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REVIEW

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Glycosaminoglycan binding by arboviruses: a cautionary tale

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Abstract

Arboviruses are medically important arthropod-borne viruses that cause a range of diseases in humans from febrile illness to arthritis, encephalitis and hemorrhagic fever. Given their transmission cycles, these viruses face the challenge of replicating in evolutionarily divergent organisms that can include ticks, flies, mosquitoes, birds, rodents, reptiles and primates. Furthermore, their cell attachment receptor utilization may be affected by the opposing needs for generating high and sustained serum viremia in vertebrates such that virus particles are efficiently collected during a hematophagous arthropod blood meal but they must also bind sufficiently to cellular structures on divergent organisms such that productive infection can be initiated and viremia generated. Sulfated polysaccharides of the glycosaminoglycan (GAG) groups, primarily heparan sulfate (HS), have been identified as cell attachment moieties for many arboviruses. Original identification of GAG binding as a phenotype of arboviruses appeared to involve this attribute arising solely as a consequence of adaptation of virus isolates to growth in cell culture. However, more recently, naturally circulating strains of at least one arbovirus, eastern equine encephalitis, have been shown to bind HS efficiently and the GAG binding phenotype continues to be associated with arbovirus infection in published studies. If GAGs are attachment receptors for many naturally circulating arboviruses, this could lead to development of broad-spectrum antiviral therapies through blocking of the virus–GAG interaction. This review summarizes the available data for GAG/HS binding as a phenotype of naturally circulating arbovirus strains emphasizing the importance of avoiding tissue culture amplification and artifactual phenotypes during their isolation.

INTRODUCTION

Arbovirus attachment receptor utilization

Arboviruses are viruses that infect and replicate in the cells of hematophagous arthropod vectors (e.g. ticks, flies, mosquitoes) and are spread to vertebrates during blood meals. Clinically important members of these viruses are found in the *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Arenaviridae* and *Reoviridae* families of RNA viruses and the *Asfarviridae* family of DNA viruses (classical swine fever virus only). A subset of vertebrate species serves as reservoirs for arboviruses that include reptilian, amphibian, avian and mammalian members. These species can become infected by virus present in the arthropod vector's saliva and generate sufficient serum viremia for acquisition of the virus by another blood-feeding arthropod, thus perpetuating the transmission cycle [1]. In addition to arthropod vector feeding preferences, the balance between high and sustained viremia levels and severity of viral illness is considered critical for the functioning of a particular species as an efficient reservoir host [1, 2]. Therefore, the ability of an arbovirus to generate sufficient viremia to achieve vector infection is an important aspect of its sylvatic transmission cycle. Given the evolutionary diversity of hosts in which these viruses replicate, two predominant theories have been promoted regarding the nature of cell attachment structures to which they bind to initiate infection: (i) arboviruses bind to highly evolutionarily conserved structures in multiple hosts; or (ii) arboviruses utilize different attachment receptors in different hosts and possibly, on different cell types within a single host. Interestingly, the identification of arbovirus attachment receptors has generally lagged behind those for non-arboviruses, even for closely related viruses within the same family [1]. Although, more recently, advanced techniques for genetic manipulation of protein expression in cells have greatly accelerated the discovery of arbovirus cell attachment/entry factors [3–13].

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Abbreviations: CHIKV, Chikungunya virus; CSFV, classical swine fever virus; DENV, dengue virus; DLN, draining lymph node; EEEV, eastern equine encephalitis virus; GAG, glycosaminoglycan; HS, heparan sulfate; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; RRV, Ross River virus; sc, subcutaneous; SINV, Sindbis virus; TBEV, tick-borne encephalitis virus; VEEV, Venezuelan equine encephalitis virus; WEEV, western equine encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.

As might be expected, the results of these studies have indicated that both of the above theories are partially correct: receptor utilization by arboviruses may simultaneously be highly divergent in different hosts and/or tissues but also involves evolutionarily conserved structures. For example, flaviviruses, alphaviruses and bunyaviruses can bind to C-type lectin receptors on specialized cells through lectin binding to conserved carbohydrate modifications of the viral glycoproteins [3, 4, 14, 15]. Studies also have been published providing evidence for infection dependence or binding of alphaviruses [Sindbis virus (SINV) [16] and Venezuelan equine encephalitis virus (VEEV) [17]], flaviviruses [Dengue virus (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV) [18–23]] and classical swine fever virus (CSFV) [24] through the high-affinity laminin receptor (HALR), which is a highly conserved ribosomal and cell surface protein [25, 26].

Since the 1990s, many publications have implicated host-expressed and highly conserved sulfated glycosaminoglycans (GAGs) as universal attachment factors for viruses from each of the arbovirus families (Table 1) (earliest reports for each family [27–32]). Clearly, identification of a common cell surface molecule that is conserved between reservoir, arthropod and dead-end hosts and utilized by many arboviruses could lead to development of broad-spectrum antiviral therapeutics that interrupt the infection process. Such drugs could significantly improve treatment of these infections, which plague millions of humans each year. However, in the case of GAG binding, careful attention must be paid to the problem of acquisition or enhancement of this phenotype as an artifact of conventional *in vitro* passage of virus isolates and the relationship of laboratory arbovirus strains to those circulating in nature. The subject of this review is a consideration of existing literature on the interactions of arbovirus strains with GAG molecules as attachment receptors, focusing initially on differences and similarities between GAG interaction studies *in vitro* with different arbovirus types and subsequently summarizing the evidence for these molecules functioning as receptors in the natural arbovirus transmission cycle.

Glycosaminoglycan structure and binding by microbial pathogens

Many reports have been published that demonstrate a role for GAGs, particularly heparan sulfate (HS) for *in vitro* cellular attachment of both DNA and RNA viruses (reviewed in [33–35]). GAGs are long chain polymers of hexuronic acid (D-glucuronic acid or L-iduronic acid) and D-glucosamine or D-galactosamine heterodimers with the specific heterodimer distinguishing the different GAG groups, of which there are four [36] (Fig. 1). As a post-translational modification, these chains are variously sulfated at the 3-O and 6-O positions leading to a net negative charge on the molecule (reviewed in [37]). GAG chains are displayed as post-translational modifications of cell membrane-bound (integral membrane or glycophosphatidyl inositol-linked) or secreted core proteins resulting in their abundant expression on most cell-surfaces and in the extracellular matrix as proteoglycans [38–40]. Although specific patterns of GAG sulfation can vary between tissues and hosts, GAG primary structures are highly conserved between diverse organisms, being found in invertebrate and vertebrate animals and plants [37].

The interaction of protein ligands with GAGs plays a role in cell adhesion, tissue structure, anticoagulation, sequestration and concentration of cell signaling factors, regulation of transcription factor activity, and, particularly with HS proteoglycans, *in vitro* infection of cells by a number of bacterial, protozoan and viral pathogens including representatives of essentially all arboviruses (reviewed in [33, 36, 41–43]). In most instances, it is unclear whether or not the proteoglycan molecule can serve as a stand-alone entry receptor or if it acts as a pathogen concentration factor, handing organisms off to other structures that directly promote infection (Fig. 2). This latter model was proposed originally by Spear and colleagues in their pioneering work on HS utilization by herpesviruses (reviewed in [44]).

