

Heparan sulphate mediates swine vesicular disease virus attachment to the host cell

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Heparan sulphate (HS) has been found to serve as receptor for initial cell binding of numerous viruses. Different glycosaminoglycans (GAGs), including heparin and HS, were analysed for their ability to bind swine vesicular disease virus (SVDV), a picornavirus with close homology to human coxsackie B5 virus. Binding of SVDV was established by heparin-affinity chromatography. In addition, infection of IB-RS-2 epithelial porcine cells was inhibited by treating the virus with soluble HS, heparin, and chondroitin sulphate B (CS-B), as well as by enzymic digestion of cell surface GAGs. Analysis of the infection course showed that SVDV uses cellular HS for its binding to the cell surface and that this interaction occurs during attachment of the virus, prior to its internalization into the cell. Sequence analysis of SVDV variants selected for their lack of sensitivity to heparin inhibition *in vitro* led to the identification of two residues (A2135V and I1266K) potentially involved in heparin/HS interaction. The location of these residues in a three-dimensional model shows that they are clustered in a well-exposed region of the capsid, providing a physical mechanism that could account for the heparin-binding phenotype.

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INTRODUCTION

Heparan sulphate proteoglycans (HS-PGs) are common cell-surface constituents of most cells and participate in numerous biological functions (Garret & Grisham, 1995; Kjellen & Lindahl, 1991). There is a growing list of viruses that have been shown to bind to HS. In most cases, this interaction provides an increased efficiency of viral attachment to host cells by facilitating the binding to another receptor. Thus, HS is considered as an initial receptor for a number of viruses (Compton *et al.*, 1993; Mettenleiter *et al.*, 1990; Okazaki *et al.*, 1991; WuDunn & Spear, 1989; Zhu *et al.*, 1995). While many enveloped viruses have been associated with HS binding (Asagoe *et al.*, 1997; Bernard *et al.*, 2000; Byrnes & Griffin, 1998; Chung *et al.*, 1998; Hulst *et al.*, 2000, 2001; Jusa *et al.*, 1997; Krusat & Streckert, 1997; Mondor *et al.*, 1998; Summerford & Samulski, 1998), the number of non-enveloped viruses reported to use HS for attachment to the host cell is limited. Among the *Picornaviridae* family, it has been shown that foot-and-mouth

disease virus (FMDV) binds to HS, although it reflects an adaptation to *in vitro* cultures (Fry *et al.*, 1999; Jackson *et al.*, 1996; Sa-Carvalho *et al.*, 1997). Within this family, there is evidence that coxsackie virus B3 (CVB3) (Zautner *et al.*, 2003), Theiler's murine encephalomyelitis virus (Reddi & Lipton, 2002) and certain echovirus serotypes (Goodfellow *et al.*, 2001) also bind cell-surface HS. However, many others (poliovirus 3, coxsackie B2 viruses and most echovirus serotypes) do not seem to interact at all with these compounds (Goodfellow *et al.*, 2001). The HS binding site has been characterized for FMDV (Fry *et al.*, 1999), Japanese encephalitis virus and Murray Valley encephalitis virus (Lee & Lobigs, 2002).

Binding of viruses to HS is usually electrostatic in nature and of low specificity. Viral proteins typically bind to HS through positively charged amino acid residues, and in some instances the HS-binding domain has been characterized (Flynn & Ryan, 1996; Trybala *et al.*, 1998). Frequently, in addition to a low-affinity co-receptor that initiates the cell attachment, virus entry into the cell depends on high-affinity receptors (Chen *et al.*, 1997; Hung *et al.*, 1999; Qiu *et al.*, 2000). The role of HS as a co-receptor has been suggested in several viral infections. Hence, there are

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examples of viruses that can bypass the binding to HS by using other alternative receptors and co-receptors (Baranowski *et al.*, 2000).

Swine vesicular disease virus (SVDV) is a picornavirus of the enterovirus genus that causes an emerging disease of pigs (SVD) whose symptoms are similar to those caused by FMDV (Nardelli *et al.*, 1968). The comparison of the complete genome sequences of SVDV and coxsackie B5 viruses (CVB5) reveals a close relationship between these two viruses (Zhang *et al.*, 1999). It has been demonstrated that SVDV is a subspecies of human CVB5 that arose as a result of an adaptation to swine. The divergence from a common ancestor has been estimated by phylogenetic studies to have occurred between 1945 and 1965 (Zhang *et al.*, 1999).

The initial events in the cycle of SVDV infection are not yet well characterized. It has been recently shown that, as in the case of coxsackievirus B1-6, the coxsackievirus-adenovirus receptor (CAR) is a functional receptor for SVDV (Martino *et al.*, 2000). Also, the decay-accelerating factor (DAF), used as co-receptor for coxsackievirus A21, B1, B3, B5, echovirus 6, 7, 11, and enterovirus 70 appears to have a role in SVDV entry into cells (Martino *et al.*, 2000). However, it has been shown that HS can be used as an alternative receptor for some picornaviruses, such as FMDV (Baranowski *et al.*, 2000) and CVB3 (PD strain) (Zautner *et al.*, 2003), in some conditions or when the 'classic' receptor is absent, a fact that supports the flexibility in picornavirus receptor usage. To investigate whether HS plays a role in SVDV infection, we have analysed the interaction of SVDV with HS and other glycosaminoglycans (GAGs).

METHODS

Viruses and cells and chemicals. IB-RS-2 pig kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 2 mM glutamine and 5% fetal bovine serum. IB-RS-2 monolayers were infected at various m.o.i. with SVDV (depending on the assay), strain SPA/2/93, and incubated at 37 °C for 24–48 h. The virus isolate was obtained from a vesicle of a naturally infected pig during the 1993 Spanish outbreak. To amplify the virus, this material was clarified and used to infect IB-RS-2 monolayers (passage 1). Virus passages were performed in IB-RS-2 monolayers and kept to a minimum (≤5) throughout this study in order to minimize adaptation to *in vitro* cell cultures. All HS binding and sequence analyses were performed with virus collected between 2 and 5 passages. Supernatant containing the virus was collected from the flasks when cytopathic effect (CPE) was maximum (24–48 h) and clarified by centrifugation at 8000 g for 20 min. The virus was then concentrated by ultracentrifugation through 2 ml 20% sucrose for 3 h at 110 000 g. For BIAcore analysis, the virus was purified on 10–35% sucrose gradients as described by Seechurn *et al.* (1990). SVDV was inactivated with 6 mM bromoethylamine (BEI) for 30 h at 28 °C (Bahnmann, 1975). Heparin, heparan sulphate (HS), chondroitin sulphate A, B and C, platelet factor 4 (PF4), and heparinases I and III were purchased from Sigma. 2-Bromoethylammonium bromide was purchased from Merck.

