Chikungunya virus strains from each genetic clade bind sulfated glycosaminoglycans as attachment factors

Nicole McAllister, a,b Yan Liu, c Lisete M. Silva, c,d Anthony J. Lentscher, a,b Wengang Chai, c Nian Wu, c,e Kira A. Griswold, a,b Krishnan Raghunathan, b,f Lo Vang, g Jeff Alexander, g Kelly L. Warfield, g Michael S. Diamond, h,i Ten Feizi, c Laurie A. Silva, b,f,# Terence S. Dermody a,b,f,#

a Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.
b Center for Microbial Pathogenesis, UPMC Children’s Hospital of Pittsburgh, Pittsburgh, Pennsylvania, USA.
c Glycosciences Laboratory, Department of Metabolism, Digestion and Reproduction, Imperial College London, London, UK.
d LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Aveiro, Portugal.
e Division of Histology and Embryology, Medical College, Jinan University, Guangzhou, China.
f Department of Pediatrics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.
g Emergent Travel Health, Emergent BioSolutions Inc., San Diego, USA.
h Department of Pathology and Immunology, Washington University School of Medicine, Saint Louis, Missouri, USA.
Department of Medicine, Washington University School of Medicine, Saint Louis, Missouri, USA.

Department of Molecular Microbiology, Washington University School of Medicine, Saint Louis, Missouri, USA.

Corresponding authors:

Laurie A. Silva, UPMC Children’s Hospital of Pittsburgh, Rangos Research Center Office 9121, 4401 Penn Avenue, Pittsburgh, PA 15224. Phone number: (412) 692-5691. E-mail address: laurie.silva@pitt.edu

Terence S. Dermody, UPMC Children’s Hospital of Pittsburgh, Administrative Office Building Suite 5300, 4401 Penn Avenue, Pittsburgh, PA 15224. Phone number: (412) 692-8071. E-mail address: terence.dermody@chp.edu

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CHIKV strains bind sulfated GAGs

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Chikungunya virus (CHIKV) is an arthritogenic alphavirus that causes debilitating musculoskeletal disease. CHIKV displays broad cell, tissue, and species tropism, which may correlate with the attachment factors and entry receptors used by the virus. Cell-surface glycosaminoglycans (GAGs) have been identified as CHIKV attachment factors. However, the specific types of GAGs and potentially other glycans to which CHIKV binds and whether there are strain-specific differences in GAG binding is not fully understood. To identify the types of glycans bound by CHIKV, we conducted glycan microarray analyses and discovered that CHIKV preferentially binds GAGs. Microarray results also indicate that sulfate groups on GAGs are essential for CHIKV binding and that CHIKV binds most strongly to longer GAG chains of heparin and heparan sulfate. To determine whether GAG-binding capacity varies among CHIKV strains, a representative strain from each genetic clade was tested. While all strains directly bound to heparin and chondroitin sulfate in ELISAs and depended on heparan sulfate for efficient cell-binding and infection, we observed some variation by strain. Enzymatic removal of cell-surface GAGs and genetic ablation that diminishes GAG expression reduced CHIKV binding and infectivity of all strains. Collectively, these data demonstrate that GAGs are the preferred glycan bound by CHIKV, enhance our understanding of the specific GAG moieties required for CHIKV binding, define strain differences in GAG engagement, and provide further evidence for a critical function of GAGs in CHIKV cell attachment and infection.
IMPORTANCE

Alphavirus infections are a global health threat, contributing to outbreaks of disease in many parts of the world. Recent epidemics caused by CHIKV, an arthritogenic alphavirus, resulted in more than 8.5 million cases as the virus has spread into new geographic regions, including the Western Hemisphere. CHIKV causes disease in the majority of people infected, leading to severe and debilitating arthritis. Despite the severity of CHIKV disease, there are no licensed therapeutics. Since attachment factors and receptors are determinants of viral tropism and pathogenesis, understanding these virus-host interactions can enhance our knowledge of CHIKV infection. We analyzed over 670 glycans and identified GAGs as the main glycan bound by CHIKV. We defined specific GAG components required for CHIKV binding and assessed strain-specific differences in GAG-binding capacity. These studies provide insight about cell-surface molecules that CHIKV binds, which could facilitate the development of antiviral therapeutics targeting the CHIKV attachment step.
INTRODUCTION

To initiate infection, viruses interact with a variety of cell-surface molecules, including proteins, carbohydrates, and lipids (1, 2). Binding to abundantly expressed cell-surface molecules, which are sometimes called attachment factors, concentrates viral particles at the plasma membrane, which enhances the probability of engagement with an entry receptor (2). The interaction between a virus and an attachment factor is usually of low-affinity (2). In contrast, interactions with entry receptors are usually of high-affinity and often trigger conformational changes in viral surface proteins that promote viral entry (2). Expression of attachment factors and entry receptors is often a determinant of viral tropism and can influence disease (3), making it important to identify these host factors and characterize their function in viral replication. When multiple attachment factors or entry receptors are used by a virus, defining the function of each during viral infection can be complex. Overall, the molecular mechanisms by which viruses bind to host cells and how such virus-receptor interactions influence tropism and disease are still not completely understood, especially for emerging viruses.

Mosquito-transmitted alphaviruses are a global health threat that periodically reemerge to cause epidemics of disease in many parts of the world (4). Alphavirus introductions into naïve populations have resulted in large epidemics, such as the chikungunya virus (CHIKV) epidemics that began in 2004 and 2013, which collectively resulted in more than 8.5 million cases and the spread of the virus into new geographic regions, including the Western Hemisphere (5–11). These epidemics were caused by CHIKV strains from two of the three genetically distinct CHIKV clades (the East Central South African [ECSA] and Asian clades, respectively) (12, 13), while strains from the
third clade (West African) have remained endemic to Western Africa (6). CHIKV causes disease in approximately 80% of those infected (14, 15), with manifestations commonly including fever, rash, myalgia, arthralgia, and arthritis (16, 17). CHIKV disease is usually self-limited and rarely fatal, but infection can cause acute and chronic disabilities that impair quality of life (18). Up to 60% of infected individuals experience debilitating arthralgia and arthritis that persist for months to years after infection (16, 17).

Additionally, large CHIKV epidemics have severe social and economic consequences (19). Despite the severity of CHIKV disease, there are no licensed antivirals or vaccines.

CHIKV can infect mosquitoes, nonhuman primates, and humans (20). In mosquitoes, CHIKV replicates in the midgut, salivary glands, fat bodies, and ovaries (21, 22). While CHIKV replicates in many human cell lines, including fibroblasts (23), macrophages (24), keratinocytes (25), epithelial cells (23), muscle cells (23, 26), and endothelial cells (23), the cells and tissues targeted in infected humans are less well-defined. However, studies using mice demonstrate CHIKV dissemination into a variety of tissues, including dermis, lymph nodes, spleen, muscle, joints, and tendons (27–30). The broad cell, tissue, and species tropism observed for CHIKV may correlate with the expression of attachment factors or entry receptors used by the virus.

