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Heparan Sulfate Facilitates Rift Valley Fever Virus Entry into the Cell

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Rift Valley fever virus (RVFV), an emerging arthropod-borne pathogen, has a broad host and cell tropism. Here we report that the glycosaminoglycan heparan sulfate, abundantly present on the surface of most animal cells, is required for efficient entry of RVFV. Entry was significantly reduced by preincubating the virus inoculum with highly sulfated heparin, by enzymatic removal of heparan sulfate from cells and in cells genetically deficient in heparan sulfate synthesis.

Rift Valley fever virus (RVFV) belongs to the *Phlebovirus* genus of the *Bunyaviridae* family. Its negative-stranded tripartite RNA genome is encapsidated by nucleocapsid protein and is surrounded by a lipid-containing envelope which is derived from the trans-Golgi network (36). Two membrane-anchored viral glycoproteins, Gn and Gc, assemble into capsomers that cover the viral surface, following a T = 12 icosahedral symmetry (12, 19). The glycoproteins mediate host cell attachment of the virus and its subsequent entry into the cell (36). A 78-kDa glycoprotein of unknown function, which is an N-terminally extended version of Gn, has been reported as a third structural glycoprotein, present only in minute amounts in the viral envelope (21, 39).

RVFV is responsible for severe epidemics among ruminants in Africa and on the Arabian Peninsula, manifested by abortion storms and high mortality among young animals. The virus is transmitted by a wide variety of mosquito vectors. After introduction into the body by the bite of an infected mosquito, the virus can spread and infect different organs, including the brain (32). Humans can also be infected, and a small percentage develops severe disease (31, 36). Apart from mosquitoes, ruminants, and humans, a wide variety of animal hosts can be infected with RVFV, including nonhuman primates, rodents, and pets (11, 18). The virus also efficiently infects a large collection of different cell types *in vitro* (see Fig. S1 in the supplemental material). The broad host, tissue, and cell tropism of RVFV suggests the involvement of a common cell surface attachment factor to be utilized by RVFV to establish infection.

To initiate entry into the cell, viruses need to interact with a cellular receptor, which is sometimes preceded by binding to a primary attachment factor (30). The cell surface structures which facilitate entry of bunyaviruses remain largely unknown, although some receptors have been described. Beta3 integrins and nucleolin have been reported to be involved in attachment of hantavirus and Crimean-Congo hemorrhagic fever virus (genus *Nairovirus*), respectively (14, 42). DC-SIGN, a C-type lectin primarily restricted to interstitial dendritic cells and certain tissue macrophages (33), has been identified as a receptor for some phleboviruses, including RVFV (29). The broad cell tropism of RVFV, however, suggests that other receptors are important for virus entry into cells that lack DC-SIGN expression.

All eukaryotic cells are covered by a dense and diverse array of carbohydrates. These sugars are essential for many different biological processes (40). It is not surprising that many viruses have evolved to use these ubiquitous and accessible surface glycans as part of their strategy to infect cells (26). Two types of glycans, sialylated glycans (SGs) and glycosaminoglycans (GAGs), have been particularly noted to play a role in virus entry. For example, influenza viruses specifically bind SGs, while dengue virus (7) and adenovirus (34) interact with GAGs to facilitate entry. Merkel cell polyomavirus has been reported to use both SGs and GAGs for entry (37).

We started to study the involvement of SGs and GAGs in RVFV entry by using a collection of Chinese hamster ovary (CHO) cell mutants with specific genetic deficiencies in glycan synthesis (see Table S1 in the supplemental material) (22). Thus, CHO lec1 and 15B (16, 38) mutants are incapable of synthesizing complex N-linked glycans, while the CHO lec2 mutant cells express sialic acid-free N- and O-linked glycans (9). The CHO pgsA-745 cell mutant (10) is deficient in the synthesis of GAGs. To facilitate our studies, we made use of the recently developed nonspreading RVFV (here referred to as $RVFV_{ns}$) (25). In contrast to wild-type virus, $RVFV_{ns}$ can be handled outside biosafety level 3 facilities, while the presence of the enhanced green fluorescent protein (eGFP) gene in the viral genome enables infection to be easily monitored. The mutant lec1 and 15B CHO cells and, to a somewhat lesser extent, the CHO lec2 cells were as efficiently infected with $RVFV_{ns}$ as the parental wild-type cells (pro5 and K1), suggesting that N- and O-linked SGs play a minor role in virus infection. In contrast, infection of CHO psgA-745 was dramatically reduced, indicating that GAGs are important for $RVFV_{ns}$ infection (Fig. 1).