Table 1. Synthetic oligonucleotides used for RT-PCR of SVDV genomes

Primer	Oligonucleotide sequence (5' to 3')	Orientation
615D	CATATTGCTATTGGATTGGCCACC	Antisense
1715R	GCAGTCCATTATATTCAGCGCAC	Sense
2051D	TATGCCCATTTGGTCAGGCAG	Sense
2143R	GGTGGTGAGTATGCTAGCAAGAAC	Antisense
2802R	GTTAACTCCAGATCAAATCTTGC	Antisense
3317R	GTAGTTGCCGACGTACACAGCACC	Antisense
3502R	TCAAATGTGACTGGATAGTGCTT	Antisense

Nucleotide sequence analysis of SVDV. Viral RNA was extracted by standard techniques and subjected to RT-PCR using the SuperScriptII kit (Gibco-BRL) following the manufacturer's protocol. The oligonucleotides used for the RT-PCR are shown in Table 1. PCR products were purified with the Wizard PCR Preps DNA purification system (Promega) and sequenced at the Departamento de Secuenciación, Centro de Investigaciones Biológicas (CIB-CSIC, Spain).

Heparin-binding assay. SVDV (10⁴ p.f.u.) in 2 µl was incubated with 50 µl heparin bound to Sepharose beads (1 mg gel ml⁻¹), for 30 min with gentle rocking. As a control for non-specific binding, SVDV was incubated with Sepharose beads bound to protein G in similar conditions. Dilutions of the supernatants containing the unbound virus were incubated with IB-RS-2 (10⁴ cells per well) in 96-well plates, at 37 °C for 20 h. CPE was determined after cell fixation and staining with crystal violet, and quantified by methanol elution and OD reading at 620 nm (Jiménez-Clavero *et al.*, 2001). In each experiment, control wells of 0% CPE (without virus) and 100% CPE (complete lysis due to virus infection) were included, and the results were expressed as percentage cell survival.

Inhibition of infection assay. IB-RS-2 cells (10⁴ cells per well) were infected with 10⁴ p.f.u. per well of SVDV in the presence of duplicate dilutions of each soluble inhibitor (heparin/HS/CS-A, -B, -C, PF4) at 37 °C for 20 h. The inhibition of the CPE observed was quantified as above and expressed as percentage cell survival. To further study the effect caused by heparin, we performed the inhibition of infection assay in different conditions: (1) virus was incubated with heparin for 30 min at 37 °C prior to addition to cell cultures; (2) heparin was added to cultures of virus-adsorbed cells (cells were first incubated with SVDV at 4 °C for 30 min and washed with cold DMEM) and incubated for 1 h at 37 °C; and (3) cells were incubated with heparin for 30 min at 37 °C and washed before addition of the virus. All these experiments were performed using 100 p.f.u. SVDV per well. Cells were washed with medium at 1 h post-infection to remove non-adsorbed virus and cultures were incubated overnight at 37 °C. The inhibition of the CPE was determined as above.

SVDV infection of IB-RS-2 cells treated with heparinase I or III. Confluent monolayers of IB-RS-2 cells in 12-well plates were washed twice with DMEM and incubated with 0.2 ml 1.5 × 10⁻² U ml⁻¹ or 3 × 10⁻² U ml⁻¹ of heparinase I or with 0.5 × 10⁻² U ml⁻¹ or 10⁻² U ml⁻¹ of heparinase III for 1 h at 37 °C with gentle shaking. The cells were washed twice with DMEM and approximately 50 p.f.u. of SVDV in 0.2 ml DMEM were added. Following virus adsorption for 1 h at 37 °C with gentle shaking, the cells were washed twice with medium and overlaid with DMEM supplemented with 10% FCS and containing 0.6% agarose. The plaques were detected by staining cells with crystal violet solution after 30 h incubation at 37 °C.

Selection of SVDV variants with lost affinity for heparin.

SVDV variants (heparin-resistant variants, Hep^{res}) with lost affinity for heparin were selected by serial rounds of infection in the presence of increasing inhibitory concentrations of soluble heparin, following the method described for the selection of SVDV monoclonal antibody neutralization-resistant (MAR) mutants (Borrego *et al.*, 2002). SVDV (5×10^6 p.f.u. ml⁻¹) was seeded in 96-well plates over IBRS-2 cells and in the presence of heparin. Three rounds of selection–amplification were repeated, increasing the heparin concentration up to 1.5 mg ml⁻¹. Seven wells showing cell lysis in the presence of heparin were obtained in the last round. These SVDV variants were isolated and amplified in the presence of heparin for further analysis.

Location of mutations affecting heparin binding. The amino acid substitutions found in SVDV variants lacking the heparin-binding phenotype were initially mapped using an SVDV homology model (Jiménez-Clavero *et al.*, 2000). The crystal structure of SVDV recently determined (Jiménez-Clavero *et al.*, 2003; Verdaguer *et al.*, 2003) confirmed the location of these amino acid substitutions.

Surface plasmon resonance (SPR) analysis.

Preparation of surfaces of sensor chips. HS was biotinylated as described (Lookene *et al.*, 1996) and immobilized on avidin bound to the surface of the sensor chip by injecting a solution of HS-biotin (100 µg ml⁻¹ in HBS buffer: 10 mM HEPES; 0.15 M NaCl; 3.4 mM EDTA and 0.005% surfactant P20; pH 7.2) at 5 µl min⁻¹ continuous flow. The CM-5 sensor chip carboxymethyl surface was activated by a 7 min injection of 0.2 M EDC (*N*-ethyl-*N'*-dimethylamino-propylcarbodiimide), 0.05 M NHS (*N*-hydroxysuccinimide). Finally, avidin (100 µg ml⁻¹ in 10 mM sodium acetate buffer pH 5.5) and biotin (100 µg ml⁻¹ in HBS) were consecutively injected. Surfaces were then regenerated with 0.5 M NaCl in 10 mM NaOH, and the final two-step immobilization levels were of 2.5 ng avidin mm² and 0.2 ng HS-biotin mm², respectively.