Several cell-surface molecules have been identified to facilitate CHIKV attachment and entry. CHIKV binds Mxra8 as an entry receptor (31, 32), but absent or decreased expression of Mxra8 in several cell types does not completely abrogate CHIKV infection, suggesting that CHIKV can use other entry receptors (31). Additionally, a variety of cell-surface molecules may act as attachment factors for
CHIKV (33–37), including glycosaminoglycans (GAGs) (38–41). GAGs serve as attachment factors for many pathogenic viruses (38–40, 42–53) and are expressed ubiquitously in humans and mosquitoes (54–56). GAGs are negatively charged linear polysaccharides composed of repeating disaccharide units expressed at the cell surface and in the extracellular matrix (54). Interactions with GAGs are often mediated by positively charged amino acid side chains of protein ligands (57). There are four main types of GAGs based on the differences in their repeating disaccharide units, including heparin/heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (54). With the exception of hyaluronan, the other types of GAGs are highly sulfated (54). Variations in GAG chain length and degree and pattern of sulfation are determined by the expression and relative abundance of specific GAG biosynthetic enzymes (54, 58). Although heparin and HS are structurally similar, heparin is a more highly sulfated version of HS, composed of more iduronic acid, and often used experimentally instead of HS due to accessibility and cost (54, 59). HS and CS/DS are abundantly expressed at the sites CHIKV infects. In mosquitoes, HS and CS/DS are expressed in the ovaries, midgut, and salivary glands (56, 60, 61). In mammals, HS is primarily expressed on epithelial cells, fibroblasts, endothelial cells, skin, and muscle (54, 62–64), and CS/DS is mainly found in cartilage, connective tissue, fibroblasts, macrophages, and endothelial cells (54, 65). Thus, HS and CS/DS expression overlaps with the broad cell and tissue tropism of the virus.

Cell-culture adaptation of CHIKV, which often results in mutations in the E2 attachment protein, can enhance GAG binding (66). CHIKV strain 181/25 displays increased GAG binding due to a specific mutation in E2 (G82R) (38, 39) that was
acquired after 29 passages in cell culture (67, 68). However, for at least some field-isolate strains, efficient infection in cell culture depends on GAG expression (38–41).

Accordingly, pre-incubation of some CHIKV strains with soluble GAGs prior to cell adsorption inhibits infection in vitro (38, 40). It is not clear whether CHIKV preferentially binds to different GAG types, nor whether CHIKV strains from the three genetically distinct clades differ in GAG binding. Moreover, the requirement of specific GAGs for CHIKV binding and infection of cells with varying levels of GAG and Mxra8 expression has not been defined.

In this study, we used microarrays to identify glycans bound by CHIKV. We discovered that CHIKV preferentially binds GAGs relative to other glycan types tested and identified heparin and HS to be bound by CHIKV most efficiently. We found that human- and mosquito-isolated CHIKV strains from each CHIKV clade directly bind to GAGs and require HS for efficient binding and infection. Although CHIKV directly binds to CS chains, CS is not required for infection and only influences binding for some strains in the cells tested. The requirement of sulfated GAGs for CHIKV binding and infection was inversely correlated with the levels of Mxra8 expression. Finally, strains of each CHIKV clade displayed differences in the efficiency of GAG utilization. These studies suggest that HS, and to a lesser extent possibly CS/DS, function as CHIKV attachment factors in the presence and absence of the Mxra8 entry receptor. Collectively, these data enhance our understanding of attachment factor engagement for diverse CHIKV strains.
RESULTS

CHIKV directly and preferentially binds sulfated GAGs. Some strains of CHIKV bind directly to heparin in vitro (38, 39). To identify other glycans to which CHIKV binds, we conducted glycan microarray analyses using virus-like particles (VLPs). Chikungunya VLPs are structurally indistinguishable from native chikungunya virions (69) and can be used in experiments at a lower biosafety level than pathogenic CHIKV. The VLPs used in our experiments are composed of the structural proteins of West African clade CHIKV strain 37997 (70) and are currently in advanced development as a vaccine candidate by Emergent BioSolutions (71–73). The microarray contained 672 sequence-defined lipid-linked oligosaccharides, representing the major types of mammalian glycans found on glycoproteins, glycolipids, and proteoglycans, as well as those derived from glucan polysaccharides of bacteria, fungi, and plants (Table S1 and Fig. 1). Ten heparin-derived oligosaccharides (2-mer to 20-mer chains) were included in this array as representatives of GAG-related sequences (Table S1). Chikungunya VLPs were overlaid onto the microarray, and VLP binding was detected by indirect immunofluorescence.

Among the 672 glycans tested in the microarray, approximately 30 glycans showed a VLP binding signal above background (Table S1 and Fig. 1). The ten highest VLP binding signals were produced by heparin GAGs of varying lengths (Fig. 1), suggesting that GAGs are the preferred glycan type bound by CHIKV. Binding was observed with a heparin dimer, and binding signals increased with increasing length of heparin chains (Table S1). Among the non-GAGs bound, most are negatively charged, including a ‘ring-opened’ NeuAc monosaccharide (position 637), SU-3GlcAβ-3Galβ-
4Glc (position 36), and Carra-Hexa-4S (position 669; Fig. 1 and Table S1). Collectively, these data demonstrate that GAGs are preferentially bound by chikungunya VLPs in vitro and highlight a potential role for GAG chain length in the efficiency of virus binding.

To gain additional information about the GAG binding specificities of CHIKV, we used GAG-focused microarrays. These microarrays included 15 size-defined oligosaccharides derived from different types of GAGs: heparin, HS, CS-A, CS-B (DS), CS-C, KS, and hyaluronan, which was the only non-sulfated GAG in this analysis (Fig. 2 and Table S2). Short (6- or 10-mer) and long (up to 14-mer) chains were included for each GAG type except for the hyaluronan 12-mer, HS 6-mer, and HS 8-mer (Fig. 2 and Table S2). Larger size-defined fractions of HS oligosaccharides were not available for the study due to the sequence heterogeneity of HS relative to other GAG types. Two non-GAG polysaccharides, dextran sulfate and dextran (74), also were included as controls for highly sulfated and neutral saccharides, respectively. Chikungunya VLPs were overlaid onto the GAG-focused array, and VLP binding was detected by indirect immunofluorescence.

