2 NA of A/Aichi/2/1968 (H3N2) (Aichi) were cloned into pCAGGS as described previously [85].

VSVs pseudotyped with HAs and/or NAs. Using VSV containing the GFP gene instead of the receptor-binding VSV G protein gene (VSVΔG*-G), pseudotyped viruses with HAs and/or NAs of BatIVs, WSN, and Aichi were generated as described previously [16]. VSVs pseudotyped with IAV glycoproteins were pretreated with trypsin (final concentration 0.0005%) for 30 minutes at 37°C, followed by incubation with an anti-VSV G monoclonal antibody, VSV-G(N)1-9, to abolish the background infectivity of parental VSVΔG*-G [25]. For virus titration, 10-fold diluted pseudotyped VSVs were inoculated into confluent monolayers of each cell line on 96-well plates, and the IU in each cell line was determined 20 hours later by counting the number of GFP-expressing cells under a

fluorescent microscope.

Electron microscopy. TEM was carried out as described previously [84]. Pseudotyped VSVs 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-HA2 monoclonal antibody (3N12-6-4) broadly cross-reactive to group 1 HA subtypes, anti-N10 NA mouse serum (FM0137) produced by immunization with a synthetic peptide corresponding to amino acid residues 328 to 343 (AQEKGEGGIQGFILDE) of N10 NA, 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 80kV.

SDS-PAGE and western blotting. Pseudotyped VSVs were treated with or without trypsin (final concentration 0.0005%) for 30 minutes at 37°C and then mixed with SDS-PAGE sample buffer with 5% 2-mercaptoethanol and boiled for 5 minutes. After electrophoresis on 5–20% SuperSep (Wako), separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with an anti-H3N2 chicken polyclonal antiserum or anti-HA2 monoclonal antibody 3N12-6-4, which reacts to H1, H2, H5, H6, H17, and H18 HAs, followed by incubation with peroxidase-conjugated rabbit anti-chicken IgY (H+L) or goat anti-mouse IgG (H+L) (Jackson ImmunoResearch). The bound antibodies were visualized with Immobilon Western (Millipore).

Cell treatment with enzymes and inhibitors. IndFSPT1 cells were preincubated with the medium containing an endosomal acidification inhibitor, ammonium chloride (Wako), at 37°C for 2 hours in a CO_2 incubator, and then infected with pseudotyped VSVs appropriately diluted to yield 200–1000 IUs, followed by incubation in the presence of ammonium chloride. IndFSPT1 cells were also pretreated

with pronase (a mixture of endo- and exoproteases from *Streptomyces griseus*) (Calbiochem) [86], for 20 minutes, an N-glycosylation inhibitor (tunicamycin from *Streptomyces* sp.) (Sigma) for 8 hours, which blocks the reaction of UDP-GlcNAc and dolichol phosphate in the first step of glycoprotein synthesis, thus inhibiting the synthesis of N-linked glycoproteins, or neuraminidase from *Vibrio cholerae* (Roche) [87] for 1 hour at 37°C in a CO_2 incubator. Treated cells were washed with serum free RPMI-1640 medium 3 times, and then incubated with pseudotyped VSVs appropriately diluted to yield 200–1000 IUs for 1 hour. After adsorption of the virus, the inoculum was aspirated and the growth medium (RPMI-1640 medium with 10% FCS) was added. Cells were incubated for 20 hours, and infected cells were counted under a fluorescent microscope. Cell viabilities were assessed by the alamar blue assay. After treatments of each enzyme and inhibitor, cells were incubated with FCS-free RPMI-1640 medium containing 10% Alamar blue (Biosource) for 2 hours, and fluorescence with excitation wavelength at 530-560 nm was measured using EnVision (PerkinElmer).

Results

Generation of VSVs pseudotyped with BatIV HAs and/or NAs. To investigate cellular entry mediated by BatIV glycoproteins, VSVs pseudotyped with BatIV HAs and/or NAs (VSVΔG*-H17N10, -H18N11,-H17, -H18, -N10, and -N11) were generated as described in the Materials and Methods section. We first observed the virions of these pseudotyped VSVs using TEM (Fig. 9). We found that the virions of all of these pseudotyped VSVs showed characteristic morphology (i.e. a bullet-like shape) similar to parental VSVΔG*-G. It was noted that VSVs pseudotyped with BatIV HA and NA (Fig. 9A and B), HA alone (Fig. 9D and E), and NA alone (Fig. 9G and H) all had numerous spikes on their surfaces, as was the case with VSVs pseudotyped with IAV HA (H3) and NA (N2) (Fig. 9C), H3 HA alone (Fig. 9F), and N2 NA alone (Fig. 9I). Immuno-TEM with anti-H17 HA and anti-N10 NA antibodies revealed that both BatIV HA and NA were efficiently incorporated into VSV particles (Fig. 9K and L). No difference was found in the overall morphology among these VSV virions. These data indicated that BatIV HAs and NAs were efficiently incorporated into the VSV particles.