Solutions. Serial dilutions of virus (41–1320 µg protein ml⁻¹) were prepared in HBS buffer. In samples for BIAcore analysis, heparin (1 mg ml⁻¹ in HBS) was used to dissociate the virus–HS complexes, and 1 M NaCl to regenerate the HS surfaces. Heparin solutions (0.062–16 µg ml⁻¹ in HBS) were employed in solution-affinity SPR experiments. The BIAcore 1000 instrument, the sensor chip CM-5, commercial HBS buffer, amine coupling kit NHS, EDC and ethanolamine were purchased from Biosensor AB.

Direct binding assays. All direct SPR analyses were run at a 5 µl min⁻¹ HBS flow and each virus preparation was injected at six different concentrations, ranging from 41 to 1320 µg protein ml⁻¹. Sensorgrams were generated by injections of virus solutions using 2 min association steps followed by 3 min dissociation in heparin (1 mg ml⁻¹) (co-injection mode). After an additional 3 min dissociation step in running buffer, the surfaces were regenerated by a 2 min pulse of 1 M NaCl without any measurable loss in baseline level or binding activity of the surface. Biosensor data were prepared, modelled and fitted using BIAevaluation 3.0.2 software (O'Shannessy *et al.*, 1993), and global curve fitting was done by non-linear least-squares analysis (Morton *et al.*, 1995) applied simultaneously to the entire dataset. The quality of the fitted data was evaluated by visual comparison between calculated and experimental curves as well as by the magnitudes of the chi-squared parameter. Initial binding rates were also measured from the linear slope of the sensorgrams at the initial stage of the association step (15 s after injection plug).

Competitive SPR assays. Calibration curves of initial binding rate vs virus concentration were built from data obtained in the direct SPR assay above and fitted to a four-parameter equation

(when possible) using the BIAevaluation 3.0.2 software. This equation was then used to calculate free virus concentrations in solution affinity assays. Virus–heparin interactions were determined by overnight incubation of different heparin concentrations (0.062–16 µg ml⁻¹) at 4 °C, with a constant virus concentration (660 µg protein ml⁻¹) in HBS. Virus–heparin mixtures were placed at 25 °C prior to injection on the HS surface for virus quantification. The amount of virus remaining free to bind the immobilized HS was quantified by measuring initial binding rates and extracting the corresponding virus concentrations from the relevant calibration curves. The variations of free virus with heparin concentration were plotted as inhibition curves.

RESULTS

Binding of SVDV to heparin and other GAGs

Heparin is a commercial derivative of its physiological homologue, heparan sulphate (HS). Heparin and HS have identical sugar chain composition and structure, but differ in characteristics such as the degree of sulphation, which is higher in heparin than in HS (Kjellen & Lindahl, 1991; Stryer, 1995). Heparin is thus commonly used as a substitute in HS–ligand interaction studies. To determine the ability of SVDV virions to bind heparin, a titrated virus preparation was subjected to affinity chromatography on heparin-Sepharose beads. SVDV (5×10^6 p.f.u. ml⁻¹) was incubated with heparin-conjugated beads at 4 °C for 30 min. The unbound fraction was collected and the virus titre was determined. As shown in Fig. 1, heparin-Sepharose beads retained SVDV strongly and specifically, as compared to the control using protein G-Sepharose beads. Nevertheless, some virus infectivity still remained in the flowthrough; this could have been due either to a saturation of the heparin-Sepharose beads used in these experiments, or to a subpopulation of non-heparin-binding viruses in the preparation. To address this question, the unbound fraction was rechromatographed and no infectivity was detected in the flowthrough (not shown).

In order to test the effect of heparin on SVDV infection *in vitro*, we analysed the effect of increasing amounts of soluble heparin on SVDV infection of IB-RS-2 cells. The infection was partially inhibited with 0.04 mg ml⁻¹ and completely inhibited with 0.1 mg ml⁻¹ of soluble heparin (Fig. 2A). The homologous human CVB5 (Faulkner strain) also showed an inhibition of infection like that of SVDV in similar experiments using the same cells; however, neither bovine enterovirus type 1 nor type 2 showed ability to bind heparin (not shown). This result indicated that soluble heparin completely abolishes SVDV infection *in vitro* at concentrations lower than those found for other heparin-sensitive picornaviruses, such as FMDV and echovirus (Goodfellow *et al.*, 2001; Jackson *et al.*, 1996).

The above results suggest that the inhibitory effect of heparin on SVDV infection appears to rely on its direct binding to the virus. However, to discard other possibilities, such as interaction of heparin with cell surface components or interference with cell activities necessary

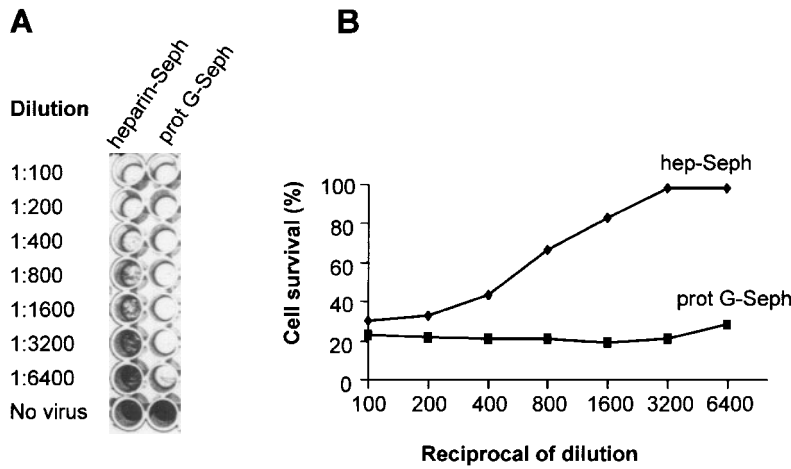


Fig. 1. Binding of SVDV to heparin-Sepharose. SVDV was incubated with heparin-Sepharose or protein G-Sepharose beads. The infectivity of the unbound fractions was titrated in IB-RS-2 cells. (A) Crystal violet staining of the cells remaining after infection. (B) The same experiment, expressed as percentage cell survival. The experiment was repeated five times, obtaining essentially the same result.

for SVDV infection, we performed *in vitro* infections with SVDV in three different conditions: (1) virus was incubated with doubling dilutions of heparin for 1 h at 37 °C in 1/10 final volume, and then added to cell cultures; (2) virus was allowed to attach to cell monolayers for 30 min at 4 °C, cells were washed and then heparin was added to the cultures; and (3) cells were incubated with heparin for 30 min at 37 °C, washed and then virus was added to the cultures. All these experiments were performed using 100 p.f.u. SVDV per well. Cells were washed with medium at 1 h post-infection to remove non-adsorbed virus and cultures were overnight incubated at 37 °C. The inhibition of the CPE was determined as described in Methods.