Positively-charged amino acids (especially Lys and Arg) either in linear HS binding consensus sequences [45–47] or in conformationally-determined three-dimensional binding sites [45, 48] on protein ligands are presumed to interact with sulfated and carboxylated components of HS chains (reviewed in [49]). While originally thought to be relatively non-specific ionic interactions, more recent work has revealed a great deal of specificity in the interaction of specific protein ligands with particular arrays of carboxyl and sulfate moieties on GAGs (reviewed in [37, 50]), although, this aspect of the arbovirus–GAG interaction remains relatively unexplored. With arboviruses, the capability to bind to HS or GAG receptors is associated with positively charged amino acids in the virus receptor attachment proteins (E2 with alphaviruses; E with flaviviruses, G with rhabdoviruses, G_N/G_C with bunyaviruses and E2 or envelope E^{rns} with CSFV) [19, 49, 51–57], Due to the fact that alphaviruses and flaviviruses were the first arboviruses shown to interact with HS/GAGs [31, 52, 58, 59] and they are the most studied with respect to this phenotype, they will be the subject of the majority of this review. Occasionally, references to non-arboviruses are included when they support conclusions drawn from studies of arboviruses.

Alphaviruses are mosquito-borne arboviruses that cause two major types of human disease. The so-called Old World alphaviruses [e.g. Sindbis (SINV), Chikungunya (CHIKV), and Ross River viruses (RRV)] cause arthritis and arthralgia while the New World alphaviruses [e.g. eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV)] cause encephalitis [1]. Laboratory strains within each group of alphaviruses have been shown to interact with GAG/HS receptors on cells *in vitro* [52, 53, 57, 60, 61]. However, with the exception of EEEV, for which the attachment protein sequences of cDNA cloned viruses have been compared with those of unpassaged field samples, these phenotypes have not been directly confirmed as reflective of circulating viruses [62]. Many studies identifying GAGs as attachment receptors have also been

Table 1. Arboviruses examined for heparan sulfate binding and attachment protein residues implicated in the phenotype

Arbovirus family (genus)	Type	Arbovirus strains tested for GAG interactions* (validated wild type residues involved in GAG interactions)†	Cell amplification mutations that increase GAG interactions‡	Reference
Togaviridae (Alphavirus)				
	SINV	AR339	–	[31]
		TR339	–	[52]
		Toto1101	E2-E70K	[52]
		AR339 cell passaged	E2-S1R E2-E70K E2-S114R	[52]
	SFV	pSFV-4	–	[53]
	RRV	RRV64	–	[57]
		RRV64 cell passaged	E2-N218R	[57]
	CHIKV	L Reunion	–	[75]
		SL15629 H20235 37997	–	[61]
		181/25 Vaccine	E2-G82R	[60]
		La Reunion cell passaged	E2-G55R E2-E79K E2-S159R E2-E166Δ E2-E166K E2-E168K	[75]
	VEEV	Trinidad Donkey	–	[54]
		TC83 Vaccine	E2-E120R	[54]
		Trinidad Donkey cell passaged	E2-E3K E2-E4K E2-E76K E2-E116K E2-E209K	[54]
	EEEV	FL93-939 (E2-K71) (E2-K74) (E2-K77) (E2-R84) (E2-R119) (E2-K156) (E2-R157)	–	[62,106]
	EEEV	Various isolates§ (E2-K71Q) (E2-K71T) (E2-T72K)	–	[49]
	WEEV	McMillan	–	Gardner unpublished observations
		CBA87	–	[62]
	MAYV	Ohio	–	Klimstra unpublished observations
Flaviviridae (Flavivirus)				

Continued

Table 1. Continued

Arbovirus family (genus)	Type	Arbovirus strains tested for GAG interactions* (validated wild type residues involved in GAG interactions)†	Cell amplification mutations that increase GAG interactions‡	Reference
	YFV	Asibi	–	[55]
		17D Vaccine	E-T380R	[55]
		YF5.2iv generated from 17D	E-T380R	[93]
	DENV	DEN2 New Guinea C Cell passaged	E-E126K	[32, 65, 67]
		DEN 2 PUO-218	–	[67]
		Cell passaged DENV3 MG-20 DANV3 PV_BR	E-N124D/E202K E-T120K E-E126K E- E202K – –	[67] [130] [130]
	DENV‖	DEN 1 45AZ5 PDK-27	–	[66]
	DENV‖	DEN 1 TH-Sman	–	[66]
	WNV	NY99	–	[73]
	JEV	RP-9 cell passaged	–	[131]
		RP-2ms cell passaged	–	[131]
	JEV	Nakayama	–	[72]
		Nakayama variants isolated from mice	E- E306K	[72]
	JEV JEV JEV	SA14-14-2 FU cell passaged SA14/CDC cell passaged	E- E138K E-E128K E-E49K E-E138K E-E306K E-D389G	[94] [73] [73]
	MVEV		–	[59]
		MVE-1–51	E-D390V E-D390L E-D390G E-D390H E-D390A	[59]
	TBEV	NeuDoerfl	–	[85,68]
		NeuDoerfl	E-E201K E-E122G E-S158R/G159R	[85, 68]
		Ya10/89 clone 125	E-E122G	[95]
		80 k	E-D67N E-T68A	[95]
	ZIKV	Expressed E glycoprotein	–	[70]
	ZIKV¶	MR766	–	[90]
	ZIKV	MRS OPY		[92]
	ALFV	ALFV3929	E-L327K	[96]
Bunyaviridae			–	

Continued

Table 1. Continued

Arbovirus family (genus)	Type	Arbovirus strains tested for GAG interactions* (validated wild type residues involved in GAG interactions)†	Cell amplification mutations that increase GAG interactions‡	Reference
	RVFV	$RVFV_{ns}$	–	[74]
		MP-12	–	[88]
		ZH-501	–	[88]
		Kenya 9 800 523	–	[88]
		Kenya 2007002444	–	[88]
	TOSCV	ISS.Phi.32	–	[132]
	AKAV	JaGAr-39	–	[29]
		TS-C2	–	[29]
		OBE-1	–	[133]
		Iriki	–	[133]
	SBV	SBV	–	[133]
Arenaviridae				
	JUNV§	?	–	[30]
	TCRV§	?	–	[30]
	LUJV#	VSV-LUJV pseudotype	–	[97]
Reoviridae (Orbivirus)				
	BTV	BTV-10	–	[28]
Asfarviridae				
	CSFV	Brescia	–	[27]
		C	–	[27]
		Brescia cell passaged	E^{RNS}-S476R	[27]

*Different strains with virus types exhibit a wide range of GAG/HS interaction efficiencies. HS dependence is proposed on the basis of the results of *in vitro* infection inhibition assays using soluble heparin or other GAG mimetics or more specific assays.
†Residues in strains validated to reflect the sequence of naturally circulating unpassaged viruses. With EEEV, these comprise three separate HS binding domains.
‡Known mutations in low-passage strains adapted by cell and/or mouse brain passage that bind GAGs with higher efficiency than progenitor strains.
§Alterations in one HS binding domain of EEEV found in different isolates that alter HS interactions and virulence in animals.
||Recombinant subviral particles of high passage clinical samples were shown bind heparin, and heparin and heparan sulfate inhibited binding to cells.
¶ZIKV infectivity was enhanced by heparin interaction.
#Reduction of infection on HAPI cells following heparinase treatment.
SINV, Sindbis virus; SFV, Semliki Forest virus; RRV, Ross River virus; CHIKV, chikungunya virus; VEEV, Venezuelan equine encephalitis virus; EEEV, eastern equine encephalitis virus; WEEV, western equine encephalitis virus; MAYV, Mayaro virus; YFV, yellow fever virus; DENV, dengue virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; TBEV, tick-borne encephalitis virus; ZIKV, Zika virus; RVFV, Rift Valley Fever virus; TOSCV, Toscana virus; AKAV, Akabane virus; SBV, Schmallenberg virus; JUNV, Junin virus; TCRV, Tacaribe virus; LUJV, Lujo virus; BTV, bluetongue virus; CSFV, classical swine fever virus.