Whereas VLPs bound to dextran sulfate, binding to unsulfated dextran was not detected, and very little binding was observed to hyaluronan, an unsulfated GAG (Fig. 2A). These data suggest an important function for sulfation in CHIKV-glycan interactions. VLPs bound all sulfated GAGs above background with varying intensities (Fig. 2A). The strongest binding signals were observed with heparin, followed by CS-B, CS-C, CS-A, and weakest for KS (Fig. 2A). In general, stronger binding signals were observed with longer GAG oligosaccharides, especially with heparin 14-mer, HS 8-mer, and CS-B 14-mer, which all reached statistical significance. Interestingly, the GAGs...
bound strongest by CHIKV, including heparin, HS, and CS-B (DS), all contain iduronic acid monomers, while the other GAG types do not (54) (Fig. 2B), suggesting that iduronic acid may contribute to CHIKV binding. Overall, CHIKV binds with greatest avidity in vitro to longer, sulfated chains of GAGs, with a preference for HS and heparin.

Multiple CHIKV strains directly bind heparin and CS. To determine whether GAG-binding efficiency differs between CHIKV strains and to validate the microarray results, we assessed viral binding to heparin and CS by ELISA. Three CHIKV strains, SL15649 (29), H20235 (75), and 37997 (70), were selected to represent the three CHIKV genetic clades (ECSA, Asian, and West African, respectively) (Table 1). Importantly, the strains chosen for analysis were isolated from infected humans or mosquitoes and minimally passaged in cell culture prior to sequencing and construction of infectious cDNA clones (Table 1). We used CHIKV strain 181/25 as a positive control for heparin binding. Strain 181/25 was derived from plaque-to-plaque passaging of parental strain AF15561 of the Asian CHIKV clade (67, 68). Cell-culture adaptation of 181/25 led to mutations in the E2 attachment protein, one of which (G82R) is linked to increased heparin binding efficiency (38, 39) and attenuated virulence in mice and humans (39, 76, 77). Serial dilutions of viable virus were adsorbed to ELISA plates coated with either heparin or CS, and bound virus was quantified. We calculated a relative binding strength (RBS) for the binding of each strain to heparin and CS, where the RBS values refer to the relative concentration of virus at which 50% of GAG-binding sites are occupied.
As expected, the attenuated 181/25 strain displayed the highest-avidity binding to heparin (Fig. 3A) and had the lowest RBS value of $7.9 \times 10^6$ genomes/sample (Table 2). The other strains tested also bound to heparin in a dose-dependent manner (Fig. 3A). The second-highest heparin-binding signals were detected for the ECSA strain with a RBS value of $1.8 \times 10^7$ genomes/sample, followed by moderate binding for the Asian and West African strains (Fig. 3A). The RBS values for heparin binding for the Asian strain was $2 \times 10^7$ genomes/sample and $3.6 \times 10^7$ genomes/sample for the West African strain (Table 2). In addition, all strains except the attenuated 181/25 strain bound to CS in a dose-dependent manner (Fig. 3B). For this reason, an RBS value for 181/25 binding to CS could not be calculated (Table 2). A similar preference for HS binding relative to CS binding by 181/25 is observed during in vitro binding and infection of mutant Chinese hamster ovary cells (38). Similar to heparin binding, the highest binding signals to CS were detected for the ECSA strain, followed by moderate binding for the Asian and West African strains (Fig. 3B). The RBS values for CS binding were $1.4 \times 10^7$ genomes/sample for the ECSA strain, $2 \times 10^6$ genomes/sample for the Asian strain, and $10^7$ genomes/sample for the West African strain (Table 2). Notably, binding signals were generally lower in the CS binding assays relative to the heparin binding signals (Fig. 2). Collectively, these data indicate that CHIKV strains from each clade directly bind in vitro to heparin and, to a lesser degree, CS, validating the microarray results that used CHIKV strain 37997 VLPs. These data also demonstrate strain-specific differences in GAG binding with the ECSA strain binding to heparin and CS with the highest avidity and the Asian strain binding to heparin and CS with the lowest avidity.
Enzymatic removal of cell-surface HS reduces CHIKV binding and infection.

Results obtained thus far demonstrate that multiple CHIKV strains bind GAGs in vitro.

To determine whether CHIKV-GAG interactions contribute to binding and infection of cells, we treated human osteosarcoma (U-2 OS) cells with a combination of heparinases (HSase) or chondroitinases (CSase) and assessed the cells for GAG expression, virus binding, and virus infectivity. U-2 OS cells were chosen for these experiments because they express higher levels of HS and CS compared with other cell types commonly used to study CHIKV replication, such as mouse 3T3 fibroblasts, baby hamster kidney (BHK) fibroblasts, and African green monkey kidney epithelial (Vero-81) cells (Fig. 4A and C). U-2 OS cells also express relatively high levels of Mxra8 (Fig. 4B and D), an entry receptor for CHIKV and other arthritogenic alphaviruses (31).

Treatment with HSase I, II, III or CSase ABC specifically and efficiently reduced levels of cell-surface HS and CS, respectively (Fig. 5A and B). Following GAG cleavage, Mxra8 expression did not change (data not shown). HS was required for efficient cell binding, as cleavage of HS reduced binding for all CHIKV strains studied (Fig. 5C). As expected, binding of the attenuated 181/25 strain, which has enhanced HS binding capacity (38, 76), was reduced by 95% following HS cleavage (Fig. 5C). Binding of the mosquito and clinical CHIKV strains was reduced by 23 to 44% following HS cleavage (Fig. 5C). Cleavage of CS decreased binding of some CHIKV strains, with the ECSA strain reduced by 29%, a reduction greater than that observed for the other strains (Fig. 5C). Additionally, cleavage of HS diminished infectivity of all CHIKV strains by 34 to 55% (Fig. 5D). Cleavage of CS did not affect infectivity (Fig. 5D), suggesting an importance of HS, but not CS, for CHIKV infection of U-2 OS cells. These data indicate...
that all strains tested depend on HS to bind to cells, while some strains also depend on CS for efficient cell attachment. Efficient infection of U-2 OS cells requires HS binding.

