



Heparan Sulfate Proteoglycan Is an Important Attachment Factor for Cell Entry of Akabane and Schmallenberg Viruses

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ABSTRACT *Akabane virus* (AKAV) and *Schmallenberg virus* (SBV) are members of the genus *Orthobunyavirus*, which are transmitted by arthropod vectors with a broad cellular tropism *in vitro* as well as *in vivo*. Both AKAV and SBV cause arthrogryposis-hydranencephaly syndrome in ruminants. The main cellular receptor and attachment factor for entry of these orthobunyaviruses are unknown. Here, we found that AKAV and SBV infections were inhibited by the addition of heparin or enzymatic removal of cell surface heparan sulfates. To confirm this finding, we prepared heparan sulfate proteoglycan (HSPG)-knockout (KO) cells by using a clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system and measured the quantities of binding of these viruses to cell surfaces. We observed a substantial reduction in AKAV and SBV binding to cells, limiting the infections by these viruses. These data demonstrate that HSPGs are important cellular attachment factors for AKAV and SBV, at least *in vitro*, to promote virus replication in susceptible cells.

IMPORTANCE AKAV and SBV are the etiological agents of arthrogryposis-hydranencephaly syndrome in ruminants, which causes considerable economic losses in the livestock industry. Here, we identified heparan sulfate proteoglycan as a major cellular attachment factor for the entry of AKAV and SBV. Moreover, we found that heparin is a strong inhibitor of AKAV and SBV infections. Revealing the molecular mechanisms of virus-host interactions is critical in order to understand virus biology and develop novel live attenuated vaccines.

KEYWORDS Akabane virus, heparan sulfate, Schmallenberg virus, cell entry

A *kabane virus* (AKAV) and *Schmallenberg virus* (SBV) belong to the Simbu serogroup of the arthropod-borne *Orthobunyavirus* genus of the family *Bunyaviridae*. AKAV and SBV are phylogenetically closely related, and they also possess similar biological characteristics: (i) both cause abortion, stillbirth, premature birth, and congenital deformities in cattle, sheep, and goats; (ii) both primarily infect the central nervous system (CNS) of the fetus; and (iii) both are difficult to control, because they are transmitted by biting midges of the genus *Culicoides* (1). Both AKAV and SBV cause “abortion storms” that result in considerable economic losses to the livestock industry (2–4).

Despite these similarities, there are important features that distinguish these two viruses. AKAV is endemic throughout Australia, Southeast Asia, East Asia, the Middle East, and Africa, whereas SBV has emerged and has dispersed across a large area of

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Europe since 2011. AKAV comprises four genogroups (I to IV), whereas SBV comprises a single genotype. No differences in the pathogenicities of different SBV strains have been described. On the other hand, the OBE-1 strain of AKAV [AKAV(OBE-1)] (genogroup II) causes severe fetal malformation, whereas the Iriki strain [AKAV(Iriki)] (genogroup I) also causes fatal nonsuppurative encephalomyelitis in newborn cattle. Molecular determinants distinguishing the pathogenicities of these two different strains are unknown (5).

Orthobunyaviruses carry a tripartite, single-stranded, negative-sense RNA genome. The L segment encodes the L protein, a viral RNA-dependent RNA polymerase; the S segment encodes the N protein and the nonstructural protein NSs, both transcribed from an overlapping open reading frame; and the M segment encodes NSm and the two major viral envelope proteins, Gn and Gc (Gn/Gc), which form heterodimeric spikes on the virus particle. Gn/Gc are the proteins on the surface of the virion that bind to cell surface molecules in the initial step of orthobunyavirus infection (6, 7). Relatively few studies have investigated orthobunyavirus entry. La Crosse and Germiston neurotropic orthobunyavirus entry into the cell has been described to be promoted by DC-SIGN (8, 9). However, DC-SIGN is probably not the main attachment factor of ruminant orthobunyaviruses, because it is expressed on macrophages and dendritic cells, not in the CNS. Heparan sulfate proteoglycan (HSPG), one of major negatively charged transmembrane protein-linking glycosaminoglycans, is expressed by almost all cells, including neural cells. HSPG is involved in cell attachment of many viruses (e.g., herpes simplex virus [10, 11], adenovirus [12], respiratory syncytial virus [13, 14], human papillomavirus [15], foot-and-mouth disease virus [16], hepatitis B virus [17], hepatitis C virus [18], Ebola virus [19, 20], dengue virus [21], and human immunodeficiency virus [22]). In addition, HSPG is involved in cell attachment of phleboviruses in the family *Bunyaviridae*, including Rift Valley fever virus and Toscana virus (23–25). In a previous study, hemagglutination of AKAV was inhibited by the addition of heparin, a form of heparan sulfate (26). Therefore, it is possible that HSPGs are involved in AKAV and/or SBV infection, similarly to other viruses.

In this study, we examined the role of HSPGs in AKAV and SBV replication.

RESULTS

Heparin or heparinase treatment inhibits AKAV and SBV infections. Heparin is a highly sulfated form of heparan sulfate (27) and is a known inhibitor of infection by various viruses. To test whether heparin inhibits AKAV or SBV replication, AKAV and SBV were incubated with different concentrations of heparin for 30 min, and the titers of the viruses neutralized by heparin were measured by plaque reduction assay in HmLu-1 cells (Fig. 1A), which support efficient replication of the Akabane and Schmallenberg viruses and abundantly express heparan sulfate. The number of plaques induced by either AKAV or SBV was reduced in a heparin concentration-dependent manner. These data suggest that heparin affects the replication cycles of both viruses.

