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Role of heparan sulfate for attachment and entry of tick-borne encephalitis virus

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Abstract

Attachment of the flavivirus tick-borne encephalitis (TBE) virus to different permissive cell lines was investigated by a newly established quantitative assay using fluorescence-labeled virus. Previous work had shown that BHK-21 cell-adapted mutants of TBE virus had acquired potential heparan sulfate (HS) binding sites on the outer surface of protein E. Quantitative analysis of one of these mutants indicated that it attached to HS-expressing cell lines with a 10- to 13-fold higher affinity than wild-type TBE virus strain Neudoerfl. CHO cells deficient in HS synthesis bound less than 5% of the amount of wild-type or mutant virus that could attach to HS-containing CHO cells but were nevertheless found to be highly susceptible to infection with both viruses. Thus, even though HS is a major determinant of TBE virus attachment on HS-expressing cells, our findings suggest the existence of an alternative host cell receptor that is less abundant than HS.

Keywords: Flavivirus; Tick-borne encephalitis (TBE); Attachment; Receptor; Affinity; Heparan sulfate

Introduction

The genus *Flavivirus*, family *Flaviviridae*, includes many important arthropod-transmitted human pathogens, such as *Yellow fever virus*, *Japanese encephalitis virus*, *Dengue virus*, *West Nile virus*, and *Tick-borne encephalitis virus* (TBE virus). Flaviviruses are small enveloped particles that contain only three structural proteins, i.e., the capsid protein C, the small membrane protein M, and the large envelope protein E. They are believed to enter cells by receptor-mediated endocytosis and subsequent acid-induced fusion of the viral and endosomal membranes (Lindenbach and Rice, 2001). The atomic stucture of the ectodomain of protein E, which mediates viral attachment (Wang et al., 1999) and fusion (Allison et al., 2001), has been solved for TBE virus by X-ray diffraction analysis (Rey et al., 1995). For the understanding of the life cycle and complex pathogenesis of these viruses, the identification of host-cell receptors and the elucidation of their roles during viral uptake are of utmost importance. However, many questions concerning attachment and entry of flaviviruses are still unresolved. A number of different proteins have been identified as potential flavivirus receptors, but there is no direct evidence for a role of any of these during host cell entry and some of the results are controversial (Lindenbach and Rice, 2001). Flaviviruses can infect a wide range of different cell types and most flaviviruses circulate in nature between arthropod vectors (mosquitoes or ticks) and their vertebrate hosts. This suggests that either the receptor molecule(s) recognized by flaviviruses are ubiquitous or the flaviviruses can utilize a range of different molecules as receptors.

Glycosaminoglycans (GAG), such as heparan sulfate (HS) and chondroitin sulfate (CS), are linear, polyanionic carbohydrate chains that are expressed in a specifically regulated manner on different tissues and throughout different developmental stages (Bernfield et al., 1999; Kjellen and Lindahl, 1991). They are highly conserved throughout evolution (Williams and Fuki, 1997) and mediate a wide variety of important functions including cellular adhesion, regulation of motility and growth, and intercellular signaling. HS is present abundantly on the surface of most animal cells and is known to serve as a receptor for various ligands, such

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as growth factors and antithrombin (Bernfield et al., 1999). There is increasing evidence that HS-containing proteoglycans can mediate internalization of physiological ligands by both clathrin-coated pits and membrane rafts associated with caveolae (Bernfield et al., 1999). HS may also play an important role during infection by many viral and nonviral pathogens (Rostand and Esko, 1997). During the past few years, members of several viral families have been shown to interact with HS (Spillmann, 2001). The functional importance of this interaction is often not well understood and may vary from case to case. Frequently, HS appears to serve as a primary attachment molecule that concentrates viral particles on the cell surface and thus may facilitate the subsequent binding of more specific receptor molecules. In some instances, HS has been shown to be necessary for viral attachment and infection of cells (Byrnes and Griffin, 1998; Chen et al., 1997; Hsiao et al., 1998; Hung et al., 1999; Jan et al., 1999). In one particular case (herpes simplex virus type 1), HS carrying a specific sulfation pattern could even substitute the function of a high-affinity protein receptor (Shukla et al., 1999). Furthermore, it was shown for a range of viruses that propagation of these viruses in certain cultured cells selected for mutations that generated a HS-dependent phenotype (Heil et al., 2001 and references therein). Although these HS-binding mutants apparently had a decisive advantage over the wild-type viruses during growth in cell culture, in a number of cases they were shown to exhibit attenuated phenotypes in animal hosts due to restricted spreading and accelerated clearing in vivo (Bernard et al., 2000; Byrnes and Griffin, 2000; Klimstra et al., 1998; Lee and Lobigs, 2002; Mandl et al., 2001; Sa-Carvalho et al., 1997).