published with flaviviruses [e.g. dengue virus (DENV) [32, 63–66], yellow fever virus (YFV) [55, 63], West Nile virus (WNV) [67], Murray Valley encephalitis virus (MVEV)] [67], tick-borne encephalitis virus (TBEV) [68], Japanese encephalitis virus (JEV) [65, 69] and Zika virus (ZIKV) [70]] which, using the nomenclature of the field, also can be broadly segregated into encephalitis-causing (e.g. MVEV, JEV, TBEV, WNV) and viscerotropic (e.g. DENV, YFV, ZIKV) groups [2, 71].

As with most RNA viruses, arboviruses possess an error-prone polymerase and highly adaptable genomes, making use of HS as a receptor controversial, as this phenotype can be rapidly acquired during amplification of virus isolates *in vitro* [48, 52, 53, 72].

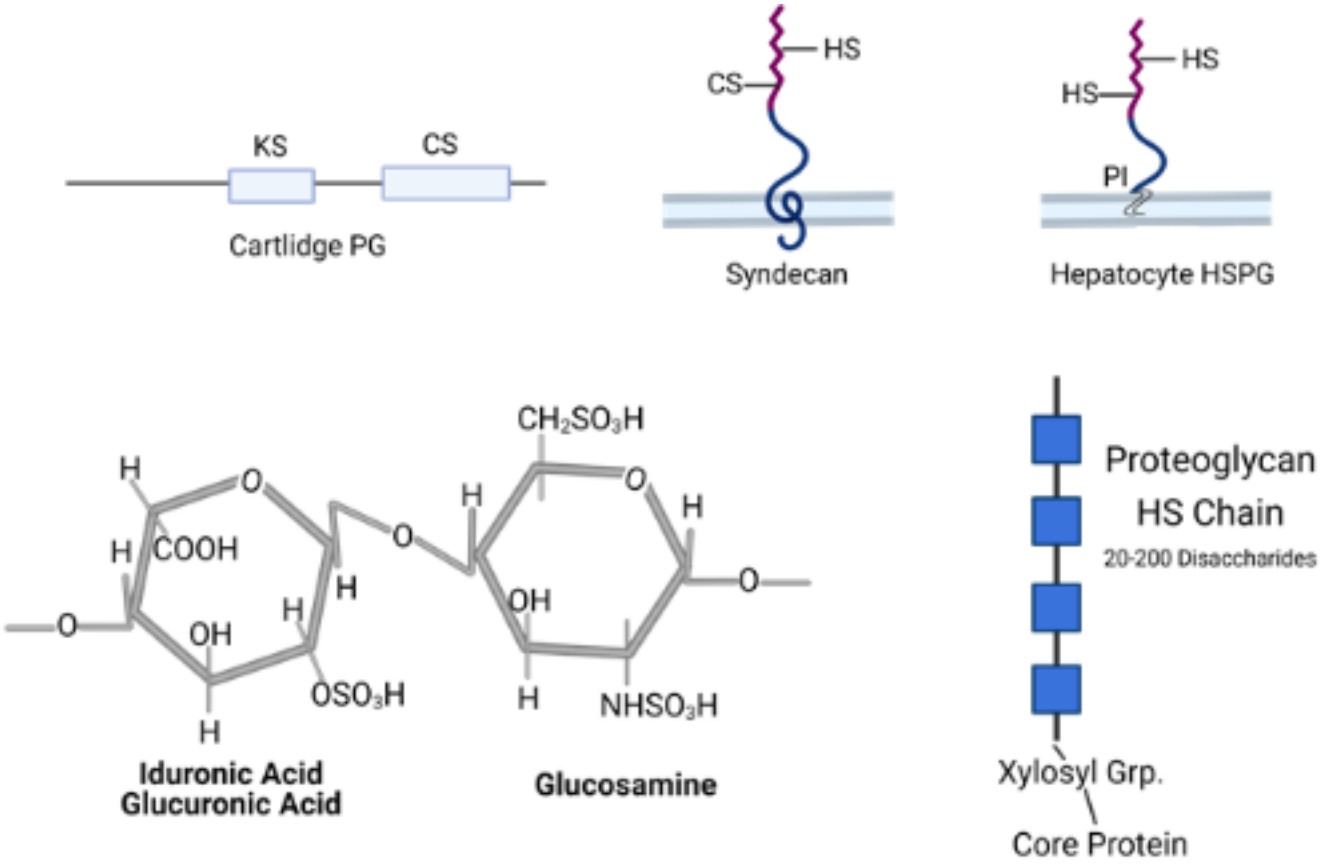

Fig. 1. Top panels: Schematic showing general arrangement GAGs on proteoglycans, including cartlidge PG, syndecan and hepatocyte HSPG. GAGs in proteoglycans can consist of long chains of keratan sulfates (KS), dermatan sulfate (DS), chondroitin sulfates (CS), or heparan sulfates (HS). PI, phosphatidyl inositol linkage to cell membrane in hepatocyte PG. Bottom panels: The core disaccharide unit of HS (sulfonated uronic acid and glucosamines) is shown on the left. On the right is depicted a canonical proteoglycan HS modification showing the disaccharide chains linked with a xylosyl group to a core protein. Sulfonation of HS chains is not uniform, and the blue boxes represent highly sulfated regions of HS chains that may interact strongly with protein ligands. Figure generated using BioRender (www.biorender.com).

Early studies with the alphavirus, SINV, demonstrated that within as few as three high multiplicity passages in cultured fibroblast cells, a positive charge mutation in the E2 protein became predominant in the population that conferred efficient (used as a measure of affinity and/or avidity) HS attachment to a virus that initially did not exhibit significant interaction with HS and, in fact, bound to multiple cell types very poorly (e.g. less than 5% of added radiolabeled virus bound to an excess of cells [52]). An analogous strain has not been available for other arboviruses, although, one study utilized observed HS binding among cloned DENV human clinical isolates which were minimally passaged in cell culture [66]. Other studies of flaviviruses have compared very low passage viruses (e.g. fewer than four) to highly cell-adapted viruses [73] as has been done with CHIKV [61] leading to the conclusion that HS binding can be a phenotype of low-passage strains. Perhaps more definitively, one study with EEEV alphavirus and one with Rift Valley fever bunyavirus have compared the attachment protein sequences found in unpassaged field samples with those of laboratory strains shown to interact with HS during infection [62, 74]. In both cases, the sequence in the unpassaged primary samples was either identical to [62] or did not contain additional positively charged residues [74] with respect to the laboratory strain.