As shown in Table 2, the infection was completely inhibited, in a dose-dependent manner, in experiment (1), whereas no inhibition was observed in experiments (2) and (3). These results indicated that the heparin-mediated inhibition of SVDV infection was caused by a direct interaction with the virus, and that the interaction of heparin with the cells, if it occurs, had no effect on SVDV infection. Moreover, they showed that the presence of heparin inhibits the attachment of the virus to the cell surface, but once the virus is allowed to bind to the cell, the addition of heparin has no effect on the infection. Taken together, these results suggest that the effect of heparin on SVDV infection is a result of a competitive inhibition, hampering

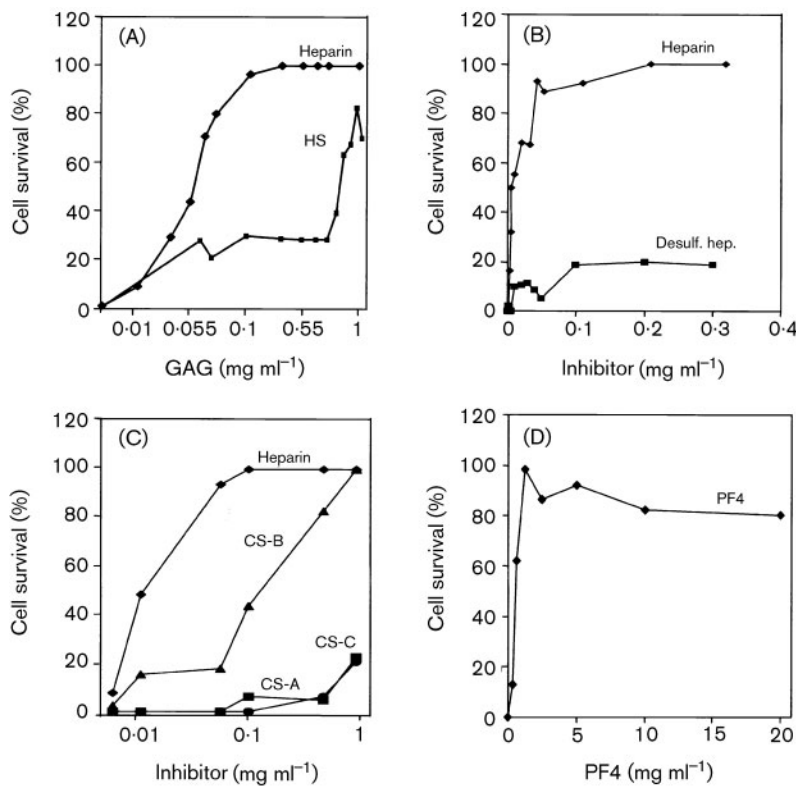


Fig. 2. Effect of GAGs on SVDV infection of IB-RS-2 cells. IB-RS 2 cells were treated with various concentrations of HS/heparin and infected with SVDV (A). SVDV was treated with various concentrations of: (B) heparin or de-*N*-sulphated heparin; (C) heparin, chondroitin sulphate A (CS-A), chondroitin sulphate B (CS-B) and chondroitin sulphate C (CS-C); (D) platelet factor 4 (PF4). The virus was incubated with the inhibitors prior to the infection of IB-RS-2 cells. Results are expressed as percentage of cell survival in absence of inhibitor. Values represent the mean of duplicate measurements.

Table 2. Effect of heparin on the SVDV infection of IB-RS-2 pig kidney cells

Heparin ($\mu\text{g ml}^{-1}$)	Cell survival (%)		
	Expt 1*	Expt 2†	Expt 3‡
0.5	94	0.05	8.6
0.25	73	3.9	4.6
0.125	60	0	8.1

*Incubation of heparin with the virus prior to the addition to IB-RS-2 cells.

†Addition of heparin to virus-adsorbed cells.

‡Incubation of heparin with cells prior to addition of the virus.

SVDV interaction with cell surface GAGs analogous to heparin, such as HS proteoglycan. To confirm this point, we carried out *in vitro* SVDV infections in the presence of different dilutions of soluble HS or heparin. As shown in Fig. 2(A), both HS and heparin inhibited SVDV infection, with HS requiring concentrations nine times higher than heparin (0.9 mg ml^{-1} vs 0.1 mg ml^{-1}) to obtain the same inhibitory effect. As these GAGs have the same basic sugar chain structure but differ in their degree of sulphation, it seemed likely that the differences in the inhibition potency observed between them were related to this characteristic. To assess this hypothesis, we performed the same assay using desulphated heparin in parallel with normal heparin. Irrelevant (<20% cell survival) inhibition was observed when adding up to $300 \mu\text{g ml}^{-1}$ of desulphated heparin, whereas a typical inhibition curve (>90% cell survival at $40 \mu\text{g ml}^{-1}$) was observed in the presence of normal heparin (Fig. 2B). To further analyse the effect of different GAGs on the inhibition of SVDV infection, we carried out the same inhibition assay in the presence of chondroitin sulphate (CS)-A, CS-B (also known as dermatan sulphate) and CS-C. All of them are sulphated, but in different positions and/or with different sugar chain constituents. Besides heparin, only CS-B (dermatan sulphate) caused a relevant inhibition of the SVDV infection, similar to that obtained with HS. Neither CS-A nor CS-C showed any effect on SVDV infection at the concentrations tested (Fig. 2C). To further determine the physiological relevance of cell surface HS for SVDV binding we studied the effect of platelet factor 4 (PF4), a small basic growth factor that binds to heparin and to cell surface HS, on SVDV infection. As shown in Fig. 2(D), the infection was strongly inhibited in the presence of increasing amounts of PF4, indicating that cell surface glycoconjugates such as HS were needed for SVDV infection *in vitro* in IB-RS-2 cells.