Next, we pretreated HmLu-1 cells with heparinase to remove HSPG from the cell surface and subsequently infected them with AKAV(OBE-1), AKAV(Iriki), and SBV. We also used VSV- Δ G-GFP, a vesicular stomatitis virus (VSV)-based vector expressing green fluorescent protein (GFP) that can complete only a single replication cycle within the cell (28), because this virus does not utilize HSPG during the early events of infection (23, 29). At 8 h postinfection (hpi), cells were fixed and stained with anti-AKAV or anti-SBV antibodies for the detection of AKAV- or SBV-infected cells, respectively. GFP-positive cells were instead counted to determine the number of VSV- Δ G-GFP-infected cells. As shown in Fig. 1B, VSV- Δ G-GFP was not susceptible to heparinase treatment, whereas the numbers of cells infected by AKAV and SBV were reduced in a heparinase concentration-dependent manner. SBV was more susceptible to heparinase treatment than the AKAVs ($P < 0.05$ at 20 and 78 μ U/ μ l of heparinase). These data suggest that HSPG plays important roles in AKAV and SBV infections.

AKAV and SBV replication in HSPG-KO cells. In order to further validate the data shown above, we established HSPG-knockout (HSPG-KO) cells using a clustered regu-

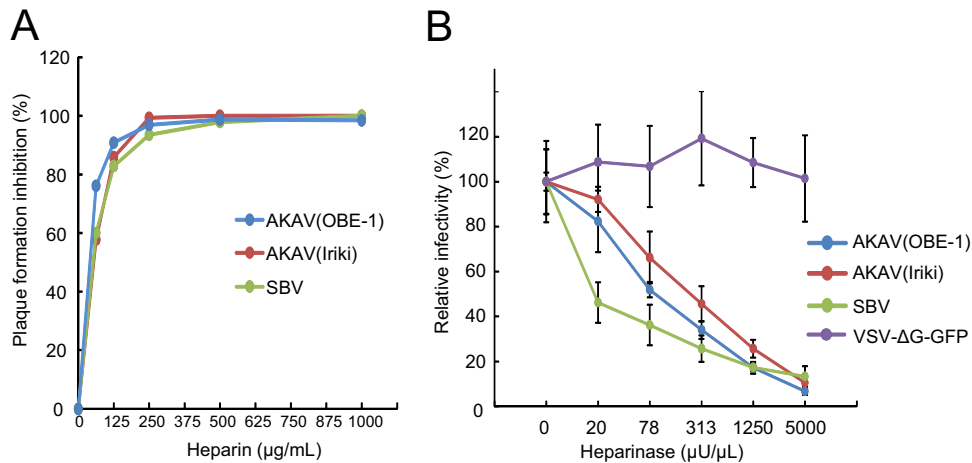


FIG 1 Effects of heparin and heparinase treatment on AKAV and SBV infections. (A) Effects of heparin on AKAV and SBV plaque reduction. Various concentrations of heparin were incubated for 30 min at room temperature with 100 PFU of AKAV(OBE-1), AKAV(Iriki), or SBV, and the ability of heparin to reduce plaque formation was assessed. (B) Effects of heparinase treatment on AKAV and SBV infectivity. HmLu-1 cells were treated with various concentrations of heparinase II, followed by AKAV or SBV infection. Cells were stained for AKAV or SBV antigen, and positive cells were counted under a fluorescence microscope. For VSV-ΔG-GFP-infected cells, GFP-positive cells were counted under a fluorescence microscope. Results are expressed in percentages relative to cells that were not treated with heparinase. The data are reported as the mean value with standard deviations for three independent experiments.

larly interspaced short palindromic repeat (CRISPR)-Cas9 system (30) disrupting the *EXT2* gene, which encodes one of the HSPG-synthesizing enzymes (27). We designed two guide RNA (gRNAs) (*EXT2*-1 and *EXT2*-2) targeting different positions in the *EXT2* gene and obtained three clones for each target (*EXT2*KO-1-1, -1-2, -1-3, -2-1, -2-2, and -2-3 cells). We also established control “random-KO” HmLu-1 cells by introducing a 20-nucleotide (nt) random target sequence in the gRNA with the CRISPR-Cas9 system and obtained three clones of the random-KO cells (random-KO-1, -2, and -3). Lack of HSPG expression in HSPG-KO cells, but not in random-KO or wild-type cells, was confirmed by flow cytometry analysis (Fig. 2A). In the HSPG-KO cells, titers of AKAV(OBE-1) and AKAV(Iriki) were about 100-fold lower than that in random-KO cells at 24 hpi but were at similar levels at later time points (Fig. 2B). SBV titers were instead between 10 and 1,000-fold lower in HSPG-KO cells than in random-KO cells throughout the course of the experiment (Fig. 2B). Next, we examined AKAV and SBV infectivity in the *EXT2*-KO cells and random-KO cells. *EXT2*-KO cells or random-KO cells were infected with AKAV(OBE-1), AKAV(Iriki), SBV, or VSV-ΔG-GFP (multiplicity of infection [MOI] of 0.1). At 8 hpi, AKAV antigen-positive cells, SBV antigen-positive cells, or GFP-positive cells were counted (Fig. 2C). Control VSV-ΔG-GFP-infected cell numbers were not significantly different between random-KO and HSPG-KO cells. Five to 10 times lower numbers of AKAV and SBV antigen-positive cells were detected in *EXT2*-KO cells than random-KO cells. To eliminate the possibility that the replication step of AKAV affected the results shown in Fig. 2C, we used a VSV-pseudotyped virus bearing AKAV glycoproteins (VSV-ΔG-GFP/AKAV). VSV-ΔG-GFP/AKAV or VSV-ΔG-GFP was inoculated into *EXT2*-KO cells or random-KO cells. At 8 hpi, GFP-positive cells were counted (Fig. 2D). As shown in Fig. 2C, control VSV-ΔG-GFP-infected cell numbers did not show a significant difference between random-KO and HSPG-KO cells. However, three-times-fewer VSV-ΔG-GFP-infected cells were detected in *EXT2*-KO cells than in random-KO cells ($P < 0.01$). These data indicated that HSPG was important for AKAV and SBV infections.