Yet, in cases where it has been examined, efficient binding to HS has been identified as an attenuating factor in live attenuated arbovirus vaccines (LAVs) including YFV 17D, VEEV TC83, CHIKV 181/25, and JEV SA14-14-2 [54, 55, 75–78], all of which were created by blind passage of virulent parental virus stocks in cultured cells. While the passage history and GAG binding phenotype of parental strains from which the LAVs were derived is frequently unclear, the vaccine strains show an increased efficiency of attachment to and dependency of infection on GAGs versus parental strains. Since nearly all studies assessing HS dependence of infection have utilized cell-adapted laboratory strains, and efficient HS binding is rapidly acquired during cell passage, rigorous assessment of the effects of cell passage on virus phenotypes should be applied to both *in vitro* and *in vivo* studies of HS dependence as is discussed below.

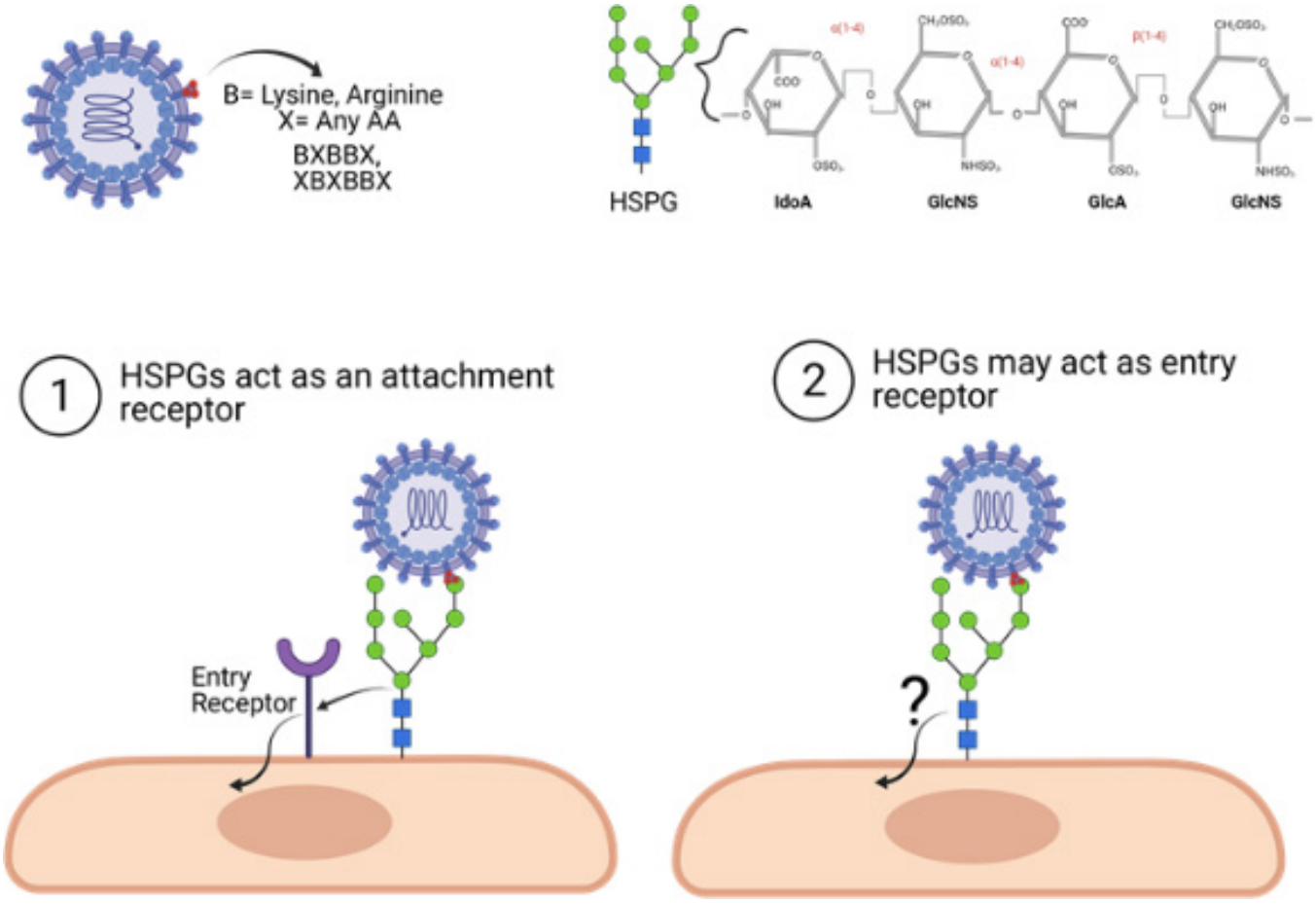

Fig. 2. Positively charged amino acids on the virus surface will interact with the highly sulfated uronic acid and glucosamines on cellular HS proteoglycans (HSPGs), probably via HS binding motifs (indicated in the top panel). These interactions can lead to virus entry by (1) the HSPG acting as an attachment receptor only, potentially by increasing the amount of virus particles interacting with the cellular surface, thus increasing the probability of virus interaction with a proteinaceous entry receptor or leading to recruitment of entry receptors to a site of virus binding. (2) HSPGs may also act as both attachment and entry receptors, promoting entry without the recruitment or interactions of any other receptor. Figure generated using BioRender (www.biorender.com).

Studies of the arbovirus GAG binding phenotype *in vitro*

Traditional techniques for assessment of GAG interactions

Typically, *in vitro* HS dependence is evaluated by examining the effects upon cell infectivity of treatment with high salt (typically NaCl) concentration, which disrupts ionic interactions, direct competition of binding and/or infectivity with soluble heparin, a highly sulfated HS analogue, or other GAG molecules, heparin lyase (heparinase) digestion of cell surface HS structures or lyases specific to other GAG molecules, and comparing relative infectivity for wild type and HS- or GAG-deficient cells (e.g. CHOK1 mutants created by Jeffrey Esko and colleagues [79]) (e.g. [32, 48, 52–54, 57, 58]). In general, a virus for which HS is a cell attachment factor, will exhibit decreased infectivity for cells with all of the treatments with the exception of lyases specific to other GAGs. However, it has been shown with a number of viruses that other GAGs such as chondroitin sulfate or dermatan sulfate may be involved in cell attachment to some degree, with the phenotype varying even between different isolates of a given virus [60, 61, 75, 80]. Furthermore, we have observed significant differences in the susceptibility of genetic variants of a single alphavirus type to the three different heparinases [52, 81], which have specificity for different HS sulfation patterns and structures [82], indicating that there can be divergence in the particular HS structure bound even by very closely related viruses. With different HS binding alphaviruses and flaviviruses, twofold to 100-fold decreases in infection efficiency are typically observed when using these treatments [52, 54, 72, 81], which may reflect underlying efficiency of binding or dependence upon a particular GAG structure for infectivity. The infectivity of most viruses can be affected by one or more of the above experimental treatments, for example SINV strain TR339, derived from a cDNA clone generated from an original 1952 SINV isolate with no history of *in vitro* passage [52] (R. Swanopoel and R.E. Johnston, personal communication), shows infectivity reduction of approximately

10–30% with heparin competition and also significant infectivity reduction with increased ionic strength [52]. However, the virus binds poorly to cells and infectivity is not appreciably reduced when infecting GAG-deficient CHOK1 cells [52]. Therefore, it is possible that other ionic interactions may be affected by some experimental conditions used to identify HS binding. Ultimately the degree to which infection is inhibited by treatments designed to disrupt ionic interactions or specific GAG binding is probably related to the efficiency of GAG binding by the virus and may be a critical factor in assessing the influence of the phenotype on virus transmission and disease processes (e.g, is binding sufficiently strong to significantly alter virus spread, tissue infectivity or other aspects of pathogenesis in animals or replication/transmission efficiency with arthropod vectors?).