**Genetic ablation of GAG biosynthesis reduces CHIKV binding and infection.** To investigate the requirement of HS for efficient CHIKV cell binding and infection when Mxra8 and CS are absent, we used human haploid Hap1 cells. Wild-type (WT) Hap1 cells abundantly express HS and have low to no expression of CS and Mxra8 (Fig. 6A-D). These features make Hap1 cells suitable for studies to determine whether HS is required for CHIKV binding and infection. Due to their haploid nature, Hap1 cells also are more amenable to genetic alteration. We used B3GAT3\(^{-/-}\) Hap1 cells, engineered using CRISPR-Cas9 technology (41), that have a targeted disruption of the B3GAT3 gene, which encodes beta-1,3-glucuronyltransferase 3 (B3GAT3). B3GAT3 catalyzes the transfer of glucuronic acid to galactose, which is a required step in the biosynthesis of heparin, HS, and CS/DS (54). Compared with WT Hap1 cells, B3GAT3\(^{-/-}\) cells exhibit diminished GAG expression (Fig. 6A and C). However, neither WT Hap1 cells nor B3GAT3\(^{-/-}\) cells express Mxra8 (data not shown). B3GAT3\(^{-/-}\) cells complemented with a B3GAT3-expressing plasmid display GAG expression comparable to WT levels (Fig. 6A and C). WT, B3GAT3\(^{-/-}\), and complemented B3GAT3\(^{-/-}\) cells were tested for CHIKV binding and infection. Binding to B3GAT3\(^{-/-}\) cells of all CHIKV strains tested was reduced by 74 to 97% compared with binding to WT cells, and complementation of the B3GAT3\(^{-/-}\) cells restored binding by 43 to 82% (Fig. 6E). Infection of B3GAT3\(^{-/-}\) cells by all CHIKV strains tested was diminished by 92 to 100% relative to WT cells (Fig. 6F). Complementation of B3GAT3\(^{-/-}\) cells with B3GAT3 partially...
restored infection (Fig. 6F). The lack of full restoration of binding and infection to WT levels after complementation of $B3GAT3^{-/-}$ cells may be due to differences in HS expressed by WT and complemented $B3GAT3^{-/-}$ cells (Fig. 6A). Overall, these data indicate that CHIKV requires HS for binding to and infection of Hap1 cells and emphasize the importance of HS as a CHIKV attachment factor when other ligands like CS or Mxra8 are absent.
DISCUSSION

The specific glycans used by different strains of CHIKV as attachment factors have not been well understood. In this study, we found that sulfated GAGs are the glycans preferentially bound by CHIKV. The strongest binding occurred with HS and heparin, followed by CS. All human- and mosquito-isolated CHIKV strains tested directly bound to heparin and CS. HS was required for efficient binding and infection of U-2 OS and Hap1 cells, while CS was required only by some strains to efficiently attach to U-2 OS cells. Moreover, the requirement of GAGs for CHIKV binding and infection inversely correlated with levels of Mxra8 receptor expression. Collectively, these data suggest that HS, and to a lesser extent CS, function as attachment factors for several CHIKV strains.

CHIKV displays broad cell, tissue, and species tropism (8, 78), which may correlate with the attachment factors or entry receptors used by the virus. Previous studies, as well as this work, identified sulfated GAGs as CHIKV attachment factors (38, 40, 41) (Fig. 5 and 6). These glycans are ubiquitously expressed in humans and mosquitoes (54–56), including the specific cells and tissues that CHIKV infects. Many pathogenic viruses, including viruses in the alphavirus family (42–45, 52, 79) as well as other virus families (46–51, 53, 80–83), bind GAG attachment factors to attach to cells. For example, enterovirus 71 (EV-71), which displays broad tissue tropism (84) like CHIKV, specifically binds HS as an attachment factor (53). An alphavirus, eastern equine encephalitis virus, also binds HS attachment factors (43). Strains of both EV-71 and eastern equine encephalitis virus with enhanced HS binding capacity display
broadened tissue tropism and enhanced virulence (42, 43, 85). Thus, GAG attachment factor binding can influence viral tropism and virulence.

Although GAGs are CHIKV attachment factors, the specific GAG structures required for CHIKV binding had not been defined. GAG types and structures vary in different cells, tissues, and organisms, and the interactions between GAGs and proteins are often mediated by the structural characteristics of GAG chains. GAG types differ in their composition of repeating disaccharide units, which can facilitate specific interactions with chemokines, growth factors, enzymes, and viral proteins (86–88). The glycan microarray analyses we conducted identified sulfated GAGs as the primary glycan type bound by chikungunya VLPs (Fig. 1) with HS and heparin most strongly bound (Fig. 2). Similarly, binding signals were generally lower in the CS ELISAs relative to the heparin ELISAs, suggesting a preference of CHIKV for binding to heparin (Fig. 3). On the glycan microarrays, some weak binding also was detected to non-GAG glycans, which may prompt further investigation into these CHIKV-glycan interactions.

Interestingly, the iduronic acid-containing GAGs, HS, heparin, and CS-B (DS), which are abundantly expressed in cells and tissues infected by CHIKV (54–56, 60–64), had the highest binding signals with VLPs relative to other GAGs tested (Fig. 2). This is reminiscent of the GAG binding properties of respiratory syncytial virus (RSV), which require iduronic acid-containing GAGs for in vitro infection (51).

GAG oligosaccharide chain length is another important structural characteristic that influences binding to many ligands, including chemokines, growth factors, tau aggregates, and viral proteins (57, 89). We found that longer, sulfated GAGs are generally bound more efficiently by CHIKV (Fig. 1 and 2). VLPs bound more efficiently
to longer rather than shorter chains of almost every GAG type (Fig. 2). The requirement of longer GAG chains for virus binding has been observed for many viruses (82, 83, 90–92). For example, RSV requires heparin with a minimum 10-mer chain for efficient binding (82), and Zika virus preferentially binds 8- to 18-mer heparin chains (83). Further investigation is required to determine the optimum chain length required for each GAG type to promote binding of different CHIKV strains.

Sulfation modifications along the GAG chain also regulate ligand binding (93). Our studies indicate that the degree of sulfation is an important factor in CHIKV-GAG binding, which is consistent with previous findings, demonstrating that N-sulfation of HS chains is required for CHIKV infection in vitro (41). VLPs bound to all sulfated GAGs and dextran sulfate but not to hyaluronan or dextran, which are unsulfated glycans (Fig. 2). GAG sulfation also influences the binding of several other viruses (80–82, 94–96). In fact, specific sulfation modifications on HS chains are important for virus-GAG interactions, such as 3-O sulfation for herpes simplex virus 1 (95, 96) and N-sulfation for RSV (82). Although we found that sulfation of GAG chains is required for CHIKV binding, the specific sulfation patterns necessary for CHIKV engagement remain unknown. Given that expression of many sulfation-modifying enzymes is tissue-specific (58, 97, 98), identifying the specific modifications necessary for CHIKV binding could enhance our understanding of its tropism and help define more specific cell-attachment inhibitors. Collectively, our glycan microarray analyses suggest that CHIKV most efficiently binds longer, sulfated GAGs with a preference for HS and heparin. As GAG mimetics are a possible therapeutic for alphavirus and flavivirus disease (99–102),
understanding the unique GAG sequences required for efficient CHIKV binding may foster development of new classes of GAG-based antiviral agents. In addition to identifying specific GAGs bound by CHIKV, we evaluated strain-specific differences in GAG attachment during infection of cells. Strain differences have been observed in CHIKV tropism and virulence (39, 103, 104). Therefore, it is important to know whether CHIKV strains also differ in attachment factor binding, which often is a determinant of tropism and virulence. Several cell-culture-adapted alphaviruses (105–107), including CHIKV strain 181/25 (38, 39, 66), bind to GAGs. GAG binding was previously thought to be attributable to a cell-culture adaptation that was dispensable for infection by naturally circulating alphavirus strains. However, evidence has accumulated supporting a role for GAG binding by clinically relevant, non-culture-adapted alphaviruses (38, 39, 43–45, 52). Using viruses that were minimally passaged in culture, we discovered that the ECSA strain bound most efficiently to heparin and CS (Fig. 3) and was the only strain that required both HS and CS expression to efficiently bind to U-2 OS cells (Fig. 5C). In contrast, the Asian strain bound less efficiently to GAGs (Fig. 3), and virus binding was least affected by HS cleavage on U-2 OS cells (Fig. 5C) and the absence of HS on Hap1 B3GAT3−/− cells (Fig. 6E). These results parallel the requirement for Mxra8 utilization for infection of fibroblasts in vitro, with Asian and ECSA strains showing full and partial Mxra8-dependence for infection, respectively (31). Similarities between the strains also were observed. All CHIKV strains tested required HS to efficiently bind and infect U-2 OS and Hap1 cells (Fig. 5 and 6). Interestingly, following HS cleavage of U-2 OS cells, residual CHIKV binding (56–77%) and infection (44–66%) was observed (Fig. 5 C and D). However, residual CHIKV binding to (19–26%) and...
infection of (1-9%) Hap1 $B3GAT3^{-/-}$ cells was significantly less (Fig. 6E and F). The low
expression of Mxra8 and CS on Hap1 cells compared to U-2 OS cells may influence the
observed differences in residual binding and infection. These data suggest that although
HS is required for efficient CHIKV binding and infection, the magnitude of the
requirement is inversely correlated to the abundance of entry receptor expression.
Additionally, the residual binding to and infection of Hap1 $B3GAT3^{-/-}$ cells, which
express little to no GAGs or Mxra8, suggests the presence of an unidentified cell-
surface molecule engaged by CHIKV or a route of viral entry other than receptor-
mediated endocytosis.