An involvement of HS during attachment and entry of flaviviruses has been demonstrated by several researchers (Chen et al., 1997; Germi et al., 2002; Hilgard and Stockert, 2000; Hung et al., 1999; Lee and Lobigs, 2000; Mandl et al., 2001; Martinez-Barragan and del Angel, 2001), but many questions concerning the specific functional role played by HS during infection remain unresolved. In the case of dengue viruses, some studies indicated an essential role of HS for both viral attachment and uptake (Chen et al., 1997; Hilgard and Stockert, 2000; Hung et al., 1999), whereas in another study HS was not found to be necessary for entry, and enzymatic removal of HS actually increased viral attachment to certain cells (Bielefeldt-Ohmann et al., 2001). Mutants of another mosquito-borne flavivirus, Murray Valley encephalitis virus, carrying substitutions in a putative receptor-binding area within domain III of protein E exhibited an altered host range and revealed a HS-dependent phenotype (Lee and Lobigs, 2000). Using TBE virus, we recently observed the emergence of HS-binding mutants during propagation of wild-type strain Neudoerfl (a tick isolate with only a short passage history in primary chicken embryo cells) in BHK-21 cells (Mandl et al., 2001). These mutants carried point mutations at various positions of protein E that created local areas of increased positive surface charge which probably represented newly formed HS-binding sites.

Here we report on the analysis of cell-attachment properties of one of these BHK-21 cell-adapted mutants in comparison with the parental TBE virus strain Neudoerfl. A new assay using fluorescence-labeled virus was established, and this allowed a quantitative determination of binding affinities. We provide evidence that HS can function as an attachment receptor for both mutant and wild-type virus, but the affinity of this interaction is significantly higher in the case of the BHK-21-adapted mutant. However; we also observed that mutant CHO cells deficient in HS synthesis were capable of binding much less virus but were still highly susceptible to infection by both viruses. This finding suggests the existence of a host-cell receptor other than HS that can mediate entry of TBE virus.

Results

Establishment of a quantitative binding assay

To analyze and compare on a quantitative basis the cell-binding properties of wild-type and BHK-21-cell-adapted mutant TBE virus, a new assay was established that was based on the use of fluorescence-labeled virus and measurement of cell-bound virus by FACS analysis. Virus was labeled metabolically with a fluorescent fatty acid analog that was incorporated into the viral membrane to avoid the need to modify the envelope proteins and thereby potentially disrupt binding sites. Addition of various amounts of labeled virus to constant numbers of cells and subsequent measurement of cell-bound fluorescence by FACS analysis yielded hyperbolic binding curves from which the dissociation constant (K_d) and a value corresponding to maximum (saturated) virus binding (B_{max}) could be determined (Fig. 1A and B). Experimental details of this assay and its evaluation are given under Materials and methods. In essence, the K_d value is obtained as a protein concentration (μg/ml) which can be transformed into molar concentration. The B_{max} value is expressed in arbitrary units of fluorescence. It cannot be used to calculate the amount of virus bound per cell in absolute numbers, but the relative amounts of virus bound to different cell types and thus the relative abundance of attachment molecules can be compared using this value.

In a control experiment, constant amounts of virus consisting of variable proportions of labeled and unlabeled virus were subjected to the binding assay. Measurement of cell-bound fluorescence was strictly proportional to the percentage of labeled virus in these mixtures (Fig. 1C), demonstrating that labeled virus bound to cells in the same way as unlabeled virus. In another control experiment, virus was digested with various concentrations of trypsin prior to addition to the cells. As shown in Fig. 1D, this degradation of viral surface proteins almost completely abolished the transmission of fluorescence to the cell. This result indicated