Studies with glycanases were undertaken as an additional approach to assess the physiological relevance of cell surface HS for SVDV attachment. IB-RS-2 cells were treated with heparinase I, which degrades heparin and HS, or with heparinase III, which degrades only HS. After the enzyme treatment the cells were washed and incubated with SVDV.

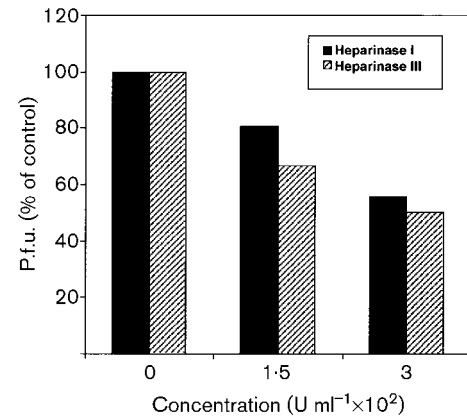


Fig. 3. Relative effect of heparinase I and III on SVDV infection to IB-RS-2 cells. Duplicates of heparinase-treated or untreated cells were washed and infected with SVDV. Viral plaques were counted after incubation of cells for 12 h. The number of p.f.u. formed on heparinase-treated cells is expressed as a percentage of untreated controls. Values shown correspond to results of one experiment representative of five independent experiments.

Cell treatment with heparinase I reduced viral infectivity by up to 45% and treatment with heparinase III resulted in a 50% decrease in infection (Fig. 3). The heparinase effect on cell infection was eliminated by addition of soluble HS during the treatment (not shown), indicating that the reduction in infectivity was caused by the degradation of cellular HS.

Characterization of the heparin-resistant variants

Like other RNA viruses, SVDV has a high mutation rate that allows it to adapt rapidly to changing environments. Based on this characteristic, we wanted to obtain viral variants lacking the ability to bind heparin. With this aim, we followed a protocol similar to the method used to select MAR mutants. This approach involved three steps: (1) to select SVDV variants lacking the ability to bind – and thus be inhibited by – heparin/HS (henceforth referred to as ‘heparin resistant variants’, Hep^{res}); (2) to determine the nucleotide sequences of the variants and identify those amino acid changes – if any – in the capsid proteins, that could be associated with this heparin-resistant phenotype; and (3) to locate these amino acid changes in the three-dimensional (3D) structure of the virus.

Phenotypic selection of variants not inhibited by heparin was carried out by infecting IB-RS-2 cells with SVDV in the presence of increasing concentrations of soluble heparin, as described in Methods. After three rounds of selection – the last of them in the presence of 3.25 mg ml^{-1} – seven SVDV variants showing a complete lack of susceptibility to inhibition by soluble heparin (Hep^{res} phenotype) were isolated. Nucleotide sequence

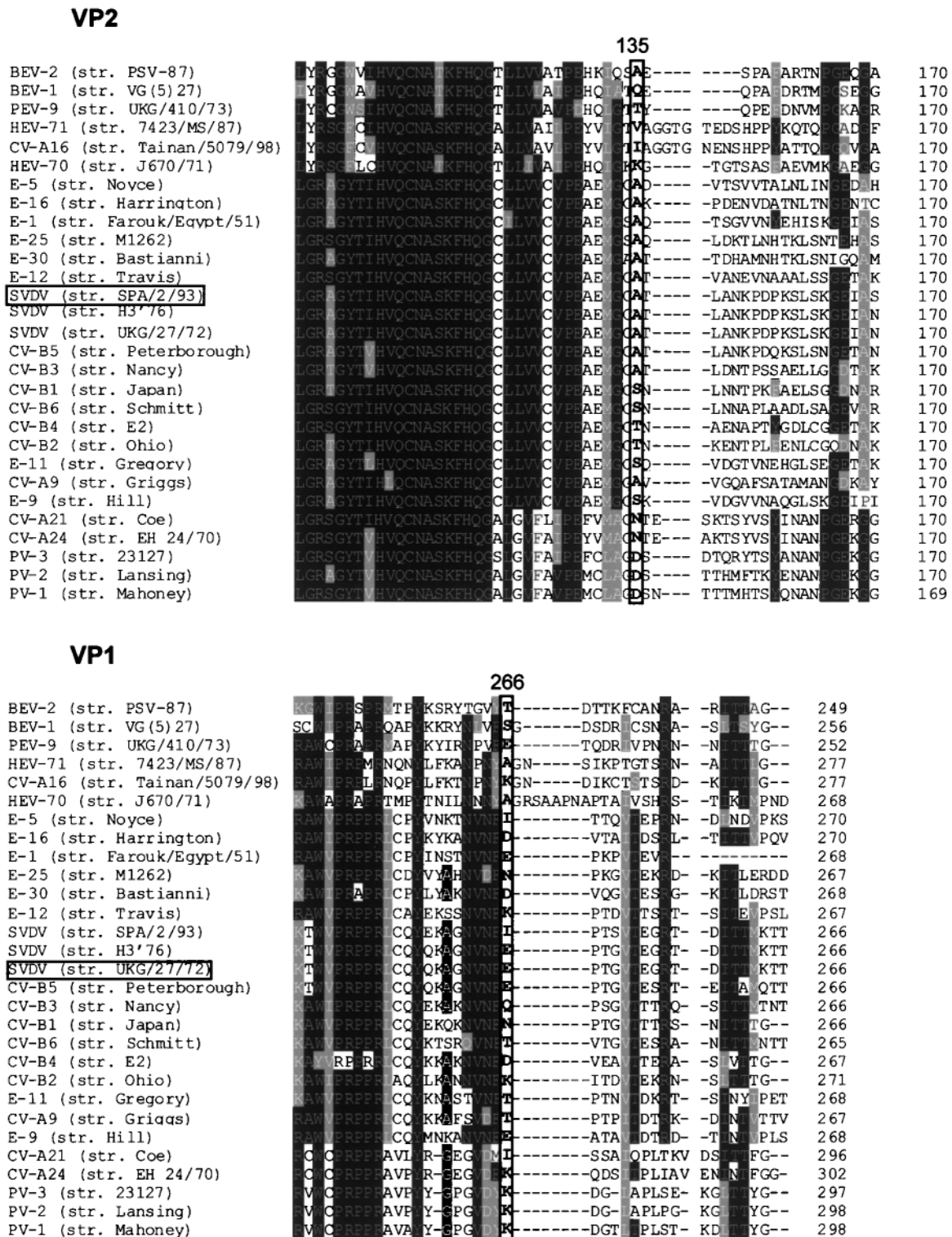


Fig. 4. Amino acid sequence alignments of different SVDV isolates, and the Hep^{res} SVDV described in this work. The amino acid sequences of the regions surrounding the substitutions found in the Hep^{res} SVDV variants are shown, including viruses representative of almost every species of the genus *Enterovirus*: poliovirus (PV-1, PV-2, PV-3), human enterovirus A (CV-A16, HEV-71), human enterovirus B (CV-B1 to CV-B6, SVDV, CV-A9, E-1, E-5, E-9, E-11, E-12, E-16, E-25, E-30), human enterovirus C (CV-A21, CV-A24), human enterovirus D (HEV-70), bovine enterovirus (BEV-1, BEV-2) and porcine enterovirus B (PEV-9).