GAGs are linear polysaccharides that can be attached to proteins to form proteoglycans. There are five classes of GAGs, heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate, and hyaluronic acid (28). Of these GAGs, HS has been identified as an attachment factor for a number of viruses and, unlike other GAGs, is abundantly expressed on most cell types (28). We first evaluated whether $RVFV_{ns}$ infection could be inhibited by inclusion of soluble heparin, a GAG analogue of HS, as a competitor in the inoculum (23). As a control virus, we used a nonspreading vesicular stomatitis virus (here referred to as VSV_{ns}), a VSV-ΔG/GFP recombinant virus pseudotyped with its

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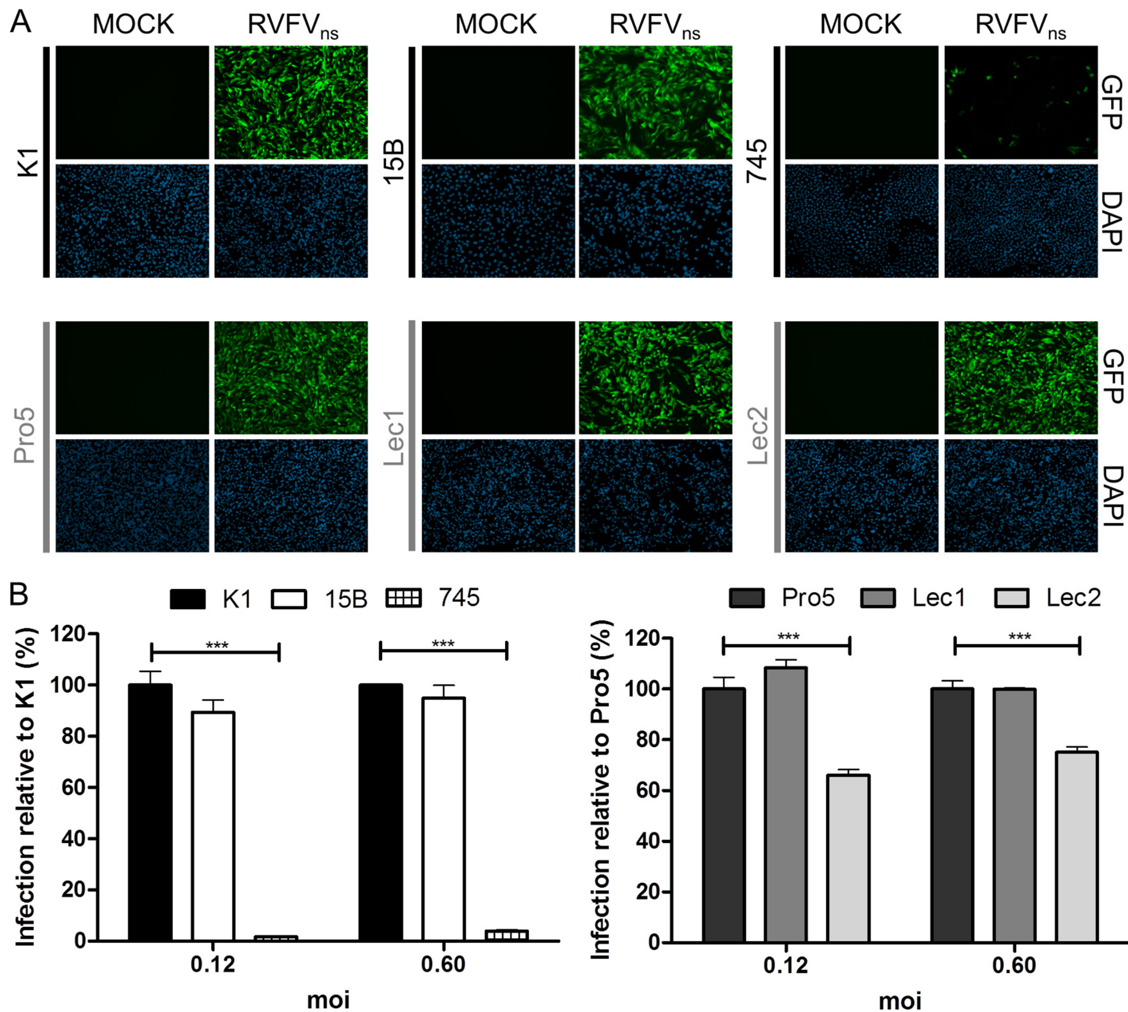