Our studies contribute to an understanding of the interactions between CHIKV
and the cell-surface molecules that promote virus attachment. We have identified
specific GAG types to which CHIKV binds as well as differences in the binding efficiency
of CHIKV to specific GAGs. Using clinically relevant CHIKV strains, we discovered
strain-specific differences in GAG binding and the requirement of GAGs for attachment
and infection of cultured cells. Our data demonstrate that multiple strains of CHIKV bind
HS and CS as attachment factors, likely promoting initial cell attachment and allowing
the virus to concentrate at the cell surface before engaging entry receptors. CHIKV
interactions with widely expressed GAGs may contribute to the broad cell, tissue, and
species tropism observed for CHIKV. Overall, findings reported here define critical
interactions between CHIKV and GAG attachment factors and improve understanding of
the multistep process of cell attachment for CHIKV.
MATERIALS AND METHODS

Cells. Baby hamster kidney cells (BHK-21; ATCC CCL-10) were maintained in alpha minimal essential medium (αMEM; Gibco) supplemented to contain 10% fetal bovine serum (FBS; VWR) and 10% tryptose phosphate (Sigma). Vero 81 cells (ATCC CCL-81) were maintained in αMEM supplemented to contain 5% FBS. Human osteosarcoma cells (U-2 OS; ATCC HTB-96) were maintained in McCoy’s 5A medium (Gibco) supplemented to contain 10% FBS. Culture media for BHK-21, Vero-81, and U-2 OS cells also were supplemented with 0.29 mg/mL L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 25 ng/mL amphotericin B (Sigma). WT and B3GAT3-/- human Hap1 cells (41) were provided by Yusuke Maeda (Osaka University) and Atsushi Tanaka (Thailand-Japan RCC-ERI). Hap1 cells were maintained in Iscove’s modified Dulbecco’s medium (Gibco) supplemented to contain 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were cultivated at 37°C in an atmosphere of 5% CO2.

VLPs and viruses. Chikungunya VLPs of the 37997 strain were prepared by Emergent BioSolutions as described (108). Suspension-adapted, serum-free human embryonic kidney 293 cells were transfected with an expression plasmid containing strain 37997 structural genes. Supernatants were collected and clarified by centrifugation. VLPs were purified using chromatography and sterile filtration, suspended in 10 mM potassium phosphate, 218 mM sucrose, and 25 mM sodium citrate, and stored at -80°C prior to use. Virus stocks were recovered from infectious cDNA clone plasmids for each CHIKV strain (Table 1), including 181/25 (67, 68), SL15649 (29), H20235 (75), and
Plasmids were linearized with NotI-HF (NEB) and transcribed in vitro using an mMessage mMachine SP6 transcription kit (Ambion). BHK-21 cells (1.19 x 10^7 cells) were electroporated with in vitro transcribed RNA using a Gene Pulser Xcell electroporator (Bio-Rad) and the square wave protocol with 2 pulses at 1000 V for 2.5 ms and 5 s between each pulse. Cells were incubated at 37°C for 48 h. Supernatants were collected and clarified by centrifugation at 1,500 x g at 4°C for 10 min to remove cell debris. Remaining supernatant was added to a 20% sucrose cushion in TNE buffer (PBS^- supplemented to contain 50 mM Tris-HCl pH 7.2, 0.1 M NaCl, and 1 mM EDTA) and centrifuged at ~ 115,000 x g for ~ 16 h in a Beckman 32Ti rotor. Pellets containing virus were resuspended in virus dilution buffer (VDB; RPMI medium supplemented to contain 20 mM HEPES [Gibco] and 1% FBS), aliquoted, and stored at -80°C. Titers of virus stocks were determined by plaque assay. Genome copy numbers of virus stocks were determined by RT-qPCR.

Viral plaque assays. Confluent monolayers of Vero-81 cells were adsorbed with serial dilutions (10-fold) of virus stocks in VDB at 37°C for 1 h. Cells were overlaid with 0.5% immunodiffusion agarose (VWR) in αMEM supplemented to contain 10% FBS, 10% tryptose phosphate, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C for ~ 48 h. Plaques were visualized following staining with neutral red (Sigma) at 37°C for 4 to 6 h. Plaques were enumerated in duplicate and averaged to calculate plaque forming units (PFU).

Viral RT-qPCR. Viral RNA was extracted from 10 μl of purified virus stocks using 490 μl TRIzol reagent (Thermo Fisher Scientific), purified using the PureLink RNA Mini Kit (Invitrogen), and eluted into a final volume of 100 μl. Viral genomes were quantified.
using the qScript XLT one-step reverse transcription-quantitative PCR (RT-qPCR) ToughMix kit (Quanta Biosciences). Reactions were conducted in 20 μl, containing 5 μl viral RNA, 500 nM forward primer (5'-AGACCAGTCGACGTGTTGTAC-3'), 500 nM reverse primer (5'- GTGCGCATTTTGCCTTCGTA-3'), and 250 nM fluorogenic probe (5'-/56-FAM/ATCTGCACC/ZEN/CAAGTGATCCA/3IABkFQ/-3'), targeting an amplicon in the nonstructural protein 2 (nsp2) coding region. Standard curves for each virus strain were prepared using in vitro transcribed viral RNA. RT-qPCR was conducted using a ViiA 7 Real-Time PCR system (Thermo Fisher Scientific) under the following conditions: 50°C for 10 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s, with data acquisition in the FAM channel during the 60°C step. RNA concentrations were determined by comparing the C_T values of each sample to an appropriate standard curve. RT-qPCR to determine genome copy numbers of virus stocks (genomes per mL) were conducted in triplicate.