Cell lines susceptible to VSVs pseudotyped with BatIV glycoproteins. Since previous studies have suggested that cell lines commonly used for IAV propagation are nonpermissive for BatIVs [10], we screened various cell lines, including bat-derived cells, for susceptibility to pseudotyped VSVs (Table 2 and Fig. 10). VSVs pseudotyped with HAs and NAs of BatIVs and well-characterized IAV strains, A/WSN/1933 (H1N1) (WSN) and A/Aichi/2/1968 (H3N2) (Aichi), were generated and treated with trypsin before use, since BatIV HAs, like WSN and Aichi HAs, have a cleavage site potentially recognized by trypsin-like proteases [10,11]. We found that VSVΔG*-WSN, -Aichi, and -VSV G infected all cell lines tested (Fig. 10A, B, and E). On the other hand, VSVΔG*-H17N10 and -H18N11 infected bat cell lines YubFKT1, IndFSPT1, and SuBK12-08, but not the other cell lines tested, except

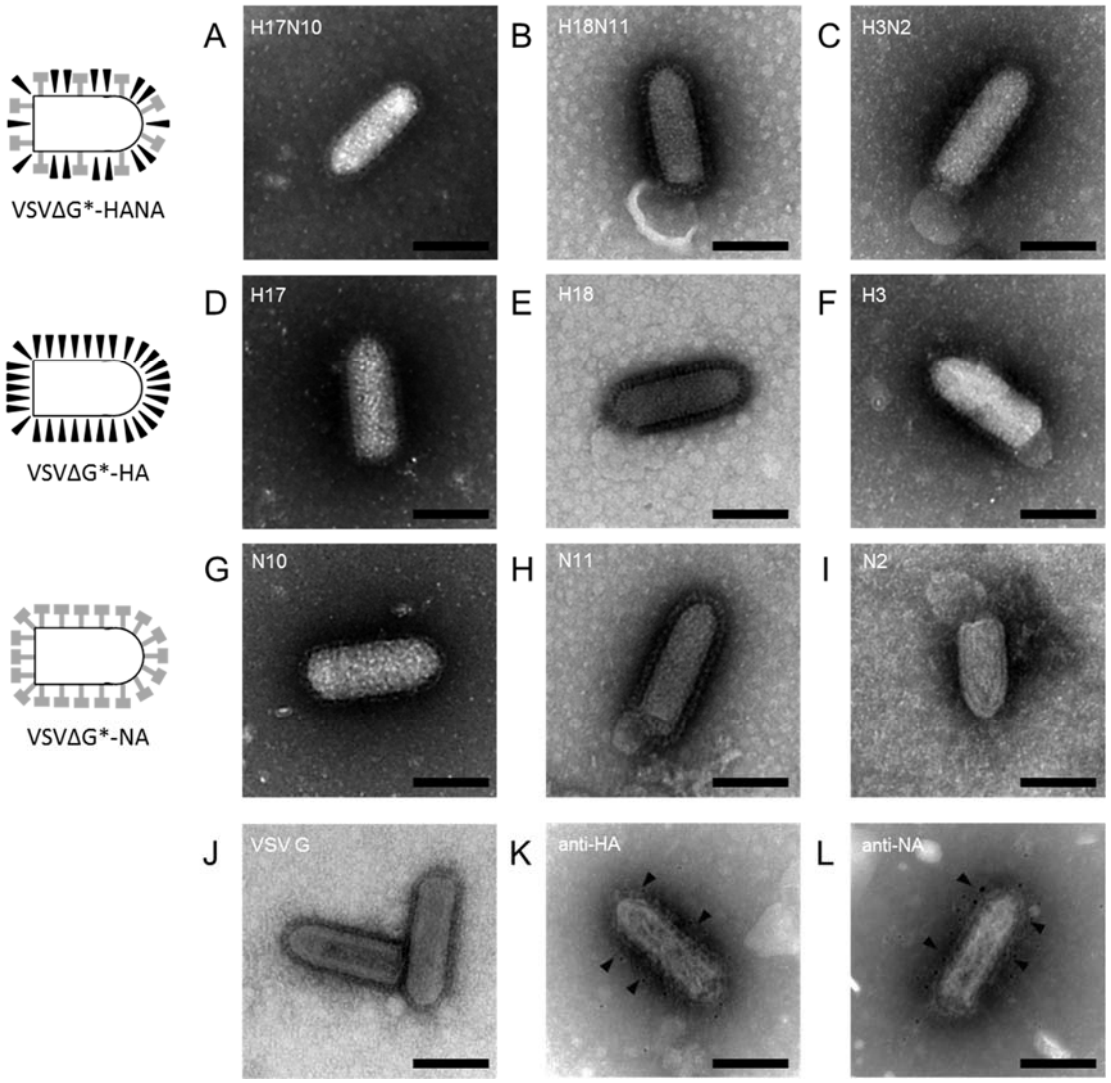

Fig. 9 Transmission electron microscopy of pseudotyped VSVs. VSVΔG*-H17N10 (A), -H18N11 (B), -H3N2 (C) -H17 (D), -H18 (E), -H3 (F), -N10 (G), -N11 (H), -N2 (I) and VSVΔG*-G (J) were fixed and stained as described in Materials and Methods. For immune transmission electron microscopy of VSVΔG*-H17N10, anti-HA2 monoclonal antibody (K) and anti-N10 NA mouse serum (L) were used. Scale bars represent 100 nm. Arrowheads indicate gold particles.