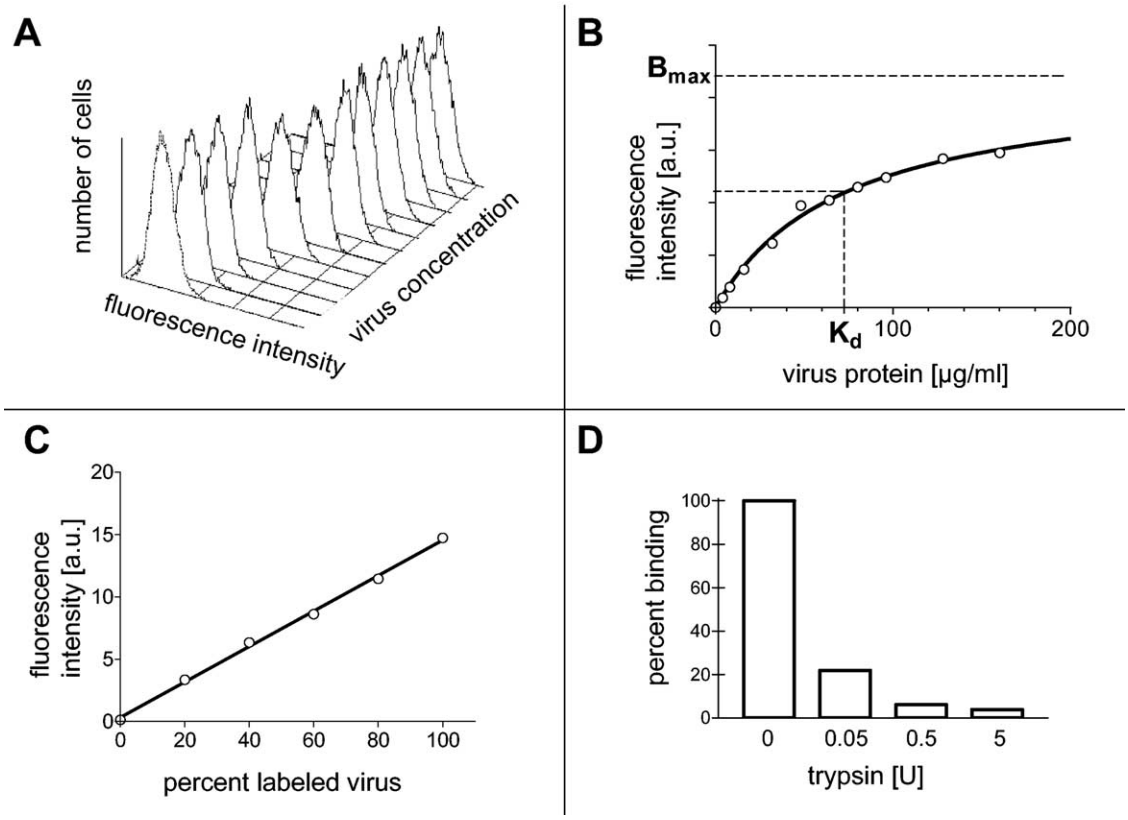

Fig. 1. Cell-binding assay using fluorescent-dye-labeled TBE virus. (A) Example of primary binding data. Equal numbers of HeLa cells were incubated with various virus concentrations and cell-associated fluorescence was measured by FACS analysis. (B) Evaluation of binding data. Median fluorescence intensitities derived from the data shown in (A) were plotted as a function of virus concentration and these data were fitted to a one-site binding hyperbola. This allowed the maximum fluorescence intensity (B_{max}), a parameter of the amount of virus bound at saturating concentrations, as well as the dissociation constant (K_d), which equals the virus concentration at half-maximum binding to be calculated. (C) Samples containing the same total virus concentration (20 μg/ml) but containing various proportions of dye-labeled and unlabeled TBE virus were added to HeLa cells and cell-associated fluorescence was determined by FACS analysis. (D) Trypsin digestion of viral surface proteins. Fluorescence-labeled TBE virus (0.5 μg) was incubated with various amounts of trypsin as indicated, then added to HeLa cells and cell-associated fluorescence was determined by FACS analysis. a.u., arbitrary units.

that binding was protein-mediated and that there was no relevant nonspecific transfer of dye from the virus preparation to the cells. These controls confirmed that the dye was integrated stably into the viral membrane and did not measurably alter the cell binding properties of the virion.

Dissociation constants (K_d) for wild-type and mutant TBE virus attachment

The binding assay was applied to determine K_d values for the attachment of wild-type TBE virus and a BHK-21 cell-adapted mutant, E(E122G), to different cells. Mutant E(E122G) carries a single-point mutation that was previously shown to create a potential HS-binding site on the outer surface of protein E (Mandl et al., 2001). Three common cell lines competent for HS synthesis and susceptible to infection with TBE virus, BHK-21, HeLa, and CHO-K1, were used in the TBE virus-binding assay. Fig. 2 shows examples of binding curves obtained for the attachment of each virus to BHK-21 cells. Both data sets indicated saturable binding and could be fitted equally well to hyperbolic binding curves. However, saturation was approached by the mutant virus at much lower protein concentrations, indicating that the mutant virus bound BHK-21 cells with a higher affinity than wild-type TBE virus. This is reflected by a smaller K_d value (0.46 nM as compared to 5.8 nM for wild-type virus). Similar results were obtained for the other two cell lines, HeLa and CHO-K1. Table 1 summarizes the mean K_d values determined for both viruses and each of the three cell lines from at least four independent experiments. These results yielded a consistent picture. The K_d values of wild-type virus attachment to these cells for all three cell lines ranged between 5.5 and 6.4 nM, whereas in the case of mutant E(E122G) it was between 0.42 and 0.62 nM. Thus, the cell-culture-adapted mutant was found to attach to these HS-containing cell lines with an approximately 10-fold higher affinity than the parental strain.

Analysis of virus binding to GAG-deficient CHO cell lines

To investigate to what extent binding was dependent on the presence of HS on the cell surface, we took advantage of