FIG 1 $RVFV_{ns}$ infection is drastically reduced in the absence of GAGs. The CHO 15B and CHO 745 mutant cells derived from the CHO K1 cell line and the CHO lec1 and CHO lec2 mutant cells derived from the CHO Pro5 cell line were cultured in Ham's F-12K medium (Invitrogen) supplemented with 10% fetal calf serum (FCS). Subconfluent monolayers were infected with $RVFV_{ns}$ at different multiplicities of infection (MOIs) (0.12 and 0.6). At 20 h postinfection (p.i.), the cells were washed once with phosphate-buffered saline (PBS) and prepared for fluorescence microscopy (A) or fluorescence-activated cell sorter (FACS) analysis (B). (A) Cells were fixed with 3.7% formaldehyde–PBS for 20 min at room temperature, and representative pictures were taken using an Evos fl microscope (AMG) (magnification, ×4; data shown refer to infections at an MOI of 0.6). Nuclei were counterstained with 4′,6′diamidino-2-phenylindole (DAPI). MOCK, mock-infected cells. (B) Cells were trypsinized and fixed with 3.7% formaldehyde–PBS for 20 min at room temperature, and $RVFV_{ns}$-infected (GFP-positive) cells were quantified by FACS (FACSCalibur). Graphical data shown are normalized to the infectivity of CHO K1 or CHO Pro5 cells and are representative of the results of two independent experiments performed in triplicate. Significant differences between conditions are indicated (analysis of variance [ANOVA]-Bonferroni); ***, $P < 0.001$. Error bars represent standard deviations (SD).

authentic fusion glycoprotein G (5). Preincubation of $RVFV_{ns}$ with heparin reduced infection of CHO K1 cells in a dose-dependent manner, whereas no such effect was observed for VSV_{ns} (Fig. 2A). To confirm the involvement of HS in RVFV entry, CHO K1 cells were treated prior to infection for 1 h at 37°C with different heparinases or chondroitinase to remove HS or CS/DS, respectively, from the cell surface (Fig. 2B). Enzymatic treatment of CHO K1 cells with heparinase caused a marked increase of infection with VSV_{ns} of more than 2-fold. In contrast, independently of the different heparinases used, infection of heparinase-treated cells with $RVFV_{ns}$ was reduced to about 50%. No effect of chondroitinase treatment was observed. The reduced $RVFV_{ns}$ infectivity of heparinase-pretreated cells was confirmed using six different cell lines, while the susceptibility of these cells to VSV_{ns} was not affected (see Fig. S2 in the supplemental material).

To further characterize the interaction between RVFV and highly sulfated HS polysaccharides, we analyzed $RVFV_{ns}$ infection of CHO K1 cells that were subjected to passage in the presence of 50 mM sodium chlorate ($NaClO_3$). $NaClO_3$ is known to inhibit the addition of *O*-sulfate groups to GAGs (1, 35). Importantly, we did not observe any apparent changes in growth rate or cell morphology of CHO K1 or A549 cells cultured for 7 days in the presence of up to 70 mM $NaClO_3$ (data not shown). Infection by $RVFV_{ns}$ of CHO K1 or A549 cells maintained in the presence of $NaClO_3$ was dramatically reduced (Fig. 3A and B), in contrast to infection by VSV_{ns}, suggesting that *O*-sulfation of HS is necessary for efficient $RVFV_{ns}$ infection of both cell lines.

Next we tested the susceptibility of CHO pgsD-677 cells (CHO HS[−]), which are deficient in HS synthesis (27), to $RVFV_{ns}$ and VSV_{ns} infection. Compared to infection of the parental CHO K1