Table 2 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
MDCK	Dog	*Canis lupus familiaris*	Kidney
SK-L	Pig	*Sus scrofa domesticus*	Kidney
QT6	Japanese quail	*Coturnix japonica*	Muscle
BKT1	Greater horseshoe bat[a]	*Rhinolophus ferrumequinum*	Kidney
FBKT1	Yaeyama flying fox[a]	*Pteropus dasymallus yayeyamae*	Kidney
YubFKT1	Eastern bent-winged bat[a]	*Miniopterus fuliginosus*	Kidney
IndFSPT1	Indian flying fox[a]	*Pteropus giganteus*	Spleen
DemKT1	Leschenault's rousette[a]	*Rousettus leschenaultii*	Kidney
ZFBK11-97	Gambian epauletted fruit bat[a]	*Epomophorus gambianus*	Kidney
SuBK12-08	Schreiber's bat[a]	*Miniopterus schreibersii*	Kidney
ZFBS13-75A	Straw-coloured fruit bat[b]	*Eidolon helvum*	Spleen

[a]Previously described (Table 1).

[b]Determined by habitat and morphology.

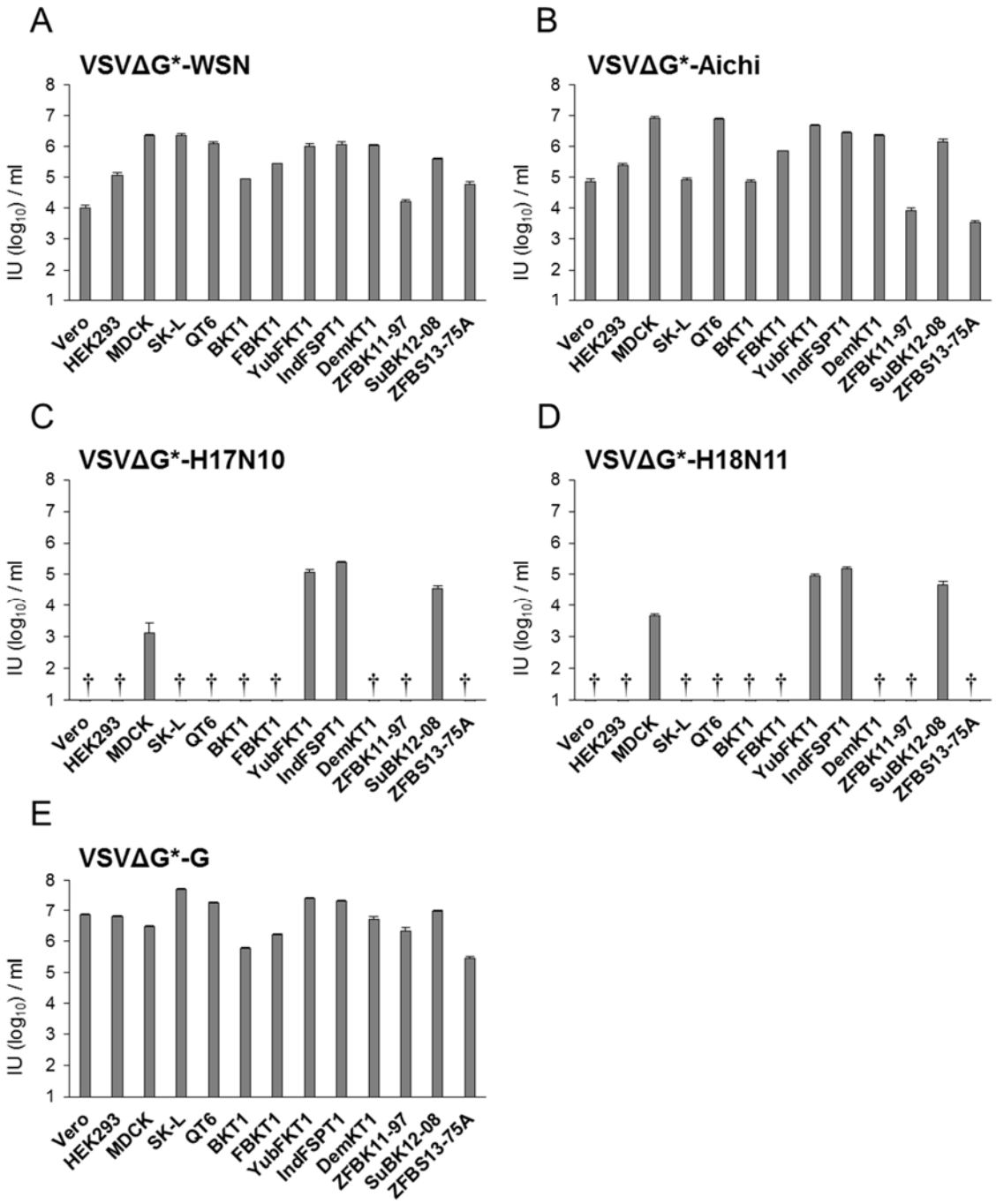

Fig. 10 Infectivities of pseudotyped VSVs in several cell lines. VSVΔG*-WSN, -Aichi, -H17N10, -H18N11, and VSVΔG*-G were inoculated into several cell lines (Table 2). IUs of each virus in different cell lines were determined by counting the number of GFP-expressing cells. Each experiment was performed three times, and averages and standard deviations are shown. Infectivities of VSVΔG*-H17N10 and -H18N11 in some cell lines were under the limit of detection (†). Significant differences (student's *t*-test) were found between MDCK and any of the bat cell lines ($p < 0.01$).