Quantification of AKAV and SBV bound to the HSPG-KO cell surface. To determine whether HSPG is important for AKAV and SBV cell surface attachment, we quantified the amounts of cell surface-bound viruses. AKAV(OBE-1) and SBV were incubated with *EXT2*-KO-1, *EXT2*-KO-2, or random-KO cells for 1 h at 4°C, and virus-bound cells were collected. RNA was extracted from the cells, and AKAV or SBV RNA (S

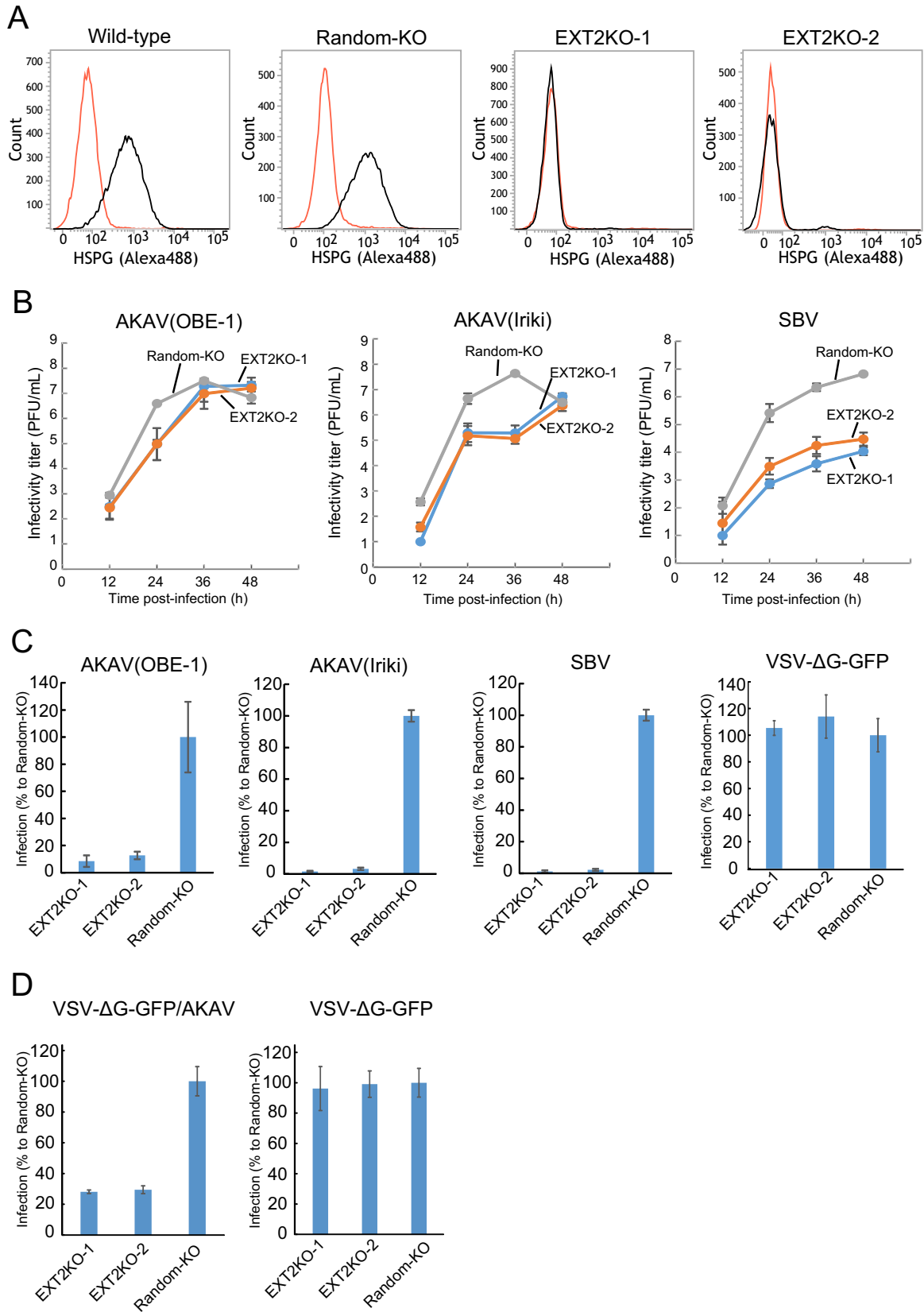


FIG 2 AKAV and SBV growth kinetics and infectivity in HSPG-KO HmLu-1 cells. (A) Flow cytometric analysis of EXT2-KO HmLu-1 cells. CRISPR-Cas9-mediated EXT2-KO cell clones (EXT2-1 and EXT2-2) were labeled with anti-heparan sulfate mouse monoclonal antibody (10E4) (black) or with isotype control (red) and analyzed by flow cytometry (FACSVerse; BD Biosciences). Representative flow data (one out of three clones of random-KO, EXT2KO-1, and EXT2KO-2) are shown. (B) Growth kinetics of AKAV or SBV in HSPG-KO cells. AKAV(OBE-1), (Continued on next page)

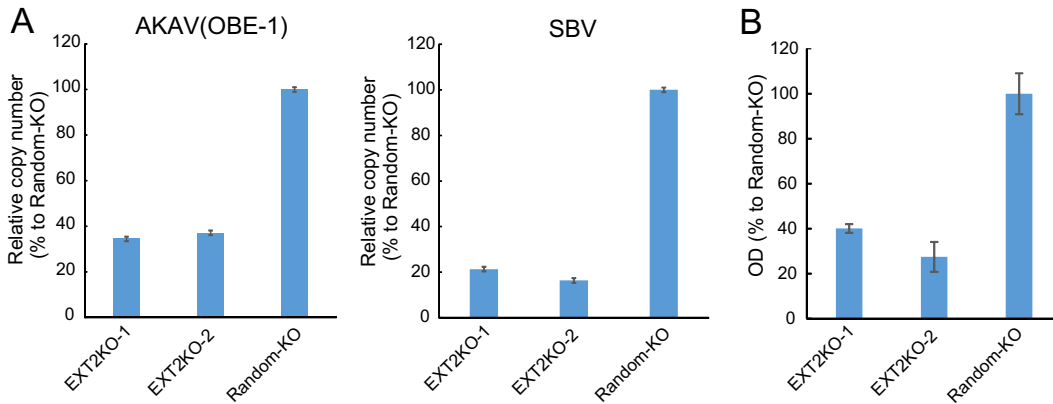


FIG 3 AKAV and SBV binding assays in HSPG-KO cells. (A) Real-time RT-PCR for the quantification of cell surface-attached viruses. AKAV(OBE-1) or SBV was incubated with HSPG-KO cells at 4°C. After a washing step, total RNAs were extracted. AKAV or SBV S RNAs were quantified by one-step real-time RT-PCR. For relative quantification, standard curves of AKAV or SBV S RNA and GAPDH were prepared by serial dilution of a mixture of total RNA from uninfected HmLu-1 cells and RNA extracted from AKAV(OBE-1) or SBV-containing supernatants. Results are expressed as the percentages relative to the levels in random-KO cells. The results are representative of three different experiments. (B) Sandwich ELISA for the detection of N proteins of AKAV attached to cell surfaces. AKAV(OBE-1) was inoculated onto HSPG-KO or random-KO HmLu-1 cells and left for 1 h at 4°C. After a washing step, the cells