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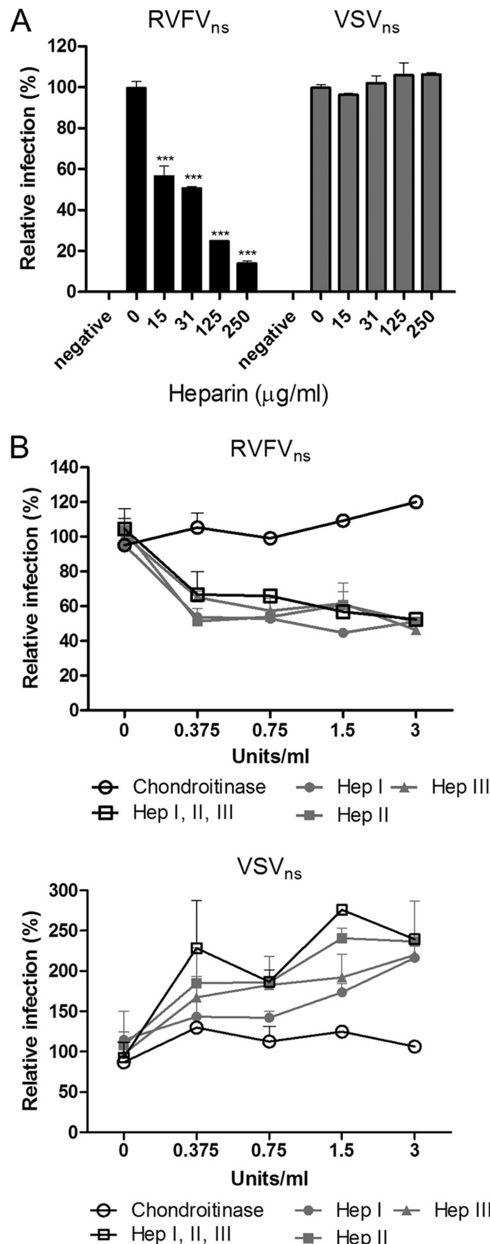

FIG 2 $RVFV_{ns}$ infection is decreased in the presence of heparin and after enzymatic removal of heparan sulfate from the cell surface. (A) $RVFV_{ns}$ and VSV_{ns} were incubated with different concentrations of soluble heparin (MP-Bio) for 10 min at room temperature in culture medium, prior to infection of CHO K1 cells. At 8 (VSV_{ns}) or 20 ($RVFV_{ns}$) h p.i., infection was quantified by FACS analysis as described for Fig. 1. The data shown correspond to the results of a representative set of two independent experiments performed in triplicate. (B) GAGs were enzymatically removed from the cell surface of CHO K1 cells. Chondroitinase ABC (specific for chondroitin and dermatan sulfate), heparinase I (specific for heparin and highly sulfated domains), heparinase II (specific for heparin and heparan sulfate), and heparinase III (specific for heparan sulfate), all purchased at Sigma, were dissolved in resuspension buffer (20 mM HEPES [pH 7.5], 50 mM NaCl, 4 mM $CaCl_2$, 0.01% bovine serum albumin [BSA]). Dilutions were prepared in digestion buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 4 mM $CaCl_2$, 0.1% BSA). CHO K1 cells were treated for 1 h at 37°C with heparinase I, II, or III, with a combination of them, or with chondroitinase at the indicated concentrations. The cells were washed twice with culture medium and then incubated with $RVFV_{ns}$ or VSV_{ns} for 30 min at 37°C. The cells were washed twice with culture medium and further incubated in culture medium at 37°C for 8 (VSV_{ns}) or 20 ($RVFV_{ns}$) h, after which infection was quantified by FACS analysis as described for Fig. 1. Data were obtained from two independent experiments performed in duplicate. Significant differences between conditions are indicated (ANOVA-Bonferroni); ***, $P < 0.001$. Error bars represent SD.

cells with $RVFV_{ns}$, infection of CHO HS^{−} cells was greatly reduced (>97%), whereas VSV_{ns} infection of these cells was enhanced (Fig. 4). To confirm HS dependency of RVFV, an autonomously replicating virus was included in this experiment. This virus expresses the eGFP reporter from its genome in a manner similar to that seen with $RVFV_{ns}$ and was rescued as previously described (25). Also, this virus displayed significantly reduced infectivity on CHO HS^{−} cells. Altogether, the observations strongly support the idea of an important role of HS for RVFV infection.

Many viruses have been reported to utilize HS for host cell attachment (reviewed in reference 28). Interactions of viruses with heparan sulfate are often based on electrostatic contacts between the negatively charged sulfate groups on HS and clusters of basic residues occurring in viral surface proteins. These clusters often comprise a BBXB or a BBBXXB motif (B, basic amino acid; X, any amino acid) (3). In analyzing the complete M segment-encoded polypeptide sequence of the RVFV used in this study (strain 35/74; GenBank accession number JF784387.1), we identified two overlapping BBBXXB HS binding motifs (116-**R**CE**<u>RRR</u>**DA<u>K</u>-124) in the pre-Gn region of the 78-kDa protein (where the boldface characters represent the first and the underlining represents the second motif), while no HS binding motifs were identified in the Gn or Gc protein sequence. The 78-kDa protein is considered to be a minor structural glycoprotein (39) and is apparently dispensable: RVFV recombinants lacking the pre-Gn region display wild-type growth kinetics in cell culture, calling into question whether the basic amino acid motifs in the protein indeed contribute to HS binding (15, 41). Alternatively, other linear or nonlinear arrangements of basic residues in Gn and/or Gc may create an HS binding motif in the tertiary structures of these glycoproteins (13, 17). Clearly, the identification of the HS binding site on the viral surface requires further study.