MDCK cells, which were much less susceptible than these bat cells. Since IndFSPT1 cells showed the highest susceptibility to VSVΔG*-H17N10 and -H18N11 (Fig. 10C and D), this cell line was used for the following experiments.

Trypsin requirement for the HA function and the dispensability of NA in virus entry. IAV HAs are known to be cleaved into HA1 and HA2 subunits by trypsin-like proteases to acquire the ability to mediate membrane fusion [88]. Western blotting revealed that both H17 and H18 HAs were cleaved into HA1 and HA2 by trypsin treatment (Fig. 11A). Thus, we investigated the requirement of HA cleavage for infectivity of pseudotyped VSVs. As expected, VSVs pseudotyped with BatIV glycoproteins did not infect IndFSPT1 cells without trypsin treatment, in a manner consistent with other IAVs (data not shown), whereas trypsin-treated viruses efficiently infected this cell line (Fig. 10). These data indicated that the HA cleavage was a prerequisite for BatIV infectivity. Next, to clarify whether BatIV HAs was responsible for virus entry, VSVΔG*-H17N10, -H18N11,-H17, -H18, -N10, and -N11 were inoculated to IndFSPT1 cells and their infectivities were compared (Fig. 11B). We found that VSVΔG*-H17 and -H18 infected IndFSPT1 cells as efficiently as VSVΔG*-H17N10 and -H18N11, whereas the infectivity of VSV pseudotyped with WSN or Aichi HA alone was much lower than that of VSVs pseudotyped with both HA and NA of the respective viruses. VSVΔG*-N10 and -N11 showed no infectivity, similarly to VSVs pseudotyped with NAs of WSN and Aichi. These results suggest that BatIV HA is the only glycoprotein mediating both virus attachment and membrane fusion and that BatIV NA is dispensable during the entry into cells.

Effects of chemical and enzymatic treatments of cells on susceptibility to VSVs pseudotyped with BatIVs. It is generally known that IAV HAs mediate membrane fusion in a low pH-dependent manner [77,89]. To investigate the requirement of endosomal acidification for BatIV HA-mediated membrane

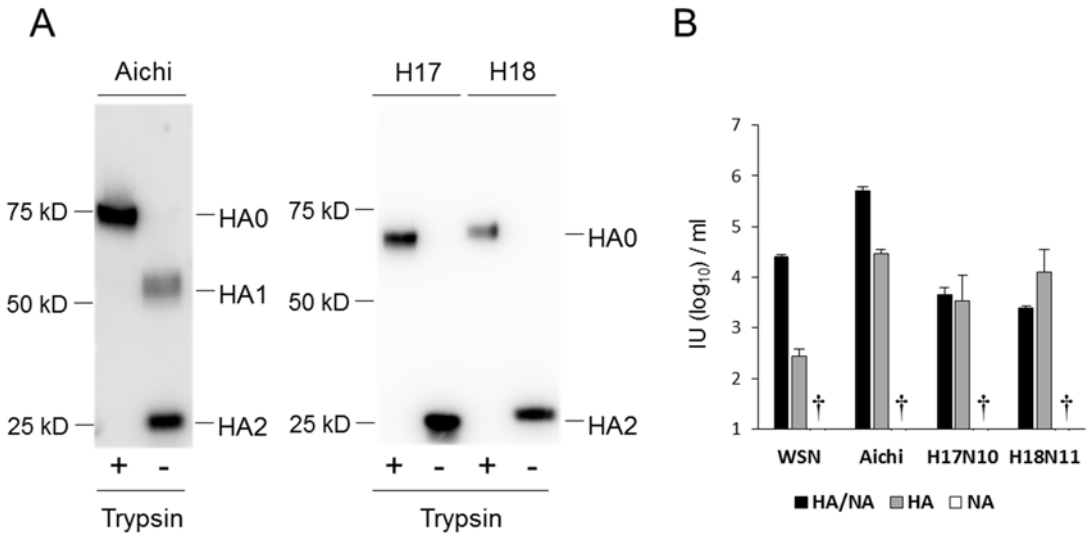

Fig. 11 Infectivities of pseudotyped VSVs with BatIV HAs and/or NAs in IndFSPT1 cells. (A) VSVΔG*-Aichi, -H17N10, and -H18N11 were treated with or without trypsin (final concentration 0.0005%) for 30 minutes at 37°C and then mixed with SDS-PAGE sample buffer with 5% 2-mercaptoethanol. After SDS-PAGE, separated proteins were detected by western blotting with anti-H3 chicken antiserum and anti-HA2 monoclonal antibody 3N12-6-4. (B) VSVs pseudotyped with HAs and/or NAs of WSN and Aichi and VSVΔG*-H17N10, -H17, -N10, -H18N11, -H18, and -N11 were inoculated to IndFSPT1 cells. Infectious units (IUs) were determined by counting the number of GFP-expressing cells. Each experiment was performed three times, and averages and standard deviations are shown. Infectivities of VSV pseudotyped with NAs alone were under the limit of detection (†). Statistical significance was calculated using student's t-test (*$P < 0.01$).