were lysed, and the lysates were added to the anti-AKAV N monoclonal antibody (5E8)-coated wells of 96-well ELISA plates (Maxisorp, Nunc), followed by incubation with biotinylated anti-AKAV mouse polyclonal antibody. Subsequently, the wells were incubated with avidin-biotinylated horseradish peroxidase (HRP) complex (Vectastain ABC kit; Vector Laboratories). A 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was used for detection, and optical density values were measured. Results are expressed as percentages relative to the number of positive random-KO cells. The results are representative of three independent experiments.

segment) was quantified by quantitative reverse transcription-PCR (qRT-PCR) (Fig. 3A). AKAV and SBV RNAs were significantly lower in HSPG-KO cells than in random-KO cells ($P < 0.01$). We also measured cell-bound AKAV by quantifying N protein using an N-detecting sandwich enzyme-linked immunosorbent assay (ELISA) (Fig. 3B). Cell surface-bound N proteins were significantly lower in EXT2-KO-1 and EXT2-KO-2 cells than in random-KO cells, confirming the qRT-PCR results. These data suggest that HSPG is an important molecule for AKAV and SBV cell surface attachment.

AKAV and SBV replication is restored in cells with *EXT2* added back. We next transduced the EXT2-KO cells with the *EXT2* gene in order to confirm that AKAV and SBV replication could be restored by simply reintroducing the *EXT2* gene back to EXT2-KO cells. We first confirmed that HSPG expression was restored in these cells by flow cytometry (Fig. 4A). Next, we examined AKAV and SBV infectivity in the *EXT2* gene transduced EXT2-KO cells and wild-type cells. EXT2-KO cells or wild-type cells were infected with AKAV(OBE-1), AKAV(Iriri), or SBV. At 8 hpi, AKAV- and SBV-infected cells were detected (Fig. 4B). As expected, infectivity of AKAV(OBE-1), AKAV(Iriri), and SBV was restored in the cells with *EXT2* added back.