analysis of the structural (P1) region of the genome of each of the seven variants revealed that they were identical in this region; thus we considered them as being the same viral variant, perhaps present in the original virus population in undetectable amounts. Comparison of nucleotide-derived amino acid sequences obtained from the parental isolate and the Hep^{res} variant revealed two amino acid substitutions in the capsid: one conservative (A/V) at position 135 of VP2 (A2135V) and one non-conservative (I/K) at position 266 of VP1 (I1266K). Multiple sequence alignments showed that there was no overall conservation of the region around these two positions in the *Picornaviridae* (Fig. 4).

The ability of the parental isolate and the Hep^{res} variant to bind HS and heparin was analysed using SPR (Biacore). The results in Fig. 5 show that the parental virus bound to solid-phase HS, being partially dissociated by heparin,

indicating a high avidity of the HS–virus interaction. The remaining virus bound to solid-phase HS was dissociated with 1 M NaCl. On the other hand, as expected, none of the Hep^{res} virus particles interacted with solid-phase HS, as shown by the absence of bound virus after sample injection and by the square-wave-shaped sensorgrams. These sensorgrams show typical bulk refractive index (RI) jumps due to sample/running buffer RI mismatch; these were equally observed on an underivatized sensor chip surface (not shown). In addition, soluble heparin was able to compete in a dose-dependent manner with immobilized HS for the binding of the parental SVDV isolate (Fig. 5D), providing further evidence that both HS and heparin share the same binding site on the SVDV capsid.

The 3D arrangement of the amino acid positions substituted in the Hep^{res} variant is shown in Fig. 6. The two positions, although in different structural chains, are

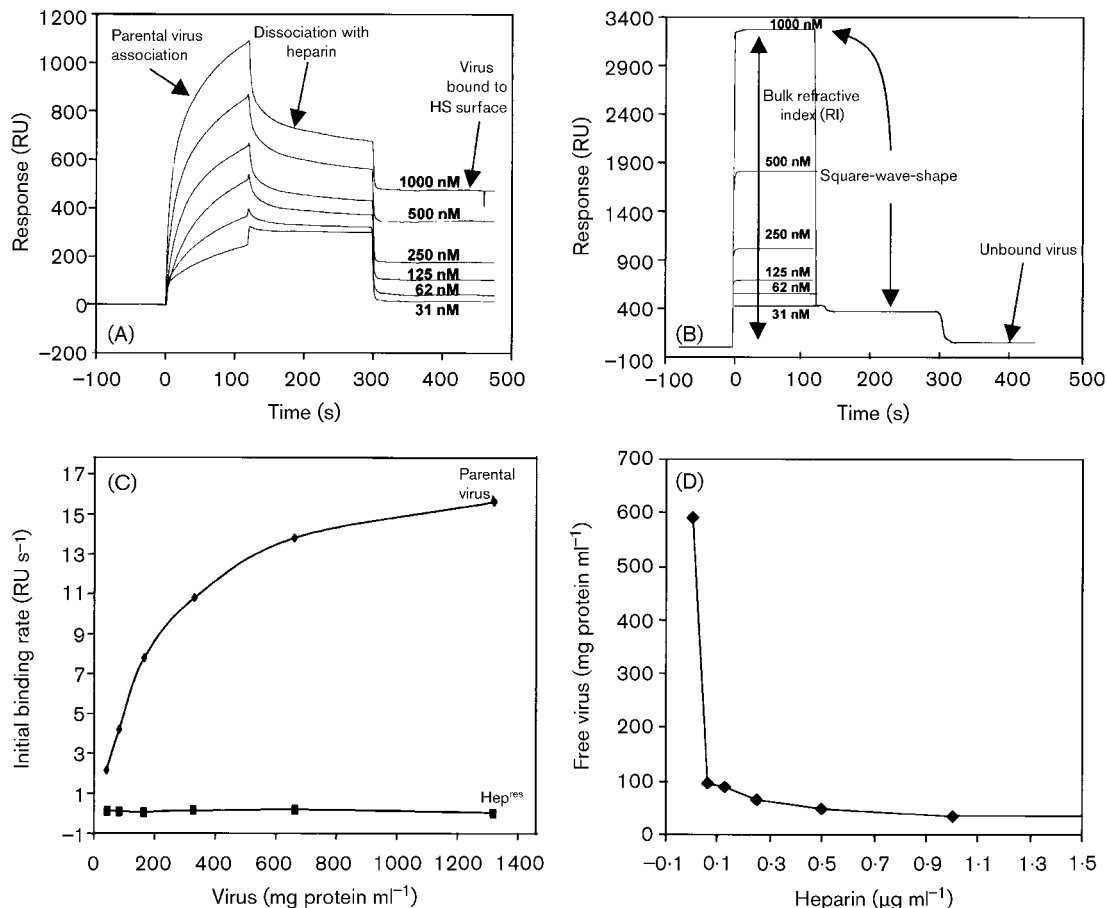


Fig. 5. Specific interaction of SVDV with solid-phase HS analysed by SPR (Biacore). (A, B) Direct binding assays. Three-step sensorgrams corresponding to an association, a dissociation with heparin and a dissociation in running buffer phases of (A) parental SVDV and (B) a Hep^{res} variant (representative of all the Hep^{res} variants). (C) Binding of virus to heparan sulphate. Only the parental SVDV isolate displayed a pronounced dependence of initial binding rate to immobilized HS. Of the seven Hep^{res} isolates analysed, only one is plotted, being representative of the result obtained with all the clones. (D) Competition binding assay. Virus was incubated with different heparin concentrations for 9 h at 4 °C. The fraction of unbound virus, able to bind the immobilized HS surface, was quantified as described in Methods. RU, resonance unit.