**Glycan microarrays.** The binding specificities of the chikungunya VLPs were analyzed using a neoglycolipid (NGL)-based microarray system (109). Two types of microarrays were used: (1) glycan microarrays composed of 672 sequence-defined lipid-linked mammalian and non-mammalian glycans as described (110) and (2) GAG-focused microarrays composed of NGL probes of 13 sized-defined glycosaminoglycan (GAG) oligosaccharides and two non-GAG polysaccharide controls. The glycan probes and sequences used in the glycan microarrays are provided in Table S1. The glycan probes and sequences used in the GAG-focused arrays are provided in Figure 2B. Information about the preparation of the glycan probes and construction of the microarrays is presented in Table S3 in accordance with the MIRAGE (Minimum Information for Scientific Articles in Glycoscience) guidelines.
Multiple analyses were conducted with the chikungunya VLPs and anti-CHIKV antibodies (Table S3). Slides were blocked at room temperature (RT) for 1 h with HBS buffer (10 mM HEPES at pH 7.4 with 150 mM NaCl and 5mM CaCl₂) supplemented to contain 0.02% (w/v) casein (Pierce) and 1% (w/v) BSA (Sigma). Microarrays were overlaid with VLP solution (50 μg/ml used in most analyses) at 4°C for 1.5 h and fixed with 4% paraformaldehyde (PFA) diluted in HPLC-grade water at 4°C for 30 min. VLP binding was detected following incubation with anti-CHIKV E2 antibody (CHK-152 (112); 1:300) or ascites fluid (ATCC VR-1241AF; 1:300) at RT for 1 h, biotinylated goat anti-mouse IgG (Sigma; 2 μg/ml) at RT for 1 h, and Alexa Fluor 647-labelled streptavidin (Molecular Probes; 1 μg/ml) at RT for 30 min. Imaging and data analysis are described in the supplementary MIRAGE document (Table S3).

**ELISAs and RBS calculations.** Pierce NeutrAvidin-coated ELISA plates (Thermo Fisher Scientific 15123B) were adsorbed with 4 ng/μl of heparin conjugated to biotin (Creative PEGWorks HP-207) or 15 ng/μl of chondroitin sulfate conjugated to biotin (Creative PEGWorks CS-106, mixture of CS-A, CS-B, and CS-C) at RT for 2 h. Wells were washed three times with wash buffer (PBS⁻ supplemented to contain 0.05% Tween 20). ELISA plates were adsorbed with serial dilutions (1:2) of virus in VDB at RT for 1 h. As a negative control, PBS was adsorbed to ELISA plates coated with heparin and CS. Wells were washed with wash buffer three times to remove unbound virus. Bound virus was detected following incubation with a mouse monoclonal anti-CHIKV E2 antibody (CHK-187 (112); 1:1000) at RT for 1 h, a horseradish peroxidase-conjugated
goat anti-mouse Ig (SouthernBiotech 2040-05) at RT for 1 h, and TMB substrate (Thermo Fisher Scientific) for up to 5 min. Absorbance at 450 nm was quantified using a Synergy H1 microplate reader (BioTek). Data were used to prepare a non-linear regression curve assuming one-site specific binding, and relative binding strength (RBS) values were calculated for each virus. RBS values refer to the concentration in genomes/mL of virus at which 50% of GAG-binding sites are occupied.

**Cell-surface glycan and protein expression.** Cells were detached from tissue-culture flasks using CellStripper Dissociation Reagent (Corning), quenched with PBS+ supplemented to contain 2% FBS, and centrifuged at 1500 x g at 4°C for 5 min. Cells (5 x 10^5 cells per sample) were stained with human anti-HS (1:750; Amsbio 370255-S), human anti-CS (1:750; Sigma C8035), human anti-Mxra8 (1 μg/mL; MBL International W040-3), or mouse anti-Mxra8 (1 μg/mL; 4E7.D10 (31)) antibodies at 4°C for 1 h. Cells were incubated with Alexa Fluor 647 antibody (1:1000; Thermo Fischer Scientific) at 4°C for 1 h. Samples were washed twice with VDB between incubations. Samples were fixed with 1% PFA at 4°C for 5 min and analyzed by flow cytometry (LSRII flow cytometer; BD Biosciences). Binding events were gated using secondary antibody-only control samples as the no-binding controls, and median fluorescent intensity (MFI) was determined using FlowJo V10 software.

**Virus binding to cells.** Cells were detached from tissue-culture flasks using CellStripper Dissociation Reagent, quenched with PBS+ supplemented to contain 2% FBS, and centrifuged at 1500 x g at 4°C for 5 min. Cells (5 x 10^5 cells per sample) were adsorbed with virus at 10^8 genomes per sample at 4°C for 2 h and washed three times with VDB. Cells were centrifuged at 1500 x g for 5 min, and pellets were resuspended in
750 μl of TRIzol. RNA was purified, and viral genomes per sample were quantified using RT-qPCR.

**Focus-forming unit (FFU) assays.** Virus was adsorbed to monolayers of U-2 OS or Hap1 cells at the MOIs indicated in the figure legends. Following incubation at 37°C for 1 h, the inoculum was removed, and cells were incubated at 37°C for 18 h in medium supplemented to contain 20 mM NH₄Cl. Cells were fixed with ice-cold methanol for 30 min and washed three times with PBS⁺. Blocking buffer (PBS⁺ supplemented to contain 5% FBS and 0.1% TX-100) was added to the plate at RT for 1 h. Cells were stained with anti-CHIKV ascites fluid (1:1500; ATCC VR-1241AF) at RT for 1 h and goat anti-mouse Alexa Fluor 488 IgG (1:1000; Invitrogen A11029) with 4′,6-diamidino-2-phenylindole (DAPI; 1:1000; Thermo Fisher Scientific) at RT for 1 h. Cells were washed with PBS⁻ three times at RT for 5 min per wash between each staining step. Infectivity was quantified by indirect immunofluorescence using the Lionheart FX automated microscope and Gen5 software (BioTek).