Cell binding versus infectivity

It is also highly relevant to *in vitro* analysis of GAG dependence to determine attachment efficiency of virus particles to cells. In general, low-passage alphaviruses bind poorly to many types of cultured cells [52, 81], while cell-adapted viruses can attach with much greater efficiency. Generally, the reductions in cell binding after the above treatments are consistent with reductions in infectivity [52], indicating that greater attachment of arboviruses mediated by HS promotes greater infection. As stated above, whether or not this is direct promotion of entry or concentration of virus particles on the cell surface is unclear and whether or not the phenotype reflects inefficient interaction with cell surfaces versus low receptor abundance is also unclear.

In vitro data generated with some flaviviruses indicate that the interaction with HS may be less efficient than that for alphaviruses (e.g. reductions in infectivity of flaviviruses may be twofold to threefold after ablation of HS structures or with heparin competition) [52, 59, 64, 72, 83], although, it is difficult to determine due to the various methodologies used to examine the phenotype. Binding efficiency differences may be due to the fact that binding sites for HS have been mapped to the disordered and flexible E3 loop region as well as the E1/E2 domain interface or E2 domain of the flavivirus E protein (Table 1, Fig. 3b), whereas, alphavirus HS binding domains are mostly present in highly structured, surface-exposed areas of the analogous E2 glycoprotein (Table 1, Fig. 3a). It is possible that the repeating structure of the alphavirus trimeric spike within which stable GAG interaction sites are positioned (resulting in 80 copies of stable E2 glycoprotein in each particle [84]), may provide a three-dimensional, multivalent and highly avid binding site. However, specific HS or GAG structures bound by individual alphaviruses or flaviviruses have yet to be identified and direct comparisons of binding efficiency are lacking. It is remarkable, however, that E2 glycoprotein sites associated with use of HS receptors by naturally circulating viruses such as EEEV can essentially be identically positioned to those acquired as a result of cell culture passage [52, 62] (Fig. 3a). Furthermore, the difference between weak to undetectable interaction with HS and efficient binding can be the result of acquisition of a single positively charged amino acid mutation in the alphavirus or flavivirus surface glycoproteins [52, 72, 73, 85].

We and others have speculated that weak cell attachment, which could be mediated by either a low-abundance receptor or inefficient binding, may be a ubiquitous feature of arbovirus particles that promotes generation of high titer sustained viremia in vertebrate hosts [4, 14, 52, 73, 85]. However, this raises a conundrum with respect to cell binding by arboviruses. In our hands, with common fibroblastic cell lines, nearly all of the measurable cell binding by laboratory strains of SINV strain AR339, for example, is dependent upon the HS interaction and is greatly reduced by manipulating the availability of HS receptors [52, 54, 81]. Therefore, at least on these cell types, it is likely that interaction of virus particles with receptors other than GAGs, at least at the virus population level measured in conventional binding assays, is of low efficiency, again either through weak interaction with a receptor or low receptor abundance. These results indicate that interactions with other identified alphavirus fibroblast receptors [e.g. high affinity laminin receptor (HALR) [16, 17], natural resistance-associated macrophage protein (NRAMP) [5], matrix remodeling associated 8 (MxrA8) [7] or low-density lipoprotein receptor class A domain-containing 3 (LDLRAD3) [6]] may either be linked to HS binding, are relatively inefficient, or receptor abundance is low on multiple permissive cell types. Notably, it has been proposed that the prion protein interacts with the HALR via binding of each protein to HS in a 'sandwich' structure [86] and this may account for its identification as an arbovirus receptor. It is also worth considering whether or not inhibitors used to block virus interactions with other attachment receptors possess properties that may non-specifically disrupt ionic interactions as this may also block GAG binding leading to reductions in cell attachment, or that HS binding and protein receptor interaction domains overlap.

In contrast, with specialized cell types such as DC- or L-SIGN expressing myeloid cells, alphaviruses that contain high-mannose carbohydrate modifications of the surface glycoproteins but do not bind HS well, can bind cells efficiently and to similar levels to HS binding viruses [4]. Alternatively, HS-independent binding of cells by different arboviruses could vary greatly between virus types. Regardless, a comparison of direct GAG and/or cell binding efficiency either between virus isolates or between representatives of different families would be of assistance in placing the GAG binding phenotype of individual viruses in context, for example, in attempts to understand the relationship of cell binding efficiency to virus phenotypes in animal models. Finally, it should be noted that HS/GAGs are generally not found to be the sole attachment/entry mediator for arboviruses on common fibroblast-derived cell lines such that a significant base-line infectivity for these cell lines cells is retained in the complete absence of GAG molecules. This confirms that other attachment/entry receptors are present on these cell types. Indeed, LDLRAD3 overexpression has been shown recently to increase binding and infectivity of VEEV in the absence of cell surface heparan sulfate [6].