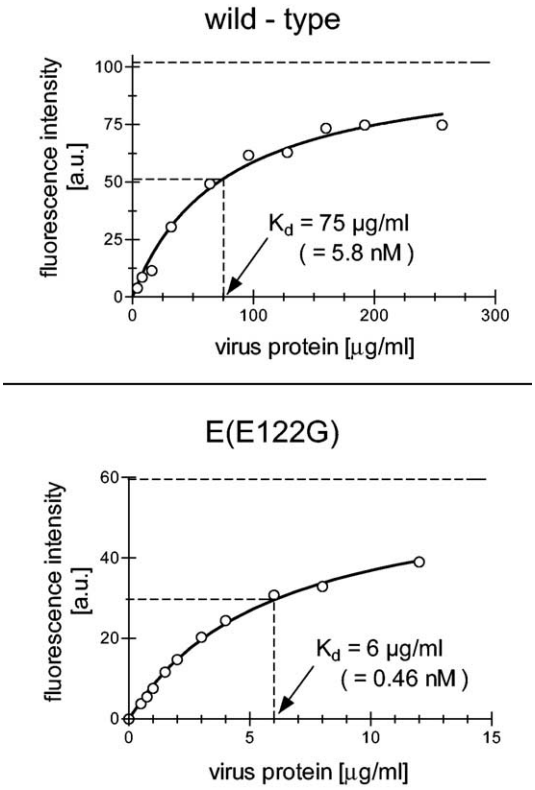

Fig. 2. Representative example of binding curves obtained for wild-type virus (top) and cell-culture-adapted mutant virus (bottom) attachment to BHK-21 cells. The dissociation constants (K_d) derived from these experiments are indicated. a.u., arbitrary units.

mutant CHO cell lines that bear defined defects in GAG synthesis. The protein-linked GAGs on the surface of CHO-K1 cells consist of approximately 70% HS and 30% CS, whereas CHO pgsD-677 cells carry only CS and no HS, and pgsA-745 cells are deficient for the synthesis of all GAGs.

When these three CHO cell lines were subjected to the binding assay using batches of virus with the same specific fluorescence, it became clear that both pgsD-677 cells and pgsA-745 cells bound significantly less of the wild-type or mutant TBE virus than the parental CHO-K1 cell line (Fig. 3, top panels). Nevertheless, plotting the data on an expanded scale of the *y*-axis revealed that binding to pgsD-677 cells was saturable and reproducibly yielded binding curves that could be evaluated as shown for an example in the lower panels of Fig. 3. In the case of pgsA-745 cells, the reproducibility of binding data was insufficient for a quantitative evaluation. B_{max} values calculated in several experiments for CHO-K1 and pgsD-677 cell-binding indicated that each cell line was capable of binding approximately the same amount of wild-type or mutant virus. The maximum number of virus particles bound by the HS-deficient cell line, however, was less than 5% of the amount that could attach to CHO-K1 cells. This is also visible in the representative experiment shown in Fig. 3. The binding curves obtained for pgsD-677 cells could be further evaluated to calculate the K_d values for attachment of wild-type and mutant virus to these cells (Fig. 3, lower panel). Mean values of several independent experiments are included in Table 1. These numbers demonstrated that both the wild-type and the mutant virus attached to pgsD-677 cells with almost the same affinity, indicating that the HS-adaptive mutation present in mutant E(E122G) had little effect on TBE virus attachment to these HS-deficient cells.

In summary, these experiments indicate that the vast majority of both wild-type virus and mutant E(E122G) particles probably attached to CHO-K1 cells via HS, because pgsD-677 cells, which differ from CHO-K1 only by the absence of HS, bound much less of both viruses. Attachment to pgsD-677 cells was apparently mediated by a less abundant molecule for which both viruses exhibited similar, relatively high binding affinities.

To obtain additional evidence for an involvement of HS in binding of TBE virus to CHO cells, heparin inhibition experiments were performed. As shown in Fig. 4, heparin inhibited binding of both wild-type and mutant virus to CHO-K1 cells in a dose-dependent manner. As reported above, pgsD-677 and pgsA-745 cells bound less virus, but this value was not further reduced by the addition of heparin. This observation was consistent with the view that binding to CHO-K1 cells, but not to the two other cell lines, was mediated by HS.

Susceptibility of CHO cells to TBE virus infection

Since the wild-type and mutant CHO cell lines were found to differ significantly in their attachment capacities for TBE virus, we addressed the question of how this correlated to their susceptibility to infection. Sequential dilutions of wild-type and mutant virus were prepared and used to infect parental CHO-K1 cells as well as pgsD-677 and pgsA-745 cells, and endpoint dilution infectivity titers were determined. The resulting titers, shown in Fig. 5, demon-

Table 1
Dissociation constants of wild-type and mutant TBE virus attachment to cells

Cell line	K_d (nM)[a]		$K_{d(wt)}/K_{d(mutant)}$[b]
	wt virus	Mutant E(E122G)	
BHK-21	6.4 ± 1.1	0.52 ± 0.10	12
HeLa	6.0 ± 0.9	0.62 ± 0.17	10
CHO-K1	5.5 ± 1.1	0.42 ± 0.07	13
pgsD-677	0.8 ± 0.1	0.37 ± 0.14	2
pgsA-745	n.d.[c]	n.d.	—

[a] Values represent mean ± SEM of results obtained from at least four independent experiments.

[b] Ratio of dissociation constants reflecting the factor by which the binding affinity of the BHK-21 cell-adapted mutant was increased compared to wild-type virus.

[c] Not determined due to insufficient reproducibility of binding data.