DISCUSSION

AKAV and SBV infect neurons (2, 4, 31) and a broad range of cells in the infected hosts. This suggests that AKAV and SBV use a cellular receptor(s) or attachment factor(s)

FIG 2 Legend (Continued)

AKAV(Iriri), or SBV was inoculated onto three clones of random-KO, EXT2KO-1, and EXT2KO-2 cells at a multiplicity of infection of 0.01. Virus titers were determined by plaque assay in normal HmLu-1 cells. The data are reported as the mean titer for three clones of each KO cell (EXT2KO-1, EXT2KO-2, or random-KO) with standard deviations. (C) Infectivities of AKAV and SBV in HSPG-KO cells. Random-KO or HSPG-KO cells were infected with AKAV(OBE-1), AKAV(Iriri), SBV, or control VSV-ΔG-GFP. Cells were stained for AKAV or SBV antigen, and positive cells were counted under a fluorescence microscope. For VSV-ΔG-GFP-infected cells, GFP-positive cells were counted under a fluorescence microscope. Results are expressed as percentages relative to the number of positive random-KO cells. The data are reported as the mean value for three clones of each KO cell (EXT2KO-1, EXT2KO-2, or random-KO) with standard deviations. (D) Infectivities of VSV pseudotyped with AKAV Gn/Gc (VSV-ΔG-GFP/AKAV) in HSPG-KO cells. Random-KO or HSPG-KO cells were infected with VSV-ΔG-GFP/AKAV or control VSV-ΔG-GFP. GFP-positive cells were counted under a fluorescence microscope. Results are represented as percentages relative to the number of positive random-KO cells. The data are shown as the mean value for three clones of each KO cell (EXT2KO-1, EXT2KO-2, or random-KO) with standard deviations.

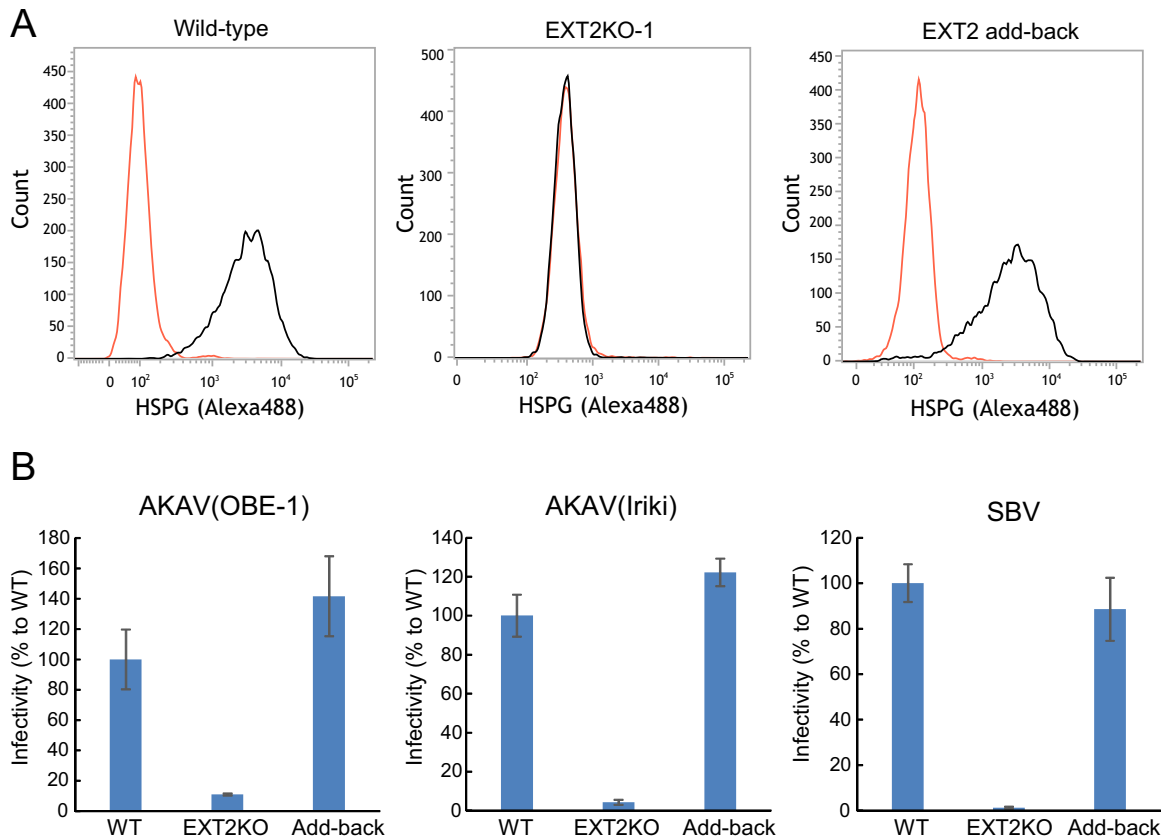


FIG 4 Rescue of AKAV and SBV infectivities by adding back the *EXT2* gene to EXT2-KO cells. (A) Flow cytometric analysis of adding back the *EXT2* gene in EXT2-KO-1 HmLu-1 cells. Cells were labeled with anti-heparan sulfate mouse monoclonal antibody (10E4) and analyzed by flow cytometry (FACSVerse; BD Biosciences). (B) Infectivities of AKAV and SBV in cells with *EXT2* added back. The cells were infected with AKAV(OBE-1), AKAV(Iriki), or SBV. Cells were stained for AKAV or SBV antigen, and positive cells were counted under a fluorescence microscope. Results are expressed as percentages relative to the number of wild-type cells. The data are reported as the mean value with standard deviations for three independent experiments.