fusion, IndFSPT1 cells were treated with ammonium chloride, which is known to neutralize the pH of acidic intracellular compartments, and then infected with VSVΔG*-WSN, -Aichi, -H17N10, -H18N11, and VSVΔG*-G. Treatment of the cells with ammonium chloride markedly reduced the infectivity of VSVΔG*-H17N10 and -H18N11, as was the case with VSVΔG*-G, -WSN, and -Aichi, in a dose-dependent manner, suggesting that BatIV HAs require a low pH for membrane fusion, consistent with the other IAV HAs (Fig. 12A). To obtain information on the biological characteristics of cellular receptors for BatIVs, IndFSPT1 cells were pretreated with tunicamycin, pronase, or neuraminidase (i.e., an N-linked glycosylation inhibitor, mixture of proteases, and sialidase, respectively), and then infected with pseudotyped VSVs (Fig. 12B to D). Tunicamycin treatment markedly reduced the infectivities of VSVΔG*-G, -H17N10, and -H18N11, but less significantly those of VSVΔG*-WSN and -Aichi (Fig. 12B). Preincubation of cells with pronase reduced the infectivities of the all pseudotyped VSVs, except for VSVΔG*-WSN (Fig. 12C). Neuraminidase treatment reduced VSVΔG*-WSN and -Aichi infectivities, but interestingly did not affect the infectivities of VSVΔG*-H17N10 and -H18N11 (Fig. 12D). We confirmed that no remarkable cytotoxicity was observed during these treatments (Fig. 12E). These results suggest that BatIV HAs do not recognize sialic acids which are critical components of the other known IAV receptor and some other molecules such as glycoproteins may serve as BatIV receptors.