**GAG cleavage assays.** U-2 OS cells were adsorbed with heparinas (HSase I, II, III; Sigma H2519, H6512, H8891, respectively) or chondroitinas (CSase ABC; Sigma C3667) at a final concentration of 2 mIU/mL diluted in digestion buffer (MilliQ water supplemented to contain 20 mM HEPES pH 7.5, 150 mM NaCl, 4 mM CaCl₂, and 0.1% BSA) at 37°C for 1 h. Cells were washed with PBS⁺ three times. Cell-surface GAG expression was quantified by flow cytometry, virus binding by RT-qPCR, and virus infectivity by FFU.

**Transient complementation of KO cells.** Hap1 B3GAT3⁻/⁻ cells were transfected with pcDNA3.1(+)−N-eGFP containing human B3GAT3 (GenScript).
OHu2110C) using Lipofectamine 3000 (Thermo Fisher Scientific L3000015) at a 3:1 transfection reagent to DNA ratio. Medium was changed at 24 h post-transfection. At 36 h post-transfection, cell-surface GAG expression was quantified by flow cytometry, virus binding by RT-qPCR, and virus infectivity by FFU assay.

**Statistical analysis.** Statistical tests were conducted using GraphPad PRISM 7 software. \( P \) values less than 0.05 were considered to be statistically significant. Descriptions of the specific statistical tests are provided in figure legends.

**Biosafety.** All studies using VLPs were conducted using biosafety level 2 conditions, and all studies using viable virus were conducted in a certified biosafety level 3 facility. Protocols used were approved by the University of Pittsburgh Department of Environment, Health, and Safety and the University of Pittsburgh Institutional Biosafety Committee.
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AUTHOR CONTRIBUTIONS

N.M., Y.L., L.M.S., and A.J.L. designed and conducted experiments, analyzed results, and wrote the manuscript. N.W. and K.A.G. designed and conducted experiments and analyzed results. K.R. analyzed results. L.V., J.A., and K.L.W. provided crucial reagents and reviewed the manuscript. W.C. and M.S.D. designed experiments, provided crucial reagents, and reviewed the manuscript. T.F., L.A.S., and T.S.D designed experiments, analyzed results, and wrote the manuscript.
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**Figure 1. Chikungunya VLPs bind specifically to GAGs.** A glycan microarray composed of 672 lipid-linked glycan probes was incubated with purified chikungunya virus-like particles (VLPs, 50 µg/mL). Bound VLPs were fixed with 4% PFA and detected using anti-CHIKV E2-specific monoclonal antibody (CHK-152), followed by biotin-conjugated IgG and streptavidin-conjugated Alexa Fluor 647. VLP-glycan binding is reported as the mean fluorescence intensity of duplicate spots of each lipid-linked glycan probe printed at 5 fmol. The glycan groups tested are arranged according to their backbone sequences as annotated. The glycans tested, probe sequences, and binding intensities are listed in Table S1. Binding data shown are representative of two independent experiments. Error bars represent half of the difference between the two values.

**Figure 2. Chikungunya VLPs bind to longer, sulfated iduronic acid-containing GAGs with a preference for heparin and HS.** (A and B) A GAG-focused microarray composed of GAGs differing in length (indicated by #-mer) and sulfation was incubated with chikungunya VLPs. Dextran and dextran sulfate, non-GAG glycans, also were included in the array to assess sulfation requirements for binding. (A) Chikungunya VLPs were incubated on the microarray. Bound VLPs were fixed with 4% PFA and detected using either anti-CHIKV E2-specific monoclonal antibody (CHK-152) or anti-CHIKV ascites fluid, followed by biotin-conjugated IgG and streptavidin-conjugated Alexa Fluor 647. VLP-glycan binding is normalized to heparin 14-mer fluorescence.
intensity signals. Fluorescence intensity was determined from duplicate spots of each glycan probe printed at 5 fmol for GAG NGL probes and 0.1 ng for dextran and dextran sulfate. Binding data shown are an average of five independent experiments, except for results with HS, which are from three independent experiments. Error bars indicate standard error of the mean (SEM). P values were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (*, P < 0.05; ***, P < 0.001; ****, P < 0.0001). Statistics presented within the graph only indicate statistical significance between samples of each glycan type. (B) The backbone sequences for each glycan probe used on the microarray are listed. GlcNAc, N-acetyl glucosamine; GlcNH₂, glucosamine; GlcA, glucuronic acid; IdoA, iduronic acid; GalNAc, N-acetyl galactosamine; dUA, 4,5-unsaturated hexuronic acid; ManA, 2,5-anhydro-mannose; DH and AO, lipid moieties of NGLs prepared by reductive amination and oxime ligation, respectively. Further details are in Table S2 and S3.

Figure 3. CHIKV strains bind directly to heparin and chondroitin sulfate. (A and B) Serial dilutions of each CHIKV strain, quantified by genome number, were adsorbed to wells of avidin-coated ELISA plates bound with (A) biotinylated heparin or (B) biotinylated CS. (A and B) PBS was adsorbed to wells coated with heparin and CS as a negative control. Following washes to remove unbound virus, virus binding was detected using mouse monoclonal anti-CHIKV E2 antibody (CHK-187), secondary goat anti-mouse HRP-conjugated antibody, and TMB substrate. Absorbance was measured at 450 nm for duplicate wells from three independent experiments. The dotted line
indicates background levels of binding, as determined using PBS control wells. Error bars indicate SEM. Data were fit using a non-linear regression curve.

Figure 4. U-2 OS cells express relatively high levels of HS, CS, and Mxra8. (A-D)

U-2 OS, 3T3, Vero, and BHK cells were stained with antibodies specific for HS, CS, or Mxra8, followed by Alexa-647 antibody. Cells were fixed with 4% PFA, and median fluorescence intensity (MFI) was quantified using flow cytometry. (A and B) Representative flow cytometric plots and (C and D) quantification of GAG and Mxra8 profiles for triplicate wells from three independent experiments are shown. Data were normalized to secondary-antibody-only negative controls. Error bars indicate SEM. \( P \)

values were determined by two-way ANOVA followed by Tukey’s multiple comparison test (**, \( P < 0.01; ****, P < 0.0001 \)).

Figure 5. Enzymatic cleavage of HS reduces binding and infection of CHIKV. (A-D)

U-2 OS cells were treated with a combination of heparinases (HSase I/II/III) or chondroitin sulfatases (CSase ABC) at a final concentration of 2 mIU/mL. (A and B) Cells were stained with antibodies specific for HS or CS, followed by Alexa-647 antibody. Cells were fixed with 4% PFA, and MFI was quantified using flow cytometry. (A) Representative flow cytometric plots and (B) quantification of GAG profiles for duplicate wells from three independent experiments. Data were normalized to secondary-antibody-only negative controls. (C) U-2 OS cells were adsorbed with \( 10^8 \) genomes/sample of the CHIKV strains shown at 4°C for 2 h and washed three times to remove unbound virus. Total RNA was purified using TRIzol, and CHIKV RNA was
quantified using RT-qPCR. (D) U-2 OS cells were adsorbed with the attenuated CHIKV strain (181/25) at an MOI of 1 PFU/cell and the ECSA (SL15649), Asian (H20235), and W. African (37997) strains at an MOI of 5 PFU/cell. Cells were fixed with methanol at 18 h post-adsorption, and the percentage of infected cells was determined using an immunofluorescence FFU assay. (C and D) Data were normalized to untreated controls. Results are expressed as (C) mean percentage of binding in triplicate wells from three independent experiments and (D) mean percentage of infected cells for four fields of view per well in triplicate wells from three independent experiments. (B-D) Error bars indicate SEM. P values were determined by (B) two-way ANOVA followed by Tukey’s multiple comparison test or (C and D) one-way ANOVA followed by Tukey’s multiple comparison test (*, P < 0.05; ***, P < 0.001; ****, P < 0.0001).