HS dependency has for some viruses been shown to be acquired after repetitive virus passage in cell culture through the acquisition of single or multiple amino acid substitutions in the surface glycoproteins, creating a positively charged HS binding motif (6, 8, 20, 24). The RVFV 35/74 strain was isolated from the liver of a sheep that died during an RVFV outbreak in the Free State province of South Africa in 1974. The virus was amplified in suckling mouse brain and subjected to three passages in BHK-21 cells (25). To study the possible acquisition of a HS-binding motif during these procedures, the M segment-encoded polypeptide sequence was aligned with those of four RVFV isolates that had been directly sequenced from serum or organ material of infected animals (2, 4). This analysis did not reveal the presence of additional basic amino acids in the 35/74 sequence (see Table S2 in the supplemental material), indicating that the requirement for HS for efficient entry of the RVFV used in this study is not likely the result of cell culture adaptation.

Although infection of RVFV in the GAG- and HS-deficient CHO cells was dramatically reduced, we observed residual infection of both cell lines. It remains to be determined whether this infection in the absence of HS is explained by the binding of RVFV

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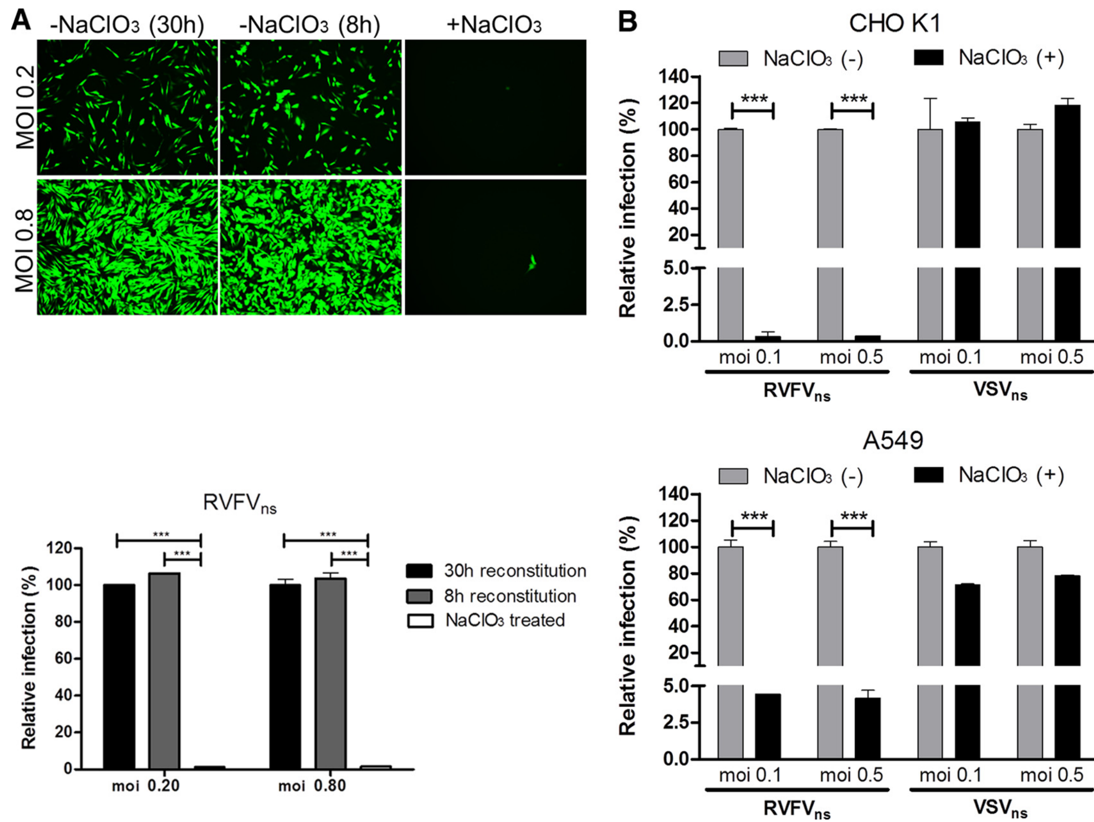