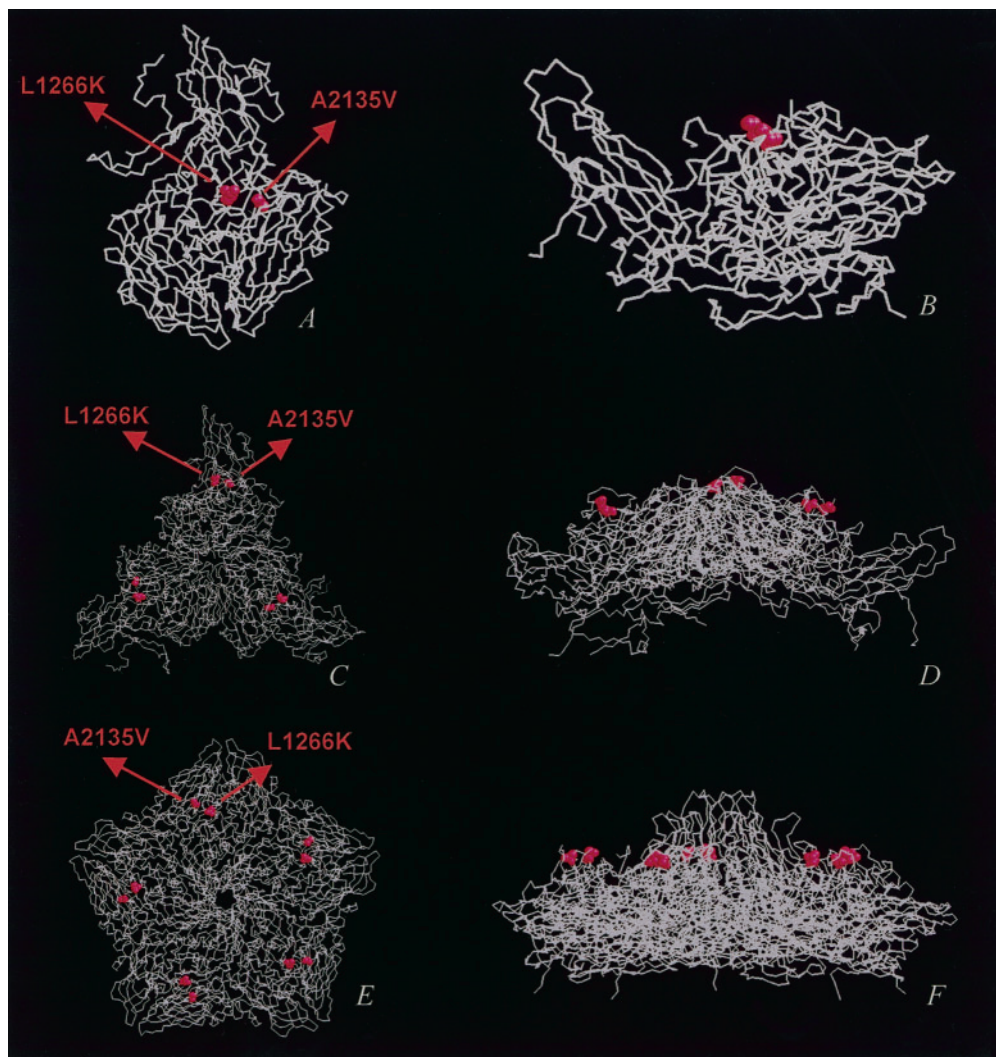


Fig. 6. Location of the amino acid substitutions found in Hep^{res}, on the 3D model of the SVDV capsid. Frontal (A) and lateral (B) view of a single protomer. Frontal (C) and lateral (D) views of three protomers centred on the threefold axis. Frontal (E) and lateral (F) views of five protomers centred on the fivefold axis. Positions of amino acid substitutions are shown as red spheres.

located close to each other in the SVDV 3D capsid structure. Furthermore, the distribution of the electrostatic potential in this region shows a strongly basic depression in the neighbourhood of the positions identified as involved in heparin/HS binding (not shown). The presence of a basic (Lys) residue protruding in close vicinity with this depression could cause a significant distortion of the interaction, resulting in a loss of binding to heparin/HS.

DISCUSSION

A large number of viruses, including several picornaviruses, utilize cell surface HS for their initial binding to the cell surface (Reddi & Lipton, 2002; reviewed by Bernfield *et al.*, 1999). Picornaviruses are known for their genetic plasticity and ability to interact with cells from different origins, facilitated by the possibility of using alternative receptors,

usually widely expressed molecules such as cell surface GAGs (Baranowski *et al.*, 2000), and adapting rapidly to different circumstances. Based on these observations, we considered that HS could be a possible receptor or co-receptor mediating the binding of SVDV to the cell surface, or have a role as an alternative receptor. To determine the role that GAGs could play in the initial steps of SVDV infection, we chose a recent SVDV isolate, SPA/2/'93, which is representative of the SVDV variants currently circulating in Europe. 'Recent' isolates have evolved from the old variants through changes in the capsid that appear to relate to a 'refinement' of the structures interacting with cell receptors; thus they are considered better adaptations to pigs than 'old' (pre-1971) isolates (Fry *et al.*, 2003). Direct binding of SVDV to heparin, a sulphated analogue of cell surface HS, was first assessed by affinity chromatography on heparin-Sepharose columns. HS binding by some viruses,

such as FMDV, has been demonstrated to be an adaptation to cell cultures (Sa-Carvalho *et al.*, 1997); however, there are examples of other enteroviruses that seem to bind HS *in vivo* (Goodfellow *et al.*, 2001). To minimize the possibilities of adaptation to cell culture, the viral isolate that we used for this study has been passaged no more than five times in IB-RS-2 cells. However, taking into account the high rate of mutation in this group of viruses, the possibility of this adaptation cannot be ruled out. Several studies have pointed out the flexibility in picornavirus receptor usage (Baranowski *et al.*, 2000; Zautner *et al.*, 2003). It seems a common characteristic of these viruses to easily adapt to bind a different receptor by replacing a few or even a single amino acid residue of a surface protein. Thus, in FMDV an amino acid replacement resulting in loss of HS recognition leads to a change in the receptor usage from HS to integrin. Similarly, amino acid substitutions in VP1 of CVB3 PD allow the virus to use both specific HS and CAR receptors, enhancing its virulence *in vitro*. The possibility to use alternative receptors for entry even into the same cell type, provides a potential advantage for these viruses for infecting their hosts. In this study, we have shown that a couple of amino acid substitutions in the capsid proteins of SVDV may result in loss of HS recognition, and these viruses are still fully infectious in the same cells under similar conditions. Therefore, it is likely that SVDV, like FMDV and CVB3, can use HS as an alternative receptor, being capable of using other mechanisms of cell entry under certain conditions.