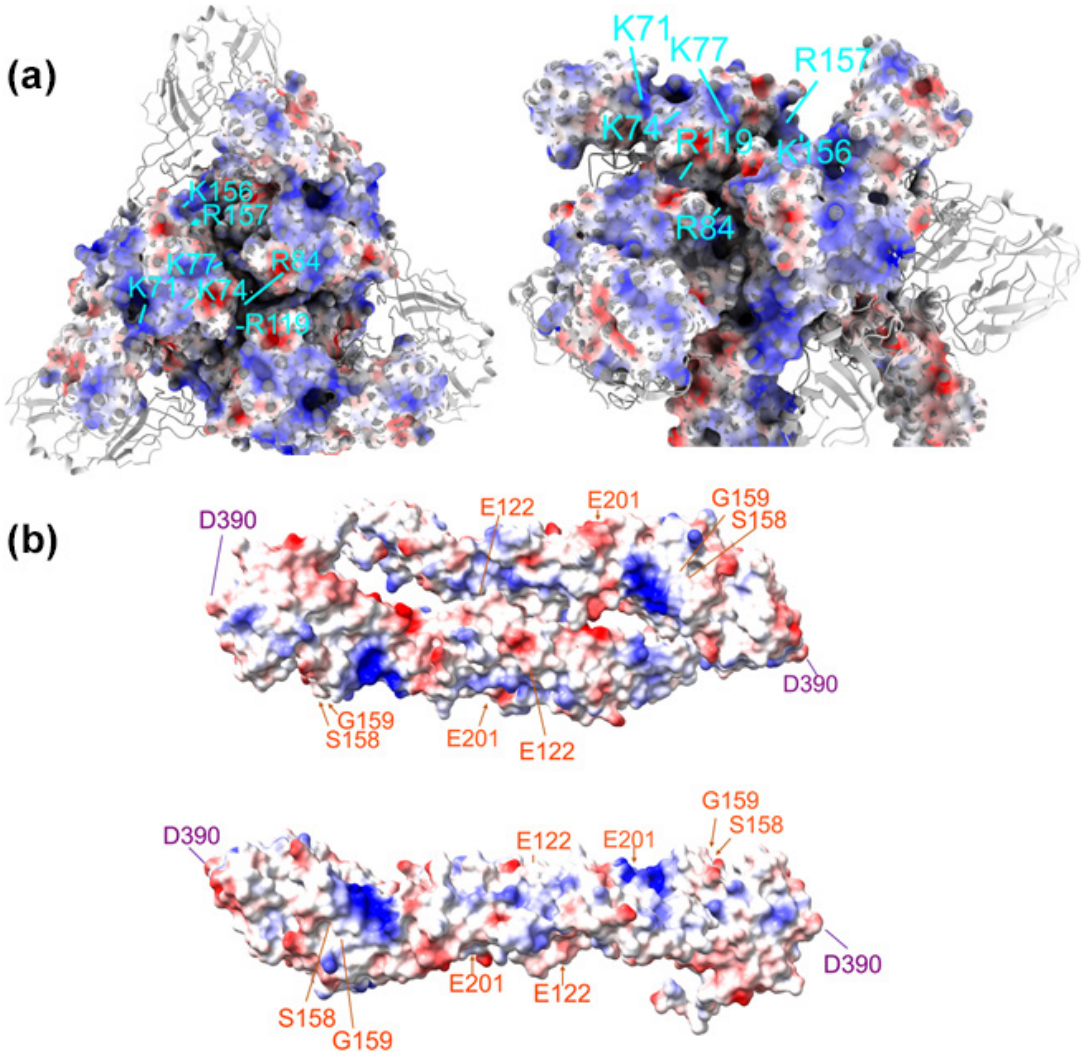

Fig. 3. (a) Top and rotated and cropped three-dimensional model of the mature trimeric spike complex of EEEV E1 and E2 heterodimers (PDB: 6M×4). E1 is shown as a ribbon diagram and E2 as a residue electrostatic map (red=negative charge and blue=positive charge). E2 residues K71/K74/K77, R84/R119, K156/R157 (identified with lines in cyan) have been shown to be three separate interaction sites involved in HS binding by naturally circulating strains. (b) Top and side view of an electrostatic three-dimensional model of the JEV E dimer ectodomain (PDB: 5MV1, dimer was reconstructed using Swiss model programming). Corresponding residues on both E proteins where mutations increase HS interactions for TBEV (E122, S158, G159 and E201) are highlighted in orange, and for MVEV (D390) is highlighted in purple. The figures were made using UCSF ChimeraX software.

HS receptor identification through global host factor knockdown

Recent attempts to identify host factors associated with arbovirus entry, studies using gene trap mutagenesis, RNAi or CRISPR/Cas9 genome knockdown have demonstrated that GAG synthesis pathways are involved in infectivity of laboratory virus strains including CHIKV or Rift Valley Fever virus in HAP1 human myeloid cells [87, 88], SINV in CHO cells [89] and DENV and ZIKV in MAGI human cervical epithelial cells [90]. These screens relied on differential survival of cells with putative essential entry mediators ablated, therefore, the infection efficiency of viruses for surviving cells was by requirement, very low to none. These results are surprising since significant infectivity of all tested alphaviruses is maintained for HS/GAG-negative CHOK1 cells. As several of these studies utilized non-fibroblast cell types, this indicates that there may be a cell type dependence of virus infection upon GAG molecules. While it is unlikely, yet possible, that these cells express a cell surface form of GAG not expressed by fibroblasts and to which arboviruses can bind, it is also possible that other pathways of cell entry are greatly reduced in comparison with fibroblast cell types, such that a GAG-dependent pathway is essential for substantial entry. However, it is also possible that GAG biosynthetic pathways are required once the cell has been entered for virus trafficking and fusion within endocytic vesicles. For instance, results from two studies have indicated that HS is not vital for ZIKV entry but is important for replication *in vitro* [91, 92]. Similarly, chondroitin sulfate enhanced CHIKV replication after infection [87]. In this case, comparative cell binding assays, as opposed to infectivity assays, are critical to distinguish between these different possibilities.

Informed passage experiments

Multiple studies have also formally examined differences in GAG interaction between low-passage arbovirus strains and those subjected to greater numbers of *in vitro* growth cycles [52–55, 57, 72, 73, 75]. These types of studies have demonstrated empirically that significant increases in GAG binding can be acquired by an arbovirus through a single amino acid substitution and that after a small number of passages at high MOI, such mutations can be predominant in a virus population and give the impression that efficient GAG binding is the phenotype of the unpassaged virus. It is sobering to consider the number of commonly used arbovirus biological stocks and the practice still common today to amplify viruses in cultured cells prior to plaque purification and/or generation of cDNA clones.

The first arbovirus study to demonstrate this effect was with SINV strain TR339 showing that particle infectivity and cell binding and competition with HS analogs increased steadily up to three passages where a plateau was observed [52]. Furthermore, sequencing of the thrice-amplified stock revealed the presence of a Glu–Lys substitution at position 70 of the E2 glycoprotein responsible for the receptor utilization change (Table 1). Other studies revealed that the Lys 70 residue was present in multiple commonly used SINV laboratory strains [51]. This phenomenon has been reproduced starting with low cell passage versions of RRV, Semliki Forest virus (SFV) and CHIKV alphaviruses and YFV, MVEV, DENV, WNV, JEV, and TBEV flaviviruses yielding a large panel of known loci associated with enhancement of GAG interaction after *in vitro* passage (Fig. 3b) (Table 1) [53, 57, 60, 66, 72, 73, 75, 85, 88, 93–97]. These findings have utility in live attenuated vaccine design and also may allow determination of differences in naturally acquired and cell-adaptive GAG binding characteristics. Recent studies with SARS CoV-2 propagation *in vitro* have reinforced the capacity of RNA viruses to mutate during the first few cell amplification steps [98, 99]. Ultimately, *in vitro* studies have yet to distinguish mechanistically between natural and cell passage-acquired HS binding and its role in cell infectivity.

Effects of HS binding upon arboviral pathogenesis in animal models

Mouse models of arboviral disease

Early studies examining the phenotypes of HS binding SINV at later stages after infection of mice or encephalomyocarditis virus in horses indicated that viruses adapted to *in vivo* replication by reducing the affinity of HS binding or losing the HS binding phenotype altogether [48, 100]. Furthermore, viruses with known cell passage-acquired HS binding mutations were attenuated in comparison with their weakly HS binding 'wild type' progenitors [48, 58, 101]. Thus, as stated above, original assessments of the GAG binding of RNA viruses were that the phenotype was an artifact of cell culture passage of virus isolates [48, 52, 53, 57]. Indeed, in virtually all circumstances where GAG binding can definitely be shown to be an artifact of cell culture amplification or the deliberate increasing of positive charge in viral attachment proteins, the phenotype is attenuating *in vivo* when the natural arbovirus infection route [e.g. subcutaneous (sc) or intradermal inoculation] is mimicked.