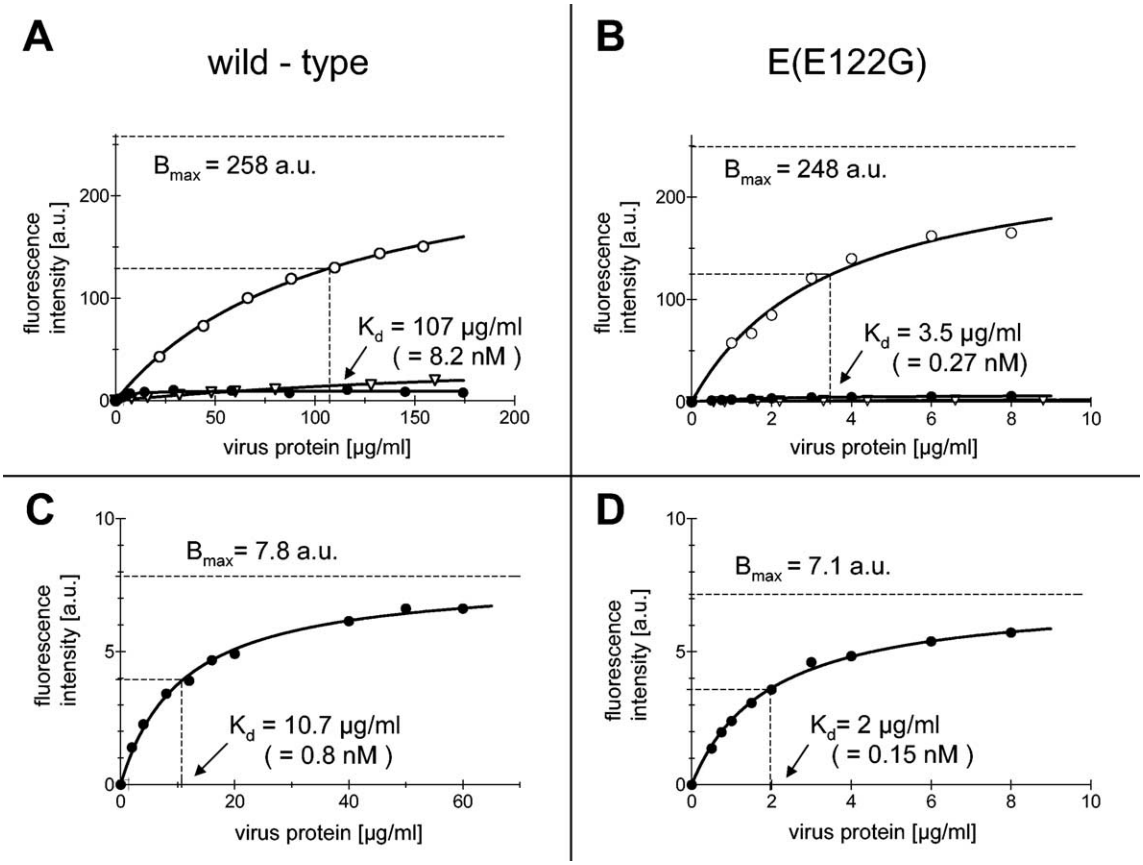

Fig. 3. Binding curves of wild-type (A and C) and BHK-21 cell-adapted mutant (B and D) virus binding to different CHO cell lines. (A and B) Comparison of binding to CHO-K1 cells (○), pgsD-677 cells (●), and pgsA-745 cells (▽) using the same batches of labeled virus. The derived B_{max} and K_d values for CHO-K1 cell attachment are indicated. (C and D) Binding curves obtained with pgsD-677 cells shown on a scale that is expanded compared to the representations above to show their hyperbolic nature. The derived B_{max} and K_d values for pgsD-677 cell attachment are indicated. a.u., arbitrary units.

strate that all three CHO cell lines were highly susceptible to infection by both wild-type and mutant TBE virus. Despite binding much less virus, the GAG-deficient cell lines were infectable with equally high or even higher dilutions of virus than CHO-K1 cells. This indicates that HS is not required for infection of CHO cells and implies the existence of another receptor that is less abundant than HS.

Discussion

Attachment and entry of viruses into their host cells are crucial steps of the viral life cycle that determine to a large extent the tropism and pathogenicity of a virus. In a classical situation, a virus binds to a specific receptor molecule with high affinity and this interaction triggers the subsequent uptake of the virus. However, these processes are frequently far more complex and involve more than one type of host cell molecule (Baranowski et al., 2001; Ugolini et al., 1999). The results presented in this article suggest that flavivirus–host interactions also involve more than a single host cell-surface molecule. HS was shown to be a major determinant of attachment, but the virus was also able to efficiently infect CHO cells lacking HS. This finding is compatible with the view that HS can serve as an attachment receptor for TBE virus, but entry itself may be mediated by other, less-abundant receptor molecules. Similar scenarios have been proposed for members of other virus families as well, e.g., in a recent study on human parainfluenza virus (Bose and Banerjee, 2002). At this time it cannot be excluded, however, that HS itself is also able to mediate entry of TBE virus. It will be necessary to carefully distinguish solely attachment-mediating molecules from receptor molecules that can mediate both attachment and entry into the host cell.