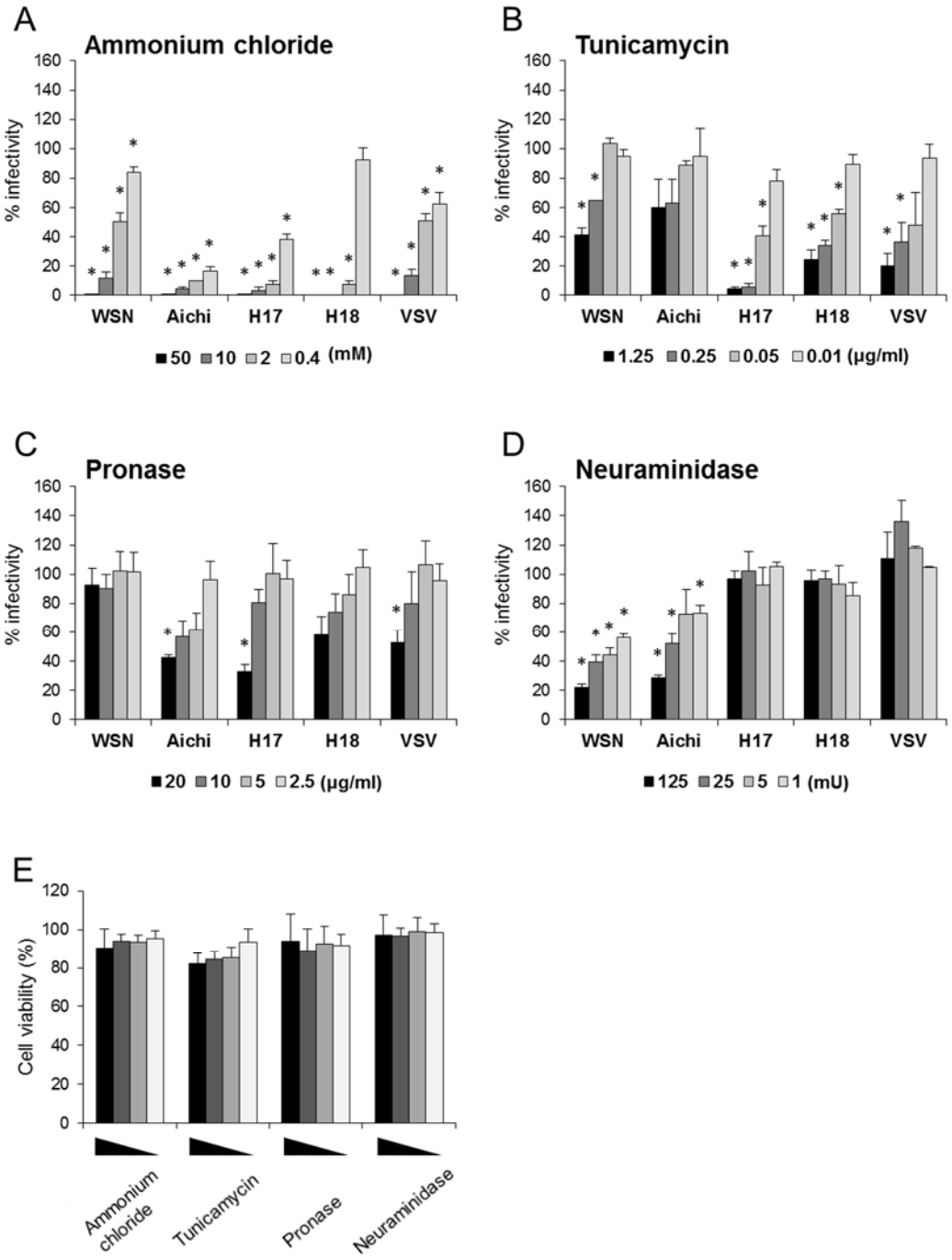

Fig. 12 Effects of chemical and enzymatic modification on infectivities of pseudotyped VSVs. IndFSPT1 cells were treated with ammonium chloride (A), tunicamycin (B), pronase (C), or neuraminidase (D) as described in Materials and Methods. Treated cells were then infected with VSVΔG*-WSN, -Aichi, -H17N10, -H18N11, and VSVΔG*-G appropriately diluted to yield 200–1000 IUs. 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. Cell viabilities were measured by the alamar blue assay (E). The percentages of fluorescence 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. Statistical significances compared to untreated cells were calculated using student's *t*-test (*$p <$ 0.01).

Discussion

In recent years, particular attention has been paid to bat-derived viruses since some species of bats have been reported to be reservoirs of several viral zoonotic pathogens (e.g., lyssavirus, henipavirus, SARS coronavirus, and Marburgvirus) [3-6]. Although the zoonotic potential of BatIVs has not been fully evaluated yet, recent studies generated reassortant viruses that had HA and NA gene segments of well-characterized IAVs (i.e., H1, H3, and H7 HAs and N1, N2, and N7 NAs) and the other gene segments derived from BatIVs, and demonstrated that the reassortant viruses replicated in cultured cells and caused severe disease in mice [79,80]. However, characterization of BatIV HAs and NAs remains an open research problem, since reassortant viruses carrying the BatIV HA and NA gene segments have not been rescued due to the lack of information on cells susceptible to this novel virus. In this study, we first determined the potentially permissive bat cell lines using VSVs pseudotyped with BatIV HAs and NAs.

We demonstrated that VSVΔG*-H17N10 and -H18N11 efficiently infected the bat-derived cell lines IndFSPT1, YubFKT1, and SuBK12-08. While IndFSPT1 was derived from *Pteropus giganteus* (family *Pterodidae*), YubFKT1 and SuBK12-08 were prepared from bats belonging to the same genus (*Miniopterus* sp., family *Miniopteridae*). Based on a phylogenetic study of bats [90], *Miniopteridae* belongs to the same cluster as *Phyllostomidae*, from which H17N10 and H18N11 BatIVs were detected, little yellow-shouldered bats (*Sturnira lilium*) and flat-faced fruit bats (*Artibeus planirostris*), respectively [10,11]. Thus, BatIV HAs appear to recognize cell surface molecules shared among the bats at least in *Miniopteridae* and *Phyllostomidae* families. IndFSPT1 should also have such molecules since it showed the highest susceptibility to BatIV HA-pseudotyped VSVs. It was noted that VSVΔG*-H17N10 and -H18N11 also infected MDCK cells, although less efficiently than these bat cell lines. This result might contradict a previous report that H17 HA did not bind to the surface of MDCK cells [91]. However, it is conceivable that the binding affinity of BatIV HA to

MDCK cell surface molecules is quite low and thus below the level of detection in the assay used in the previous study. In the present study, MDCK cells indeed showed much lower susceptibility to BatIV HA-pseudotyped VSVs than YubFKT1, IndFSPT1, and SuBK12-08. Nonetheless, it would be interesting to clarify whether MDCK cells express some BatIV receptor molecules shared with the bat cell lines.

It is also noteworthy that VSVΔG*-H17N10 and -H18N11 did not infect Vero E6, HEK293, SK-L, and QT6 cells. Previous studies show that quails can act as an intermediate host in the interspecies spread of avian IAVs [92-94]. Furthermore, pigs are thought to serve as "mixing vessels" for the production of reassortant viruses between avian and human IAVs [95-99]. Our results suggest that BatIVs do not readily infect humans, pigs, or birds and support that notion that these viruses have limited zoonotic potential [79,80].