The mechanism of attenuation of viruses that bind HS efficiently appears to be related to spread of virions as several studies have demonstrated that the *in vivo* dissemination of HS binding arboviruses is restricted. In comparisons of the blood retention time or viremic spread of HS-binding versus non-binding alphavirus, flavivirus and other RNA virus strains, efficient HS-binding viruses are generally cleared more rapidly from mouse blood and show diminished spread [48, 54, 56, 72, 102]. Similarly, viruses isolated from mouse blood later during infection with HS-binding SINV had frequently lost the HS binding phenotype, indicating that it was an inhibitor of virus spread and serum retention [100]. In general, the inhibition of spread *in vivo* for a particular virus genotype correlates with the degree of infection dependence upon or binding efficiency to HS as measured in *in vitro* assays [52, 54, 81]. This indicates that cell culture adaptive mutations may confer GAG interaction that exceeds the optimal efficiency of GAG binding naturally present in virus glycoproteins, thereby, attenuating disease *in vivo*. However, there are exceptions, for example with SINV, the E2 position 70 lysine mutation acquired after fibroblast passage, can slightly, but significantly, increase neurovirulence of the TR339 strain after intracranial infection of weanling mice although it is attenuating in suckling and adult mice [81]. In addition, this mutation is essential in combination with an *in vivo*-selected non-charged amino acid mutation for neurovirulence of an adult neurovirulent SINV mutant ([81, 103] described in more detail below). Interestingly, deletion of certain post-translational carbohydrate addition sites from the SINV E2 glycoprotein also increased HS binding and mouse virulence after intracranial infection [104]

EEEV is unique among alphaviruses examined to date in that virus access to peripheral lymphoid tissues and infection of myeloid cells is restricted by efficient binding to HS [49, 62] as well as restriction of myeloid cell replication by virus genome binding to a hematopoietic cell-specific microRNA [105]. In these studies, sequences of the E2 attachment protein of the cDNA cloned FL93-939 strain were compared with those from primary unpassaged field isolates and found to be identical in the majority of samples tested [62]. A combination of biological [62] and cryo-EM structure imaging analyses [106] has identified three domains on the native virus E2 protein capable of interaction with HS (Fig. 3a). These include lysine and arginine residues at positions 71, 74 and 77 (71 and 74 being most critical) [49, 62], of the E2 attachment protein and binding sites comprised of positively charged residues at positions 84/119 and 156/157, which were predicted to bind heparin hexamers in cryo-EM reconstruction experiments [106]. All three domains have been examined by charged-to-alanine mutagenesis and each independently affects EEEV binding to and infectivity for cells in a HS/GAG-dependent manner [62, 106]. In a mouse model, wild-type (WT) EEEV virus exhibited

significantly less replication and numbers of infected cells in the draining lymph node (DLN) after sc inoculation than an E2 71–77 charged-to-alanine mutant that no longer bound HS efficiently [62]. Elimination of the HS binding domain increased levels of serum interferon-α/β and survival times due to decreased virus replication and dissemination within the central nervous system (CNS) [49, 62]. This is similar to reports with both Theiler's murine encephalomyelitis virus [107] and neuroadapted SINV [81]. In contrast, mutation to reduce the efficiency of HS binding decreased replication and spread within the brain [62, 81]. The 84/119 and 156/157 mutations have not been examined for effects upon virulence *in vivo* (Fig. 3a). Together, these data indicate that the HS binding domain(s) of WT EEEV suppress virus access to permissive cells in DLNs while simultaneously increasing virus infection of neurons and spread in the CNS, contributing to the severe manifestations of EEEV. Furthermore, natural alteration of the HS binding site may be linked to variation in virulence of individual virus isolates [49]. Several other studies have found a positive association of HS binding with arbovirus neurovirulence specifically, especially when the requirement for viremic spread was obviated, and it has been speculated that HS binding may be a neurovirulence factor due to the close association of cells within the brain, which may promote efficient infection of neighboring cells by HS binding viruses [81, 108–110] (reviewed in [111]).

The role of GAGs in arthropod vector infection

Infection of the hematophagous arthropod vector gut epithelium from a blood meal and subsequent spread to and infection of cells in the salivary gland is a requirement for sylvatic transmission of arboviruses (reviewed in [112, 113]). Very few studies have examined the role of HS or GAG binding in this process, and it is unclear if the spread of arboviruses in arthropods is analogous to viremic spread in vertebrates. A recent study has implicated rice arbovirus binding to sperm HS in vertical transmission [114]. *In vitro*, we have passaged SINV TR339 on C6/36 mosquito cells and obtained clustered mutations in the E2 protein (Ser–Arg at amino acid 60 and Ala–Thr at amino acid 61) that, together, increase HS dependence of infection, indicating that HS proteoglycans can function as attachment receptors on mosquito cells. It has been demonstrated that glycosaminoglycans capable of binding pathogens are present in the mosquito gut and salivary gland [115–118] (reviewed in [119]), although, the diversity of core proteins in proteoglycans is limited in comparison with vertebrates [120]. One recent study has evaluated HS/GAG structures in different mosquito species, finding some differences in predominant type and structure [121].

In preliminary studies, we have determined that with EEEV, HS binding increases virus infectivity for the mosquito gut epithelium after a blood meal, indicating that, at least with a virus that expresses this phenotype in sylvatic transmission cycles, HS binding promotes mosquito infection. In addition, our data supports the notion that with EEEV, HS binding also promotes transit to and infection of the salivary gland (SC Weaver and WB Klimstra unpublished data). Similar results were obtained in a study using SINV strains with different efficiencies of GAG binding [115]. Whether or not HS binding is promoting to or inhibitory of release from the arthropod salivary gland remains to be determined.

HS binding may also play a role in mosquito–vertebrate transmission efficiency. Results from one study indicated that mosquito saliva proteases that were delivered alongside virus particles and that degraded the extracellular matrix, improved DENV engagement of HS receptors on cells [122] Amplification of CHIKV on mosquito cells reduced virion binding to HS, presumably through blockade of HS binding sites on virions by the high-mannose, glycoprotein carbohydrate modifications obtained during invertebrate replication [123]. However, in contrast, growth of a SINV cell-adapted strain in C7/10 mosquito cells increased the efficiency of virion binding to heparin bead columns [104]. While more research is a critical need, these findings indicate that HS binding of mosquito-delivered alphaviruses may be influenced by characteristics of the mosquito vector. This phenomenon may have parallels with differences in mammal- versus arthropod-derived alphavirus and flavivirus glycoprotein interactions with C-type lectins [4, 14].

The relationship between natural and laboratory acquired GAG binding in animal models

It is likely that promiscuous and efficient interaction of HS binding domains with ubiquitously expressed HS results in sequestration of virus particles to non-productive structures *in vivo*. This may lead to limitations of virus spread from initial sites of inoculation (e.g. footpad to DLN and serum in murine models) and, therefore, in the context of natural infection, may represent an adaptation of some viruses to limit immune responses by suppressing myeloid cell replication. Similar hypotheses regarding suppression of spread *in vivo* have been proposed in studies of other viruses which exhibit increased GAG binding over other strains [55, 56, 73, 85]. Furthermore, efficiency of infection of cells within isolated organs such as brain may be increased by efficient GAG binding. Therefore, the role of HS binding in suppressing virus spread *in vivo* could be important in the pathogenesis of a number of viruses.