Several previous studies have demonstrated attachment of different flaviviruses to HS, and a few studies have also provided evidence for an importance of HS for viral entry (Chen et al., 1997; Germi et al., 2002; Hilgard and Stockert, 2000; Hung et al., 1999; Martinez-Barragan and del Angel, 2001). HS dependence has also been found to be a common feature of cell-culture-adapted flavivirus mutants (Lee and Lobigs, 2000; Mandl et al., 2001). In this study we were now able to demonstrate that both prototypic Western subtype TBE virus strain Neudoerfl and a BHK-21 cell-adapted mutant that differed from the parental strain only by a single

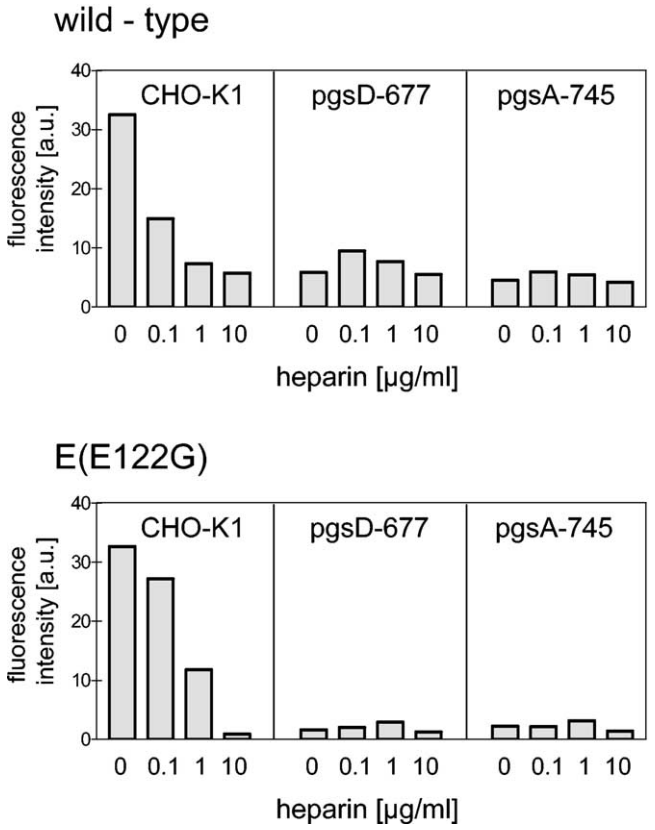

Fig. 4. Inhibition of virus binding to CHO cell lines by soluble heparin. Labeled virus was preincubated with various concentrations of heparin as indicated and then added to equal numbers of either CHO-K1 cells or the mutant CHO cell lines pgsD-677 and pgsA-745. Cell-associated fluorescence was quantified by FACS analysis. All experiments were performed using the same preparation of dye-labeled wild-type virus (top) at a concentration of 5.0 μg/ml or mutant E(E122G) virus (bottom) at a concentration of 0.5 μg/ml. a.u., arbitrary units.

amino acid substitution in protein E attached to cells mainly through HS, but the mutant bound with an approximately 10-fold higher affinity. This allows the conclusion to be drawn that the molecular basis of cell-culture adaptation was probably not the emergence of a new receptor specificity, but rather an improvement of an already existing capability. Previously applied tests, such as inhibition of growth by heparin or heparinase treatment, had not been sensitive enough to demonstrate the low-affinity HS-binding property of wild-type TBE virus (Mandl et al., 2001). The finding that mutations increasing the affinity for HS emerged rapidly during growth in BHK-21 cells indicated that they offered a decisive selective advantage in this growth environment (Mandl et al., 2001). As shown in a previous publication, these mutations increased the relative infection efficiencies in BHK-21 cells (i.e., they infected these cells at higher dilutions) but not in other cells, such as primary chicken embryo cells. The HS-adapted mutants of TBE virus also generally exhibit a lower physical stability than wild-type particles (Kroschewski and Mandl, unpublished observation). Thus, a higher affinity for HS may be helpful in certain cells for subsequent receptor interactions and may improve the kinetics of entry. The same property, however, could potentially be a disadvantage in other growth environments, impeding the release, stability, or spread of new virus particles (Bernard et al., 2000; Byrnes and Griffin, 2000; Klimstra et al., 1998; Lee and Lobigs, 2002; Mandl et al., 2001; Sa-Carvalho et al., 1997). A fine-tuning of its affinity for HS might allow a virus to optimize its evolutionary fitness in a particular growth environment. Potential low-affinity HS binding sites on the surface of wild-type protein E remain to be identified. Two potential sites have been proposed on the basis of sequence inspections for the dengue 2 virus protein E, but these predictions still await experimental verification (Chen et al., 1997). In the same study, the dissociation constant of dengue virus 2 binding to heparin was reported to be 15 nM, a value that is of the same order of magnitude as those obtained in this study for wild-type TBE virus attachment to HS. In the case of a member of a different virus family, adeno-associated virus 2, the K_d of virus–heparin interaction was determined to be 2 nM (Qiu et al., 2000), which lies between the values obtained for wild-type and mutant TBE virus in this study. Interactions of viruses with classical high-affinity receptors, such as influenza virus binding to MDCK cells (Nunes-Correia et al., 1999), usually exhibit several orders of magnitude higher affinities than the TBE–HS interaction, but in certain cases, such as poliovirus binding to HeLa cells (Bibb et al., 1994), the K_d value was found to be in the same range as that of TBE virus mutant E(E122G) attachment.