that is expressed on a variety of cells. DC-SIGN was previously shown to promote La Crosse and Germiston neurotropic orthobunyavirus entry (8, 9). However, the distribution of DC-SIGN is not consistent with the tropisms of either AKAV or SBV, because DC-SIGN is expressed on a limited number of cell types (i.e., dendritic cells and macrophages). Here, we demonstrated that HSPG plays an important role in AKAV and SBV infections as an attachment factor. Cell surface HSPGs are expressed ubiquitously throughout the body (27), including on neuronal cells (32). Therefore, our data support the correlation between HSPG distribution and AKAV and SBV tropism. However, AKAV and SBV show a strong preference for neuronal cells *in vivo*, and there are likely other reasons for this. AKAV and SBV were still able to replicate in HSPG-KO cells, although at lower levels than those in HSPG-expressing cells. These data suggest the presence of another cellular factor(s) that defines the tissue tropisms of AKAV and SBV. Further studies are needed to identify the neuronal cell-specific receptors or reveal other defining steps after cell entry.

In AKAV(Iriki)-infected HSPG-KO cells, after the virus titer reached a plateau at 24 and 36 hpi, a second AKAV(Iriki) growth wave was observed at 48 hpi (Fig. 2B). During the AKAV(Iriki) replication in HSPG-KO cells, the virus may have acquired a mutation(s) in the receptor-binding site of the glycoproteins, possibly leading to adaptation to HSPG-KO cells. Thus, to investigate whether the AKAV(Iriki) recovered from HSPG-KO cells at a later time point of infection included mutations in the glycoproteins, we sequenced the entire M segment at 60 hpi but found no mutations. Hence, the mechanism of the second growth wave of AKAV(Iriki) still remains unknown. Continu-

ous virus passage in HSPG-KO cells may result in mutations to adapt to another cellular attachment factor(s) or receptor(s).

Repetitive passage of some viruses in tissue culture induces one or two amino acid mutations in their glycoproteins, which increases their affinity toward HSPGs (33–38). This adaptation also induces viral attenuation *in vivo* (33–38). A previous study showed that cell culture-derived SBV showed slightly slowed replication in cattle; however, the involvement of HSPG underlying this is unknown (39). Rift Valley fever virus (23), dengue virus (40), and human T-lymphotropic virus 1 (HTLV-1) (41) do not acquire any mutations after passage in cell culture, which enhances their affinity toward HSPGs. This suggests that the species of the virus determines whether the virus adapts to cell culture and uses HSPGs for entry. AKAV(OBE-1), AKAV(Iriki), and SBV, used in the present study, were passaged several times in tissue culture before being cloned into reverse genetics plasmids. However, AKAV(Iriki) maintained its pathogenic potential against goat fetuses and mice (42, 43), implying that it did not undergo changes during passage in the cell culture. However, it is unclear whether serial passages in cell culture induced mutations in Gn/Gc proteins of AKAV and SBV to give them higher affinity to HSPG. Therefore, further studies are needed to compare the HSPG-binding affinities of the viral strains examined with those of our lab strains and isolates from clinical specimens which have original sequences.

In this study, we showed that the heparinase susceptibility of SBV was greater than that of AKAVs. In addition, SBV replication in HSPG-KO cells was limited compared to that of AKAVs. Moreover, AKAV(OBE-1) exhibited slightly lower HSPG dependency than AKAV(Iriki) did for virus infection and growth. One possible explanation for the difference in HSPG dependency is that AKAV(OBE-1), AKAV(Iriki), and SBV possess different sequences in their HSPG-binding domains and probably in Gn or Gc. Although HSPG-binding domains have been identified in other viral glycoproteins as well as in cellular proteins (44), we could not find known HSPG-binding motifs in AKAV and SBV glycoprotein sequences (data not shown). HSPG-binding domains are not simply defined by the secondary sequences of HS-binding proteins (44). The tertiary structure of these proteins and heparan sulfate interactions are also important for binding. Determining the three-dimensional (3D) structure of Gn/Gc proteins is likely required to define the binding site more precisely. Although the orthobunyavirus Gn/Gc 3D structure is not available currently, determining the 3D structure of Gn/Gc proteins is likely required to define the HSPG binding site.

Here, we clearly show that AKAV and SBV utilize HSPG for their initial cell surface attachment in gene-edited HSPG-KO cells. These findings further our understanding of the orthobunyavirus life cycle. Molecules inhibiting orthobunyavirus and HSPG interactions may be effective antivirals.

MATERIALS AND METHODS

Cells. Baby hamster kidney cells stably expressing T7 RNA polymerase (BHK/T7-9 cells) (45) were kindly provided by Naoto Ito (Gifu University, Japan) and cultured in Dulbecco's modified Eagle's minimum medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 10% tryptose phosphate broth at 37°C. Golden hamster lung (HmLu-1) cells were cultured at 37°C in DMEM supplemented with 5% FCS. Human embryonic kidney (HEK293T) cells were maintained in DMEM supplemented with 10% FCS.