In contrast, most studies conclude that HS or GAG binding acquired as an artifact of cell adaptation *in vitro* has an attenuating effect on virus replication and disease *in vivo* and it is clear that HS binding is an important attenuating characteristic of multiple live attenuated vaccine strains. For unknown reasons, some viruses may have adapted to achieve vertebrate–invertebrate transmission while incorporating the high efficiency HS binding phenotype. With EEEV, this may imply that other features in its attachment protein (or other viral proteins) allow the efficient binding of HS in the face of the inhibitory effects of this phenotype to spread *in vivo*. As mentioned earlier, neurovirulent Sindbis virus strain, NSV, developed by Griffin and colleagues [81, 103] which is more virulent from the natural sc route of infection in adult mice, was derived by serial passage of cell-adapted HS

binding strain in sucking and weanling mice. The adult neurovirulence phenotype is mediated by the E2 E70K HS binding mutation, probably an artifact of cell culture passage, but only in combination with an additional E2 N55H mutation [81]. As mentioned above, increased GAG binding has also been associated with enhanced neurovirulence in some flaviviruses in the absence of a requirement for viremic spread [67, 72, 95]. Therefore, replication in vertebrates can result in adaptation to retain or even enhance virulence in the presence of efficient HS binding. Considering results ablating individual HS binding sites in EEEV [62] and adding HS binding sites to other alphaviruses and flaviviruses [53, 54, 57, 73, 85], it may be that disruption of adapted homeostasis, either increasing or decreasing HS binding versus that of natural viruses, may be the primary attenuating factor *in vivo*. This can function through suppression of virus spread *in vivo* (e.g. acquisition/enhancement of HS binding) or increases in lymphotropism/innate immune responses and reduction in neurovirulence (reduction in HS binding). In addition to these points, the effects of cell type-specific expression of particular proteoglycans and/or GAG structural variants and their specific binding by viral attachment proteins must be considered when evaluating effects of GAG binding on tropism and spread *in vivo*. As mentioned above, it must be noted that these conclusions are almost exclusively derived from arbovirus disease models and do not adequately consider effects upon transmission between reservoir species and arthropod vectors.

CONCLUDING REMARKS

Arboviruses infect evolutionarily divergent hosts during their transmission cycle. This places constraints on many aspects of their replication including attachment receptor utilization. Research to date has indicated that both cell/host-specific and highly conserved receptors can be utilized for attachment, sometimes in combination as with the interaction of conserved virus carbohydrate modifications with vertebrate C-type lectin receptors. GAG molecules expressed as post-translational modifications of cell surface proteoglycans are also a class of highly conserved receptor structures to which many arbovirus strains attach, however, unlike other identified receptors, the lesser specificity of the ionic GAG–protein interaction appears to allow adaptation to efficient binding of these receptors rapidly and with as little as a single nucleotide change in viral attachment protein genes. Controversy exists because efficient GAG interactions can often be a consequence of minimal *in vitro* cell adaptation and have little relevance to naturally circulating viruses but can also be observed with truly 'wild type' strains.

The efficiency of interactions with GAG molecules during infection can exhibit a very large range, as measured by binding and GAG infection dependence assays, and increasing strength is generally associated with increasing infectivity of viruses for common fibroblastic cell lines leading to the conclusion that the primary selective advantage of *in vitro*-acquired GAGs interactions is to increase binding of arboviruses to cells and increase infectivity [52]. From this review of the literature, it seems clear that lower numbers of cell passages are most often associated with weaker interactions with GAGs and *vice-versa* (with EEEV as a potential exception). Therefore, the study of GAG binding by arboviruses has, to date, very probably been biased by the characteristics of HS interaction engaged in by cell-adapted virus strains. In particular, conclusions regarding the potential for use of HS analogs as anti-arboviral therapeutics may have been affected.

From our experience and this review of the literature, we propose that the following procedures be followed during isolation of strains for analysis of GAG interaction phenotypes to avoid acquisition of cell-adaptive GAG binding mutations. (1) attachment proteins should be sequenced directly from clinical material and the predominant sequences directly recapitulated through synthesis of viral genes when creating cDNA clones (2) cDNA cloned viruses should be passaged less than three times in cultured cells prior to evaluation of GAG interaction phenotypes (preferably, stocks would be harvested after a single electroporation of viral genomic RNA into cells), and (3) deep sequencing should be utilized to confirm that the predominant sequence of attachment proteins in virus stocks is reflective of the cDNA clone. These procedures would ensure that cell culture adaptive mutations are not incorporated into 'wild type' stocks. While these methods for generation of virus stocks from cDNA clones may constrain quasispecies diversity in the initial stages of virus production, it should be rapidly regenerated during virus production and after infection of experimental animals. Furthermore, this is presumably no less artificial than shifts in quasispecies composition imposed by repeated amplification of virus strains in cultured cells. It is well established that passage of arboviruses in single cell types alters the adaptive balance achieved through the alternating vertebrate–invertebrate replication cycle [124].

Making the assumption that lower passage viruses are genetically closer to viruses circulating in nature, a critical goal of additional research in this area is to determine if the weak interactions with GAGs achieved by low passage and/or authentic natural isolates are relevant to replication and disease *in vivo*. Furthermore, it will be important to determine if the disruption of HS interactions by HS analogs used therapeutically has a beneficial effect on disease caused by naturally GAG-binding viruses. Given that some evidence exists that closely related viruses may bind different GAG structures (e.g. differential heparinase sensitivity [49, 52, 81] and the fact that different mutations that alter HS interactions can be selected in virus glycoproteins with passage on different cell types [75]), cell type-specific aspects of virus–GAG interactions and proteoglycan core protein identity should also be explored. GAG- and HS-deficient CHOK1 cells have become the standard for assessment of virus–GAG interactions and this approach should be expanded to tissues and cell types relevant to pathogenesis *in vivo* (recently a few studies have used this approach with arboviruses [6, 7, 121, 125]). In particular, the structural and functional relationship of GAGs to other entry receptors may be

cell type-dependent. In a possible analogy with arboviruses, a diversity in function of different HS structures has been observed with herpes simplex virus (reviewed in [44, 126]) where unmodified HS, such as that expressed by CHOK1 cells, can bind viruses but only specifically modified forms of HS, such as those modified by the 3–*O*-transferases can facilitate entry [127]. A CHIKV laboratory strain, but not JEV or YFV flaviviruses, was shown to rely on N-sulfation of HS for infection of HAP1 cells indicating some similarities [87].

It will be also important to determine if proteoglycans can serve as single attachment and entry receptors or if they merely serve to concentrate viruses on the cell surface. In particular, the relationship of GAG binding and the effect of GAG deficiency upon virus utilization of other receptor structures should be investigated during the identification stage of these receptors. Highly relevant to this idea, recent cryo-EM structures of CHIKV and VEEV bound to proteinaceous receptors, Mxra8 and LDLRAD3 [128, 129], have revealed common E2 contact sites for both receptors and some of these are either immediately adjacent to or overlapping with positively charged clusters involved in HS binding [52, 62, 75, 106]. This raises the possibility that cell culture adaptation to efficient HS binding might alter interactions with other receptors.

Finally, the ease with which arboviruses can transition between GAG binding phenotypes indicates that they could seamlessly respond to an adaptive pressure for increased cell binding and infectivity during the complexities of the arthropod–vertebrate–arthropod transmission cycle or even within individual tissues of a single host. Viruses such as EEEV may express an efficient GAG binding phenotype during the majority of their transmission and disease cycles but other viruses may not. Therefore, the adaptive potential of arboviruses to GAG binding should be considered at each point in the arboviral transmission cycle (reservoir, arthropod vector and dead-end host) as well as within individual tissues within each host. Such considerations may have significant influence on the potential or context for use of GAG analogs as antiviral therapeutics. Separate from these issues, rational manipulation of the native GAG binding phenotypes of arboviruses should become an important factor in the informed design of live attenuated arbovirus vaccines.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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