The nature of the molecule(s) that mediates attachment and entry on the mutant CHO cell lines remains unknown. It is also unclear whether the same receptor(s) is present and functionally important on other host cells. Virus binding to such low-abundance receptor molecules cannot easily be assessed in the presence of large quantities of cell-surface HS. Our data indicate that the host-cell molecule that me-

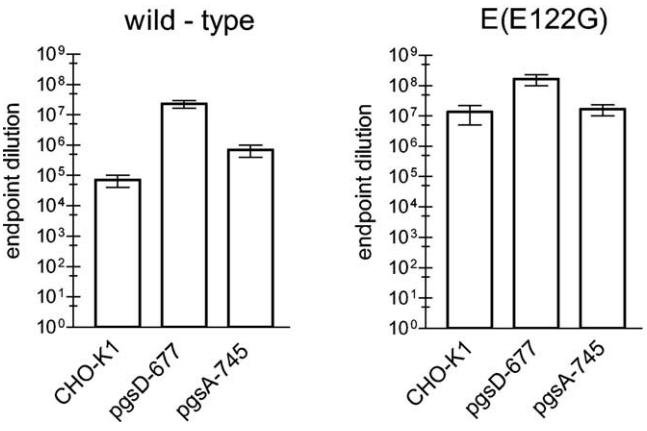

Fig. 5. Comparison of relative infectivity titers in different CHO cell lines. Titers were determined by endpoint dilution experiments (see Materials and methods). Each value is the mean of three independent experiments (error bars represent SEM).

diates attachment to pgsD-677 cells is approximately 20-fold less abundant than HS on CHO-K1 cells, but exhibits a significantly higher binding affinity for wild-type TBE virus. Interestingly, wild-type virus infected pgsD-677 cells at even higher dilutions than CHO-K1 cells (the differences in titers as shown in Fig. 5 are statistically significant), and it is tempting to speculate that this difference may be caused by the higher binding affinity of the unknown pgsD-677 receptor. Certainly, both pgsD-677 cells and pgsA-745 cells will be valuable tools in future studies for identifying host-cell-receptor molecules. The results obtained so far indicate that attachment and entry of flaviviruses is a complex process that involves more than a single type of host-cell-surface molecule.

Materials and methods

Virus strains and production of labeled and unlabeled TBE virus

Strain Neudoerfl, the prototype strain of Western subtype TBE virus, was used as the wild-type strain. It is a tick isolate with a very short passage history (Heinz et al., 1980) and its complete genomic sequence is available under GenBank Accession No. U27495. The generation and characterization of TBE virus mutant E(E122G) was reported previously (Mandl et al., 2001). This mutant differs from the wild-type strain Neudoerfl by only a single amino acid substitution (a glycine instead of glutamic acid at position 122 of protein E). The mutation was originally observed to arise during growth of TBE virus in BHK-21 cells and was introduced into the infectious cDNA clone to generate the recombinant mutant E(E122G). Among the available BHK-21 cell-adapted mutants, E(E122G) was chosen for this study because it exhibited a better physical stability than other mutants during the purification procedures. Infectivity titrations and virus productions were performed using high-titered virus stock preparations that were prepared and characterized previously (Mandl et al., 2001).

For the production of labeled or unlabeled virus, primary chicken embryo (CE) cells were infected and virus was harvested from the supernatants 48 h postinfection. Viruses were purified either by two cycles of sucrose density-gradient centrifugation (Heinz and Kunz, 1981) or by a slightly modified procedure involving polyethylene glycol precipitation and sucrose gradient centrifugation as described previously (Mandl et al., 2001). To obtain metabolically labeled virus, the fluorescent fatty acid analogue Bodipy FL C_{12} (Molecular Probes Europe BV, Leiden, The Netherlands) was added to the cell-culture medium 18 to 20 h prior to infection and maintained during the entire growth period at concentrations of 6 or 8 μM for mutant and wild-type virus production, respectively. Bodipy FL C_{12} is a highly photostable, nonpolar fluorophore that is excitable at 488 nm to green fluorescence emission with high quantum yield and low environmental sensitivity (Johnson et al., 1991). When added to the culture medium, the fatty acid linked probe is readily incorporated into phospholipids of cellular membranes (Kasurinen, 1992). The concentration of virus protein in the purified preparations was quantified by a four-layer enzyme-linked immunosorbent assay (ELISA) after sodium dodecyl sulfate denaturation (Heinz et al., 1994). In the case of wild-type virus, labeled preparations with virus protein concentrations of up to 400 μg/ml were obtained, whereas due to the lower physical stability of the mutant, protein concentrations of mutant preparations amounted to only 25 μg/ml. The fluorescence activities of these preparations were measured at 530 nm using a Perkin–Elmer LS 50B fluorescence spectrophotometer (slit width $\leq$ 15 nm, excitation at 488 nm). The specific fluorescence activity achieved by the applied labeling procedure varied among different preparations of wild-type and mutant viruses by a factor of less than 3.