Viruses. AKAV strains OBE-1 and Iriki (42, 46) and SBV (31) were generated by reverse genetics. Briefly, to recover AKAV(Iriki), 1.2 μ g of pT7riboSM2/IL, 0.6 μ g of pT7riboSM2/IM, and 1.2 μ g of pT7riboSM2/IS plasmids were mixed in 200 μ l of Opti-MEM (GIBCO, Grand Island, NY, USA) with 9 μ l of Trans-IT LT1 transfection reagent (Mirus Bio, Madison, WI, USA), incubated at room temperature for 15 min, and then added to BHK-T7 cells (45) grown in 6-well plates. To recover AKAV(OBE-1), 1.2 μ g of pT7riboSM2/OL, 0.6 μ g of pT7riboSM2/OM, and 1.2 μ g of pT7riboSM2/OS plasmids were transfected into BHK-T7 cells as described for AKAV(Iriki) generation. To recover SBV, 1 μ g each of pUCSBVST7, pUCSBVMT7, and pUCSBVLT7 plasmids and 2 μ g of a plasmid expressing T7 polymerase under the control of the chicken β -actin promoter (pCAGGS-T7pol) were mixed in 300 μ l of Opti-MEM (GIBCO) with 15 μ l of Trans-IT 293 transfection reagent (Mirus), incubated at room temperature for 15 min, and then added to HEK293T cells grown in 6-well plates. At 3 days posttransfection, the culture supernatant of transfected cells was harvested and added to HmLu-1 cells. Viruses were propagated in HmLu-1 cells cultured in DMEM supplemented with 2% FCS. Nonspreading vesicular stomatitis virus (VSV- Δ G-GFP) pseudotyped with its own glycoprotein G was propagated following transfection of the VSV G protein-expressing

plasmid (pCAGGS-VSVG) into HEK293T cells (28). VSV-pseudotyped virus with AKAV Gn and mutant Gc was also propagated in HEK293T cells following transfection of the mutant Gn/Gc/NSm of AKAV(OBE-1), which lacks a 10-amino-acid region in the C-terminal of the wild-type Gc. This mutant was made since VSV-pseudotyped virus with Gn and mutant Gc lacking the C termini of cytoplasmic tail glycoproteins of Crimean-Congo hemorrhagic fever (CCHF) virus, a member of *Bunyaviridae*, showed higher titer than VSV-pseudotyped virus with wild-type CCHF Gn/Gc proteins (47).

Production of PABs to AKAV or SBV. An anti-AKAV mouse polyclonal antibody (PAb) was prepared by two intraperitoneal injections of sucrose gradient-purified AKAV(OBE-1) into 6-week-old female ICR mice (Japan SLC, Hamamatsu, Japan) at 2-week intervals. An anti-SBV mouse PAB was prepared by two intraperitoneal injections of sucrose gradient-purified SBV in 6-week-old female ICR mice (Japan SLC) at 2-week intervals. Reactivities and specificities of both anti-SBV and anti-AKAV PABs were confirmed by immunofluorescence assay, using AKAV(OBE-1)- or SBV-infected and mock-infected HmLu-1 cells.

Plaque assay. A standard plaque assay was used to determine the infectivities of AKAV and SBV. After virus adsorption to HmLu-1 cells for 1 h at 37°C, the inocula were removed, and cells were overlaid with DMEM containing 0.6% agarose and 2% FCS. After incubation for 3 days, cells were stained with neutral red before counting PFU.

Plaque reduction assay. One hundred microliters of serial 2-fold dilutions of heparin solution were prepared in minimal essential medium (MEM) containing 0.3% bovine serum albumin (BSA/MEM). An equal volume of the suspensions containing 100 PFU/100 μ l of AKAV(OBE-1), AKAV(Irliki), or SBV was added to each dilution. After incubation for 30 min at room temperature, 200 μ l of each virus-heparin mixture (containing 100 PFU of viruses) was titrated by the plaque assay.

Heparinase treatment. Cells were seeded in 48-well plates at 24 h prior to infection. Medium was removed, and the cells were incubated with serial 4-fold dilutions of heparinase II (New England BioLabs) in BSA/MEM with 2 mM CaCl₂ for 1 h at 37°C and then washed with BSA/MEM. The plates were immediately transferred on ice to suppress the synthesis and transport of HSPG. We then added the appropriate amounts of AKAV(OBE-1), AKAV(Irliki), or SBV to 100 μ l of BSA/MEM and incubated the mixture for 1 h on ice. The supernatant was removed, and the cells were washed twice with BSA/MEM and with and DMEM with 5% FCS before incubation for 8 h at 37°C. The cells were then fixed with 4% paraformaldehyde for 15 min at room temperature. After removing paraformaldehyde, the cells were permeabilized with 0.1% Triton X-100 and incubated with an anti-AKAV N mouse monoclonal antibody (MAb) (5E8) (48) for AKAV-infected cells or an anti-SBV mouse PAb for SBV-infected cells, followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG antibody. The fluorescence-positive cells were counted as AKAV- or SBV-infected cells under fluorescence microscopy (Vert. A1; Carl Zeiss).