It is known that VSV G protein and IAV HA recognize ubiquitous cell surface molecules for virus entry. VSV G recognizes various cell surface molecules and thus VSV exhibits remarkably robust and pantropic infectivity [100-103]. IAV HAs recognize sialic acids typically occupying the terminal positions of glycoproteins or glycolipids [104,105]. Accordingly, VSVΔG*-G, -WSN, and -Aichi infected all cell lines used in this study, whereas we found that VSVΔG*-H17N10 and -H18N11 infected only particular bat cell lines and that neuraminidase treatment did not affect the infectivities of VSVΔG*-H17N10 and -H18N11. This result was in agreement with previous results based on the crystal structure analysis and surface plasmon resonance of sialylated glycans with α2,3-linkage or α2,6-linkage [83,91]. Glycan microarray analyses also showed that H17 HA did not display obvious avidity to any glycans [91]. Interestingly, we found that the infectivities of VSVΔG*-H17N10 and -H18N11 were markedly reduced by the treatment of IndFSPT1 cells with tunicamycin, which inhibits N-linked glycosylation, leading to unfolding or misfolding of proteins and inhibition of glycoprotein expression. Pretreatment of the cells with pronase also reduced the infectivities of VSVΔG*-H17N10

and -H18N11. Taken together, our data suggest that some particular glycoprotein(s) serve as receptors for BatIVs.

Previous studies indicated that N10 NA did not have sialidase activity [81,82]. It was also shown that most of the amino acid residues responsible for NA activity were substituted, and proposed that N10 NA protein should be termed an NA-like protein [82]. In this study, we found that VSVs pseudotyped with BatIV NAs alone were not infectious, confirming that NA did not play a central role in BatIV entry into cells. However, it should be noted that the production efficiency of pseudotyped VSVs bearing WSN and Aichi HAs alone was much lower than that of VSVs pseudotyped with both HAs and NAs, suggesting that NA activity facilitated virus release from infected cells and/or increased the HA function [106]. By contrast, no remarkable difference was found in the infectivity between VSVs pseudotyped with BatIV glycoproteins (i.e., HA alone vs. HA and NA). These data suggest that, unlike the other IAVs, the target molecules of BatIV HAs and NAs are different and that the "HA-NA balance" concept proposed for IAVs does not be applied to BatIVs.

Because H17N10 and H18N11 BatIVs have never been isolated, their ability to infect humans and other mammals and the pathogenic potential for these hosts can only be hypothesized. In this study, the replication-incompetent VSV pseudotype system enabled us to investigate the cellular tropism controlled by the interaction between BatIV HA and its cellular ligand, which might be some glycoproteins. Although a reverse genetics approach and *in vivo* experiments for the infectious BatIV are needed to provide direct evidence of its pathogenicity and host specificity, our data suggest that BatIV may preferentially infect particular bat species.

Summary

Recently, influenza virus-like RNA genomes were detected in bats from Central and South America. The nucleotide sequences of their HA and NA gene segments were phylogenically distinct from those of previously known IAV subtypes, and thus these newly found BatIVs have been proposed to be classified into novel subtypes. While IAVs are known to have high morbidity in humans and some domestic animals, the pathogenicities of these BatIVs are largely unknown since infectious virus strains have not been isolated yet. To gain insight into the biological properties of BatIVs, we generated VSVs pseudotyped with the BatIV glycoproteins (i.e., HA and NA) and screened several cell lines, including bat-derived cells, for their susceptibility to the viruses. We found that VSVs pseudotyped with BatIV HAs and NAs efficiently infected particular bat cell lines but not those derived from primates, and that proteolytic cleavage with a trypsin-like protease was necessary for HA-mediated virus entry. VSVs pseudotyped with BatIV HA alone infected cells as efficiently as VSVs bearing both HAs and NAs, indicating that BatIV NAs were not essential for virus entry. Modification of the susceptible bat cells with ammonium chloride, pronase, tunicamycin, and neuraminidase revealed that BatIV HAs required endosomal acidification for membrane fusion and might recognize some cellular glycoproteins as receptors rather than the sialic acids used for the other known influenza viruses. These data provide fundamental information on the mechanisms underlying the cellular entry and host restriction of BatIVs.

Conclusion

Bats have been identified or suspected as reservoirs of several zoonotic viruses. In the present study, the author focused on the novel unisolated bat-derived viruses, LLOV and BatIV, belonging to the family *Filoviridae* and *Orthomyxoviridae*, respectively, both of which include zoonotic viruses. Using the replication-incompetent VSV pseudotype system, their glycoprotein functions were analyzed in order to obtain biological properties including zoonotic potential of these bat-derived viruses.

In chapter I, it was found that LLOV GP was antigenically distinct from the other known filovirus GPs and that LLOV GP shared the overall properties for fundamental functions required for filovirus entry into cells. Cellular tropism shown by VSV pseudotyped with LLOV GP suggested that LLOV had the potential to infect many mammalian cells, including human, monkey, and pig cells, with preferential tropism for some bat cells. These results suggest that the GP-receptor interaction does not impose a barrier to potential transmission of LLOV to humans and other animals.

In chapter II, It was found that BatIV HAs required proteolytic cleavage with a trypsin-like protease and endosomal acidification for HA-mediated virus entry, similarly to HAs of the other known IAVs. On the other hand, VSV pseudotyped with BatIV glycoproteins infected particular bat cells efficiently and that BatIV HAs did not recognize sialic acids, which are known to serve as receptors of typical IAVs, but might recognize some glycoproteins expressed on the bat cells. These results suggest that BatIVs may preferentially infect particular bat species and not readily infect other animals including humans.

The present study provides fundamental information on the LLOV and BatIV glycoprotein functions and the zoonotic potential of these viruses. Moreover, the present results are helpful for future attempts to isolate and/or generate infectious strains of LLOV and BatIV. However, further experiments with infectious viruses, once in hand, are needed to provide direct evidence of their

pathogenicities and host ranges.