FIG 3 $RVFV_{ns}$ infection strongly depends on sulfation of heparan sulfate. (A) CHO K1 cells were subjected to two passages in culture medium containing 50 mM $NaClO_3$ (Sigma) and subsequently cultured in the presence of 50 mM sodium chlorate, or in chlorate-free culture medium for 30 or 8 h prior to infection, to reverse the chlorate effect. Twenty h postinfection, cells were analyzed by fluorescence microscopy or FACS analysis as described for Fig. 1. Graphical data shown are normalized and are representative of the results of two individual experiments performed in triplicate. (B) A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS). CHO K1 or A549 cells were subjected to two passages in culture medium containing 50 mM $NaClO_3$ (Sigma) and subsequently cultured in the presence of 50 mM sodium chlorate [$NaClO_3$ (+)] or in chlorate-free culture medium [$NaClO_3$ (−)] for 8 h prior to inoculation with $RVFV_{ns}$ or VSV_{ns} at the indicated MOI. At 8 (VSV_{ns}) or 20 ($RVFV_{ns}$) h p.i., cells were analyzed by fluorescence microscopy or FACS analysis as described for Fig. 1. Significant differences between conditions are indicated (ANOVA-Bonferroni; *** = $P < 0.001$). Error bars represent SD.

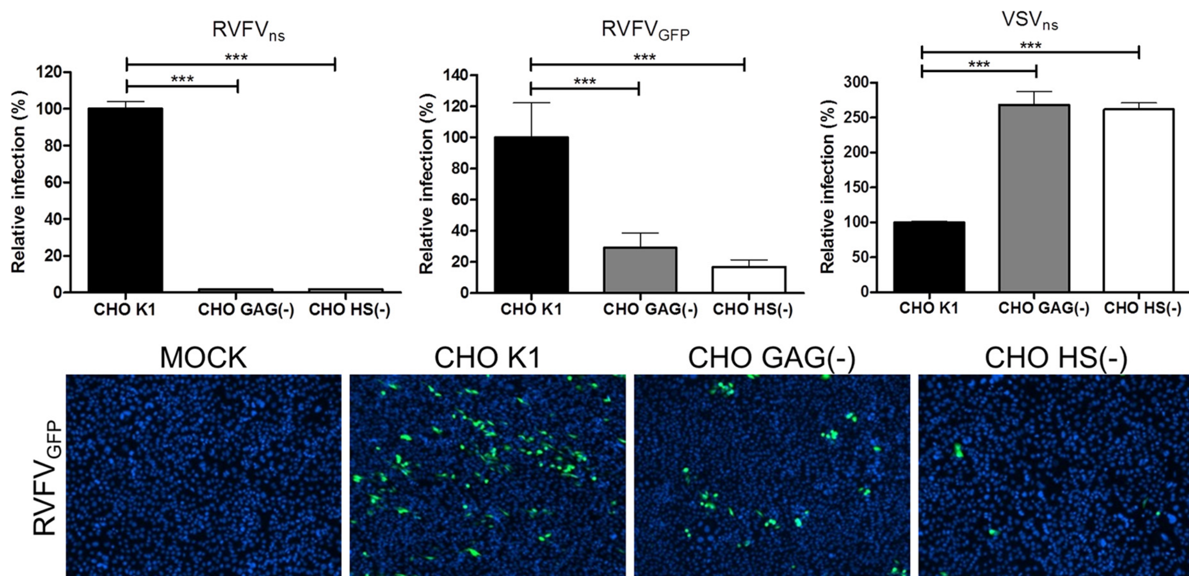

FIG 4 Entry of $RVFV_{GFP}$ into GAG-deficient CHO cells is inefficient due to the lack of heparan sulfate. Mutant CHO pgsD-677 cells (HS[−], able to express all GAGs except for heparan sulfate) and pgsA-745 cells (CHO GAG[−], deficient in expression of all GAGs) and the parental CHO K1 cells were inoculated with $RVFV_{ns}$, VSV_{ns}, or $RVFV_{GFP}$. At 8 (VSV_{ns}), 10 ($RVFV_{GFP}$), or 20 ($RVFV_{ns}$) h p.i., cells were analyzed by fluorescence microscopy and GFP-expressing RVFV-infected cells were quantified. Graphical data shown are normalized to the infectivity of CHO K1. Significant differences between conditions are indicated (ANOVA-Bonferroni); ***, $P < 0.001$. Error bars represent SD.

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to another, unidentified attachment factor or receptor present on these cells.

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