EXT2-KO cells. EXT2-KO cells were established using the CRISPR-Cas9 system. EXT2 gene target sequences (EXT2-1 [CTATCCCTGAAAAGTACG] or EXT2-2 [CTACACGGATGACATCAGCC]) containing oligonucleotides were introduced into the guide RNA (gRNA) expression cassette of the plentiCRISPR vector (a gift from Feng Zhang; Addgene plasmid 52961) (30). A random target sequence (N₂₀) containing oligonucleotides was also introduced into the gRNA expression cassette of the plentiCRISPR vector. One microgram of the plentiCRISPR plasmid containing each target gRNA sequence was transfected into HmLu-1 cells (1×10^5 cells) with TransIT-LT1 (Mirus). One day after transfection, the medium was replaced with 10 μ g/ml of puromycin-containing medium for 5 days of selection. Surviving cells were passaged, diluted, and inoculated onto fresh dishes for colony formation. Each colony was picked, propagated, and genotyped. The genomic region surrounding the CRISPR-Cas9 target site for each gene in the cloned cell was PCR amplified with KOD-FX neo (Toyobo), and the PCR products were gel extracted and sequenced using a 3130 Genetic Analyzer (ABI). Primer sequences are available upon request. Clones with indels introduced at the targeted site were picked. Genotyped clones were detached with phosphate-buffered saline (PBS) containing EDTA and incubated with anti-heparan sulfate MAb (10E4) (USBio) (49), followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgM antibody (AbCam). IgM clone MOPC 104E (Sigma, St. Louis, MO, USA) was used as an isotype control. Labeled cells were analyzed by flow cytometry (FACSVerse, BD Biosciences).

Real-time RT-PCR for the quantification of cell surface-attached viruses. For virus adsorption on cells, 1×10^7 PFU/ml of AKAV(OBE-1), AKAV(Irliki), or SBV was inoculated onto HSPG-KO or random-KO HmLu-1 cells and left for 1 h at 4°C. After 1 h of incubation, unbound viruses were washed three times with ice-cold BSA/MEM. Virus-bound cells were lysed, and the total RNA was extracted with Isogen (Nippon Gene). The extracted RNAs were assayed using RNA-direct SYBR green real-time PCR master mix (Toyobo), according to the manufacturer's instructions, in a Thermal Cycler Dice Real Time System (TaKaRa). One microgram of the extracted RNAs was amplified using the AKAV S RNA-specific primer set (forward, 5'-CCACAACCAAGTGTCGATCT-3'; reverse, 5'-AGATGCGGTGAAGCGTAAA-3') or the SBV S RNA-specific primer set (forward, 5'-GGCCAAGATGGTCTACATAAG-3'; reverse, 5'-TGCTGGCACAGGATT TGAG-3'). The RNA was normalized to host GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA using a golden hamster GAPDH-specific primer set (forward, 5'-AAGGTCATCCCAGAGCTGAA-3'; reverse, 5'-CTGCTTACCACCTTCTTGA-3'). For relative quantification, a standard curve of AKAV or SBV S RNA and GAPDH was prepared by serial dilution of the mixture of total RNA extracted from uninfected HmLu-1 cells and viral RNA extracted from AKAV(OBE-1)- or SBV-containing supernatants.

Sandwich ELISA for detection of N proteins of cell surface-attached AKAV. For virus adsorption on cells, 1×10^7 PFU of AKAV(OBE-1) was inoculated onto HSPG-KO or control HmLu-1 cells in 6-well plates and left for 1 h at 4°C. After 1 h of incubation, unbound viruses were washed three times with ice-cold BSA/MEM. Virus-bound cells were then lysed with lysis buffer containing 10 mM Tris-HCl (pH 7.4), 0.5% Triton X-100, 150 mM NaCl, and 1 mM EDTA for 10 min on ice. The lysates were collected and clarified by centrifugation ($10,000 \times g$ for 5 min at 4°C). Supernatants were then added to the anti-AKAV

N MAb (5E8)-coated wells of 96-well ELISA plates (MaxiSorp; Nunc) and incubated for 30 min at room temperature. After washing with PBS–0.1% Tween 20 (PBS-T), biotinylated (biotin labeling kit-NH₂; Dojindo) anti-AKAV mouse PAb was added to the wells and incubated for 30 min at room temperature. After washing with PBS-T, avidin-biotinylated horseradish peroxidase (HRP) complex (Vectastain ABC kit; Vector Laboratories) was added to the wells and incubated for 30 min at room temperature. A 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was used to read the assay.

EXT2 gene reintroduction into HSPG-KO cells. The golden hamster *EXT2* open reading frame sequence (GenBank accession number [XM_013118841](https://doi.org/10.1016/0378-1135(89)90024-2)) was amplified from a pool of cDNA, which was a product of reverse transcription of total RNA from HmLu-1 cells, using an *EXT2*-specific primer set. A pS lentivirus transfer vector under the control of the spleen focus-forming virus promoter was prepared by removing the Venus gene from the pSVenusfull vector (50) by BamHI and NotI digestion. Amplified *EXT2* cDNA was cloned into the pS lentivirus vector and designated pS-EXT2. The lentivirus *EXT2* expression vector was produced in HEK293T cells by cotransfection of the transfer vector pS-EXT2 and two lentivirus packaging plasmids, p8.9QV (51) and pCAGGS-VSVG. The lentivirus vector was concentrated by ultracentrifugation and inoculated onto *EXT2*-KO HmLu-1 cells. HSPG expression was confirmed by flow cytometry as described above.

Statistical analysis. All samples were compared by Student's *t* test with two-tailed analysis to determine statistically significant differences.

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