The presence of the point mutation in protein E of fluorescent dye-labeled mutant E(E122G) was confirmed by RT-PCR and direct sequence analysis as described previously (Wallner et al., 1995).

Cell cultures and determination of infectivity titers

BHK-21, HeLa, and CHO cells were grown under standard conditions. The following three CHO cell lines were used: CHO-K1 is the parental cell line that is fully competent for glycosaminoglycan synthesis. The derivative mutant cell line pgsD-677 (ATCC CRL-2244) is unable to produce HS due to genetic defects affecting the enzymes *N*-acetylglucosaminyltransferase and glucuronyltransferase (Lidholt et al., 1992). pgsD-677 cells do, however, produce high levels of chondroitin sulfate (Lidholt et al., 1992). The second mutant CHO cell line, pgsA-745 (ATCC CRL-2242), is deficient for the enzyme xylosyltransferase and therefore produces only minimal levels of glycosaminoglycans (Esko et al., 1985). Infectivity titers on all of the three CHO cell lines were determined by endpoint dilution infection experiments as described previously (Mandl et al., 2001). Virus preparations were diluted in 0.5-log steps and used to infect cells in 24-well culture plates. The culture medium was checked for virus production at 3 and 6 days postinfection by a four-layer ELISA (Heinz et al., 1986).

Quantitative cell binding assay

CHO and HeLa cells were detached with phospate-buffered saline (PBS), pH 7.4, containing 2 mM EDTA at room temperature. BHK-21 cells were rinsed with PBS, pH 7.4, containing 0.3 mM EDTA, and then harvested by scraping. Subsequently, cells were washed once with binding buffer (50 mM HEPES, 100 mM NaCl, 1 mg/ml BSA (A-6003; Sigma), pH 7.4) and then suspended in 10 ml of this buffer. Cells were counted with a Casy1 TT cell counter (Schärfe System, Reutlingen, Germany), and this procedure was also

used to confirm the viability and singularity of cells. After counting, aliquots containing equal numbers of cells (1×10^5) were mixed with various amounts of fluorescence-labeled virus to give a final volume of 50 μl. These mixtures were incubated for 1 h on ice with constant gentle agitation and under protection from light. Unbound virus was removed by a single washing step using 4 ml of binding buffer. After pelleting, cells were resuspended in 300 μl of binding buffer and the amount of cell-associated virus was quantified using a FACS Calibur flow cytometer (Becton–Dickinson; 15 mW argon laser, 488 nm) with a 530/30 nm bandpass filter (FL-1) analyzing 10,000 events per sample. Cells incubated with only buffer containing no virus were subjected to the same procedure and used as a reference for instrument settings. Autofluorescence measured from these negative control cells was subtracted from each positive sample value. Evaluation of forward-scatter versus side-scatter measurements exhibited no alteration of cell sizes, indicating that there was no relevant cross-linking of cells by the virus. The viability of cells was confirmed by checking fluorescence values at longer wavelengths (FL-2, 585/30 nm bandpass; FL-3, 650 nm longpass). FL-1 median fluorescence activities (quantitatively expressed as arbitrary units) were calculated using Cell Quest Software (Becton–Dickinson) and this parameter was used for subsequent calculations. Median fluorescence values were plotted as a function of virus concentration yielding hyperbolic binding curves. Curve fitting using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) showed that the curve was best represented by a simple single-site binding model based on the law of mass action. Attempts to apply other binding models, such as two-site binding, did not improve the fitting (as expressed by the P value calculated by the program). Applying the algorithm for one-site binding, the maximum amount of bound virus (B_{max}; expressed in arbitrary units) and the equilibrium dissociation constant (K_d; equal to the virus protein concentration at half-maximum binding) could be calculated. Protein concentrations were transformed into molar concentrations taking the protein molecular weight of a TBE virus particle to be 13,000 kDa. This value is based on the postulation that each particle consists of 180 copies of each of the three structural proteins C, M, and E (Ferlenghi et al., 2001).

Trypsin digestion and heparin inhibition

Tryptic digestion of viral surface proteins was achieved by incubating 0.5 ng of labeled virus with various amounts (0, 0.05, 0.5, and 5 units) of trypsin (Worthington Biochemical Corp., Lakewood, NJ) in a reaction volume of 50 μl buffer (50 mM triethanolamine, 100 mM NaCl, pH 8.0) for 40 min at room temperature and then the reaction was stopped on ice. Subsequently, 5×10^5 HeLa cells in 20 μl binding buffer were added and specific binding was quantified as before.

Inhibition of virus binding to cells by heparin was tested by incubating a constant amount of labeled virus (250 or 25 ng of wild-type or mutant virus, respectively) with various amounts (0, 5, 50, and 500 ng) of soluble heparin (H-3393, from bovine lung; Sigma). The mixtures were incubated in binding buffer on ice for 30 min and then added to cells to determine binding as described above.

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