Figure 6. Genetic disruption of GAG biosynthesis reduces CHIKV binding and infection. (A-D) WT, B3GAT3−/−, and complemented B3GAT3−/− Hap1 cells were stained with antibodies specific for HS, CS, or Mxra8, followed by Alexa-647 antibody. Cells were fixed with 4% PFA, and MFI was quantified using flow cytometry. (A and B) Representative flow cytometric plots and (C and D) quantification of GAG and Mxra8 profiles for triplicate wells from three independent experiments. Data were normalized to secondary-antibody-only negative controls. (E) WT, B3GAT3−/−, and complemented B3GAT3−/− Hap1 cells were adsorbed with 10^8 genomes/sample of the virus strains shown at 4°C for 2 h and washed three times to remove unbound virus. Total RNA was purified using TRIzol, and CHIKV RNA was quantified using RT-qPCR. (F) WT, B3GAT3−/−, and complemented B3GAT3−/− Hap1 cells were adsorbed with the attenuated
CHIKV strain (181/25) at an MOI of 2.5 PFU/cell and the ECSA (SL15649), Asian (H20235), and W. African (37997) strains at an MOI of 10 PFU/cell. Cells were fixed with methanol at 18 hpi, and the percentage of infected cells was determined using an immunofluorescence FFU assay. (E and F) Data were normalized to WT cells. Results are expressed as (E) mean percentage of binding in triplicate wells from three independent experiments and (F) mean percentage of infected cells for four fields of view per well in triplicate wells from two independent experiments. (C-F) Error bars indicate SEM. *P* values were determined by (C) two-way ANOVA followed by Tukey’s multiple comparison test, (D) two-tailed Student’s *t* test, or (E and F) one-way ANOVA followed by Tukey’s multiple comparison test (*, *P* < 0.05; ***, *P* < 0.001; ****, *P* < 0.0001).
Chikungunya VLP binding (fluorescence intensity)

Array position of glycan probe

- Miscellaneous
- Homo-oligomers
- GAGs
- Polysialyl
- O-glycans
- Gangliosides
- N-glycans
- PolyLacNAc li-active
- Lacto-N-neotetraose & Lacto-N-tetraose-based
- Lactose and N-acetyllactosamine-based
A

Chikungunya VLP binding (%)
(normalized fluorescence intensity)

| Dextran sulfate | Dextran 12-mer | 6-mer | 8-mer | 10-mer | 14-mer |
|----------------|---------------|-------|-------|--------|--------|
|                |               |       |       |        |        |

GAG chain length

Non-GAGs
Hyaluronan
HS
Heparin
CS-A
CS-B (DS)
CS-C
KS

B

| Position | Probe        | Sequence |
|----------|--------------|----------|
| 1        | Dextran Sulfate | Glucose residues are variously sulfated at positions 2,3 or 4 |
| 2        | Dextran       |          |
| 3        | HA 12-mer     |          |
| 4        | HS S5 mer     | May contain small amounts of GlcNS(6S) and IdoA(2S); some GlcNAc may be non-sulfated |
| 5        | HS S8 mer     | May contain small amounts of GlcNS(6S) and IdoA(2S); some GlcNAc may be non-sulfated |
| 6        | Hep 10-mer    |          |
| 7        | Hep 14-mer    |          |
| 8        | CS-A 6-mer    |          |
| 9        | CS-A 14-mer   |          |
| 10       | CS-B 6-mer    |          |
| 11       | CS-B 14-mer   |          |
| 12       | CS-C 6-mer    |          |
| 13       | CS-C 14-mer   |          |
| 14       | KS 6-mer      | Variously 6S on Gal and GlcNAc |
| 15       | KS 14-mer     | Variously 6S on Gal and GlcNAc |
**A** Heparin-ELISA

**B** CS-ELISA

- **Genomes/sample**
- **Virus binding (O.D. 450)**
  - Attenuated
  - ECSA
  - Asian
  - W. African
  - PBS

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**Figure Legend:**

- **A** Heparin-ELISA
- **B** CS-ELISA

- **Genomes/sample**
- **Virus binding (O.D. 450)**
  - Attenuated
  - ECSA
  - Asian
  - W. African
  - PBS

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**Notes:**

- The graphs show the virus binding levels at different genome concentrations for various strains.
- The x-axis represents the number of genomes/sample, while the y-axis shows the virus binding (O.D. 450).
- Each strain has a distinct line color and marker type.
- The PBS line indicates the baseline binding value.
| Clade   | Strain    | Isolation                        | Passage history                      |
|---------|-----------|----------------------------------|--------------------------------------|
| Asian   | Attenuated 181/25 | Tissue culture passage of AF15561 strain | 11 passages in GMK cells  
18 passages in MRC-5 cells |
| ECSA    | Sri Lanka SL15649 | Human patient in Sri Lanka (2006) | 3 passages in Vero cells |
| Asian   | Caribbean H20235 | Human patient in St. Martin (2013) | 3 passages in Vero cells |
| W. African | Senegal 37997 | Mosquito in Senegal (1983) | 1 passage in AP-61 cells  
2 passages in Vero cells |
| Virus       | Heparin RBS* | Heparin - 95% CI | CS RBS* | CS - 95% CI |
|------------|--------------|------------------|---------|-------------|
| Attenuated | 7.9 x 10^6   | 6.13 x 10^6 – 1.0 x 10^7 | ND      | ND          |
| ECSA       | 1.8 x 10^7   | 1.3 x 10^7 – 2.5 x 10^7 | 1.4 x 10^7 | 8.8 x 10^6 – 2.4 x 10^7 |
| Asian      | 2.0 x 10^7   | 1.4 x 10^7 – 2.9 x 10^7 | 2.0 x 10^6 | 5.9 x 10^5 – 4.4 x 10^6 |
| W. African | 3.6 x 10^7   | 2.2 x 10^7 – 5.9 x 10^7 | 1.0 x 10^7 | 4.3 x 10^6 – 2.5 x 10^7 |

*RBS values represent the concentration of virus (genomes/mL) at which 50% of GAG-binding sites are occupied.