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和文要旨

人および動物の両方に感染する人獣共通感染症病原体は、新興および再興感染症の主な原因となっており、その多くはウイルス性疾患である。哺乳動物の約 4 分の 1 を占め最も多様性に富む動物の一つであるコウモリ (翼手目) は、狂犬病ウイルス、ヘニパウイルス、フィロウイルス等の人獣共通感染症の原因となるウイルスを保有することが判明しており、人獣共通感染症病原体の自然宿主として重要視されている。さらに、昨今の次世代シークエンサー等の遺伝子解析技術の進歩によって、コウモリ由来の様々な新規ウイルスの遺伝子の検出が可能になっている。しかし、感染性ウイルスが分離されていないウイルスについては、その病原性や宿主域に関する研究は乏しい。今後も、人間の活動域がコウモリの生息域に侵入することで、コウモリ由来の未知のウイルスによる人獣共通感染症の発生が懸念されることからも、コウモリが保有するウイルスの生態や病原性に関する知見を得る事は人獣共通感染症の先回り対策として重要である。本研究では、近年コウモリから遺伝子が検出されたフィロウイルス科に属する Lloviu virus (LLOV) およびオルソミクソウイルス科に属するコウモリ由来インフルエンザウイルス (BatIV) の 2 つのウイルスに焦点を当て、ウイルスの複製サイクルおよび病原性発現に重要であると考えられるウイルス表面糖タンパク質の性状および機能を水胞性口炎ウイルス (VSV) シュードタイプを用いて解析した。

エボラウイルスおよびマールブルグウイルスに代表されるフィロウイルスは人および霊長動物に対して致死性の高い重篤な出血熱を引き起こすことで知られている。第 1 章では、まず LLOV の表面糖蛋白質 (GP) の抗原性を他のフィロウイルスと比較した。その結果、LLOV の GP は他のフィロウイルスとは交差抗原性をほとんど有しておらず、血清学的に独立していることが分かった。この結果から LLOV に対する抗体は従来の血清学的な検査では検出することが出来ない可能性が示唆された。次に、GP を持つ VSV シュードタイプを作出し、様々な動物由来細胞における感染性を LLOV および他のフィロウイルスの

間で比較したところ、LLOV の GP を持つウイルスは他のフィロウイルスの GP を持つウイルスと同様に人を含む様々な動物由来細胞に感染したが、特定のコウモリ由来の細胞に対して他のフィロウイルスに比べて高い感染性を示した。また、LLOV の GP は他のフィロウイルスと同様に、エンドソームでの低 pH およびカセプシンによるトリミングを膜融合時に必要とすることが分かった。さらに、他のフィロウイルス同様に、人由来の C 型レクチンを吸着レセプターとして利用することが確認された。これらの結果は、GP と宿主レセプターとの相互作用の観点では、LLOV は他のフィロウイルスと同様に人に感染する可能性を示唆している。

インフルエンザ A ウイルスは人、豚、馬、家禽等に呼吸器感染を引き起こす代表的な人獣共通感染症病原体の一つである。第 2 章ではシュードタイプ VSV を用い、BatIV のヘマグルチニン (HA) およびノイラミニダーゼ (NA) の機能を解析した。BatIV の HA および NA をもつ VSV シュードタイプは特定のコウモリ由来細胞に効率よく感染した一方、人、豚、鳥由来の細胞には感染性を示さなかった。HA または NA のみを持つ VSV シュードタイプの感染性の結果から、他のインフルエンザ A ウイルスと同様にウイルスの細胞内への侵入は HA が担っており、トリプシン様酵素による HA の開裂が感染性獲得に不可欠であることが分かった。一方、他のインフルエンザ A ウイルスとは異なり NA の有無は感染性に影響しなかった。また感受性細胞を様々な薬剤で処理し、感染性への影響を調べた結果から、HA は低 pH 条件下で膜融合を引き起こすことが分かった。しかし、BatIV の HA は他のインフルエンザ A ウイルスのレセプターであるシアル酸糖鎖を認識せず、ある種のコウモリに発現する糖タンパク質をレセプターとすることが示唆された。これらの結果から、BatIV は特定の種のコウモリのみに感染し、人、豚および鳥等には容易に感染しないことが示唆された。

本研究では、遺伝子のみが検出され感染性ウイルスが分離されていないコウモリ由来のウイルス（LLOV および BatIV）の細胞侵入メカニズム、組織特異性および宿主域等

を推定するために、ウイルス粒子表面糖タンパク質を解析した。糖タンパク質はウイルスの細胞侵入過程において必須であり、糖タンパク質と宿主細胞に発現するレセプターの相互作用はウイルスの病原性発現において重要な因子であると考えられるため、本研究はこれらのウイルスの人獣共通感染症病原体としてのポテンシャルを推測する上で重要な情報を提供した。また、本研究によって得られた知見は、感染性ウイルスの分離あるいはリバース・ジェネティクス法による組換えウイルスの作出に有用であり、今後は感染性ウイルスを用いた詳細な病原性解析が期待される。

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