The assays used to investigate the mechanism of the HS–virus interaction indicated that the interaction of SVDV with cellular HS occurs during cell attachment, but once the virus is bound, addition of HS does not affect the process of infection. Therefore, as proposed for other viruses, the binding of SVDV to cellular GAGs probably mediates an early step of the virus–cell interaction, facilitating the subsequent recognition of other receptors such as CAR (Martino *et al.*, 2000). However, it cannot be ruled out that HS is being used as an alternative receptor. Inhibition of viral infection by soluble heparin is a conventional approach to determine the ability to bind to heparin by many viruses. It has been recently published that among echoviruses (EV), the species most sensitive to the inhibition by heparin are EV 9 and EV 25, requiring $125 \mu\text{g ml}^{-1}$ to inhibit the infection. In contrast, some EV such as 1, 4, 12 and 13, as well as other enteroviruses like PV3, CVB2 and CVB3, are insensitive to heparin up to 2 mg ml^{-1} (Goodfellow *et al.*, 2001). Using the same method, SVDV appears to be especially sensitive to inhibition by heparin, since only $100 \mu\text{g ml}^{-1}$ completely inhibited the infection of IB-RS-2 cells in comparable conditions.

The role of cell surface GAGs in SVDV attachment was further assessed by treating IB-RS-2 cells with heparinase I and III prior to the incubation with the virus. In these experiments, the infectivity of SVDV was strongly reduced after treatment with heparinase III, whose principal

substrate is HS, and to a lesser extent with heparinase I, whose substrate is heparin. Our data therefore support the hypothesis that SVDV interacts with cell surface HS during the process of infection.

It has been shown that HS interactions with proteins are mostly electrostatic in nature, the positively charged amino acid residues interacting with the negatively charged sulphates (Fromm *et al.*, 1995). This is probably the case for HS–SVDV interaction, since there was a good correlation between degree of sulphation and inhibition of infection. Thus, the most effective substrate for binding was heparin, which is the most sulphated, followed by HS and dermatan sulphate, while chondroitin sulphates A and C were less effective. In addition, the failure of SVDV to bind desulphated heparin corroborated this hypothesis. However, the carbohydrate backbone of the GAG may add specificity to the interaction, resulting in the relatively high affinity of the SVDV–HS interaction observed in the SPR analysis.

The rapid appearance of heparin resistant (Hep^{res}) variants, after three passages in heparin-containing cultures, suggests either a high rate of the mutation(s) responsible for this phenotype, or alternatively, the presence of a minor population of a viral variant resistant to heparin, which was not detected in the heparin–Sepharose chromatography experiment. The results of sequence analysis of the seven Hep^{res} isolates, showing that all of them had the same two substitutions in the VP1 and VP2 proteins, respectively, suggest that they might be the same SVDV variant, present in undetectable amounts in the original population. The analysis of the kinetics of virus–heparin binding by SPR showed that the association and dissociation rate values of the parental SVDV corresponded to a high-affinity interaction. In contrast, the sensorgrams corresponding to the seven Hep^{res} isolates were similar and indicated a lack of interaction with heparin. Analysis of the amino acid sequences of the P1 structural polyprotein of the Hep^{res} variant revealed that the two substitutions in VP1 and VP2 proteins were located in the same region on the 3D model of the SVDV protomer, defining a potential HS-binding domain that is well exposed on the viral capsid (Fig. 6). Interestingly, this region almost overlaps with a cluster of amino acids that are changed between old and recent SVDV isolates, and located in the neighbourhood of, but not overlapping, the CAR footprint (Fry *et al.*, 2003). The region homologous to this cluster in the echovirus 7 capsid has been implicated in DAF binding (He *et al.*, 2002). Pig DAF contains homologues for only three out of the four SCRs that are encoded by human, rat and mouse DAF. Sequence comparison indicates that it is the fourth SCR that is missing. Moreover, the accumulation of changes in the putative DAF-binding site in the recent SVDV isolates suggests adaptive modifications to a different interaction, perhaps with the pig homologue of human DAF, or with a porcine molecule still not identified. In this context, cell surface HS may be a good candidate. Whether HS substitutes DAF as the attachment receptor in the pig, or

participates in the cell binding as an alternative receptor, remains to be studied.

The loss of heparin binding by the Hep^{res} variant is not straightforward, based on the electrostatic charge of the amino acid substitutions. There is a conservative substitution A2135V in the VP2 protein and a non-conservative one I1266K in the VP1 that does not imply the loss of a positively charged amino acid. The two substitutions are close to each other and adjacent to a region of relatively high concentration of basic amino acids, characteristic of the heparin-binding domains of proteins. Three lysines, K1253, K1258 and K1259, at the C terminus of VP1 and one arginine R3073 in the BC loop of VP3 contributed to this region. The crystal structure of FMDV serotype O in complex with HS (Fry *et al.*, 1999) showed the HS binding site of the virus in a depression of positive electrostatic charge on the capsid, contributed by the three surface proteins: VP1, VP2 and VP3. The predicted HS binding for SVDV maps near, but does not overlap, the FMDV HS binding site (Fry *et al.*, 1999; Verdaguer *et al.*, 2003). Among the two amino acid substitutions that seem to be involved in the HS binding site, position 1266 is relatively variable among other SVDV variants. However amino acid 2135 is conserved among old and recent SVDV isolates, including CVB5 (Verdaguer *et al.*, 2003). As indicated above, CVB5 also binds HS; therefore it seems that 2135 might be more important than 1266 for maintaining the HS binding site functional. The most likely explanation for these findings is that this basic domain is involved in the binding to HS, and that slight differences in the amino acid sequences in an adjacent region might produce a strong difference in binding activity. Taken together, these studies indicate a role for GAGs in SVDV attachment: the virus probably binds highly sulphated forms of cell surface HS, which is likely to provide the initial interaction step during SVDV infection.

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