The structure and function of a foot-and-mouth disease virus–oligosaccharide receptor complex

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Introduction

Animal viruses infect host cells by the recognition of specific receptor molecules on the cell surface, followed by internalization and the transfer of viral nucleic acid, or nucleoprotein core, to the cytosol. These processes are important determinants of tissue tropism and key points where natural selection operates, yet despite intensive studies, the underlying molecular mechanisms remain largely obscure in terms of detailed structural descriptions. The small, icosahedral picornaviruses are particularly well studied both functionally and structurally (Rueckert, 1996); little is known of the structural details of how they recognize their receptors, with the exception of a low resolution cryo-electron microscopic (EM) reconstruction of a human rhinovirus associated with a soluble fragment of its intercellular cell adhesion molecule-1 (ICAM-1) receptor (Olson et al., 1993).

Foot-and-mouth disease virus (FMDV) causes a highly infectious disease in cloven-hooved animals, which can be economically devastating. It exists in seven serotypes (O, A, C, Asia, SAT1, SAT2 and SAT3), and has the basic picornavirus structure depicted in Figure 1 (terms defined there will be used throughout this paper). FMDV enters cells through receptor-mediated endocytosis followed by acid pH-dependent release and translocation of RNA across the endosomal membrane (Carrillo et al., 1984; Mason et al., 1994; Curry et al., 1995). FMDV can attach to cells via arginine–glycine–aspartic acid (RGD)-binding integrin molecules (Surovii et al., 1988; Fox et al., 1989; Baxt and Becker, 1990; Mason et al., 1994; Berinstein et al., 1995), such as αvβ3 (Berinstein et al., 1995; Jackson et al., 1997; Neff et al., 1998). The RGD tripeptide motif that recognizes the integrin is located in a long, flexible loop on the surface of the virion (Logan et al., 1993), and is found on all seven serotypes. Whilst mutations in this RGD motif are usually lethal (Mason et al., 1994) [except for viruses repeatedly passaged in tissue culture (Martinez et al., 1997)], competition studies (Sekiguchi et al., 1982) suggested that serotypes O and A use more than one receptor for cell attachment. This was clarified by the demonstration that for certain strains of O1 FMDV, the predominant cell surface ligand is heparan sulfate (HS) (Jackson et al., 1996), and that attachment is both highly specific and necessary for efficient infection.

Heparan sulfate has an important role in cell entry by foot-and-mouth disease virus (FMDV). We find that subtype O1 FMDV binds this glycosaminoglycan with a high affinity by immobilizing a specific highly abundant motif of sulfated sugars. The binding site is a shallow depression on the virion surface, located at the junction of the three major capsid proteins, VP1, VP2 and VP3. Two pre-formed sulfate-binding sites control receptor specificity. Residue 56 of VP3, an arginine in this virus, is critical to this recognition, forming a key component of both sites. This residue is a histidine in field isolates which provides the potential for discriminating between different cell types through specific protein–sugar interactions. Residue 56 of VP3, an arginine in this virus, is a conserved characteristic of the virus, switching to an arginine in adaptation to different cell types. This residue is a histidine in field isolates which provides the potential for discriminating between different cell types through specific protein–sugar interactions. Residue 56 of VP3, an arginine in this virus, is a conserved characteristic of the virus, switching to an arginine in adaptation to different cell types. This residue is a histidine in field isolates which provides the potential for discriminating between different cell types through specific protein–sugar interactions.

Sulfated glycosaminoglycans (GAGs), such as HS, are increasingly implicated in cell adhesion, in line with their ubiquitous distribution on cell surfaces as the carbohydrate component of proteoglycans (Salmivirta et al., 1996). These polymers of disaccharide repeats are highly sulfated. The basic repeating unit of heparin is L-iduronic acid (Idu) and D-glucosamine (GlcN) joined by an α(1→4) linkage (Figure 1). HS has a complex heterogeneous structure, which provides the potential for discriminating between different cell types through specific protein–sugar interactions. These properties of HS, and the observation that FMDV O1 BFS binds HS without the mediation of any other cellular component, led to the suggestion that infection might be initiated by two-step attachment, initial contact being with a low affinity HS proteoglycan receptor, followed by transfer to the high affinity integrin receptor for endocytosis (Jackson et al., 1996). A growing list of viruses use distinct receptors for attachment and internalization, including adenovirus type 2 (Wickham et al., 1993), several herpesviruses (WuDunn and Spear, 1989; Mettenleiter et al., 1990; Compton et al., 1993) and human immunodeficiency virus type 1 (HIV-1) (Feng et al., 1996; Clapham and Weiss, 1997; Wagner et al., 1998).

To understand how HS facilitates cell entry by FMDV, we have undertaken a quantitative study of the way cellular HS binds FMDV and enhances infection, together with a structural analysis of the receptor–virus complex.
We here report high resolution crystallographic analyses for FMDV O1BFS complexed with various heparin and HS preparations. We have also extended the study to field isolates of FMDV, strain O1Kaufbeuren (O1K), closely related to O1BFS. We find that the change of a single residue has profound biological consequences, effected through modulation of HS interactions.

Results

Receptor activity of HS

Measurements of the receptor activity of cell surface HS were made with two closely related FMDV strains O1BFS and O1KB64, both of which previously have been shown to depend on HS for their ability to produce plaques in cell monolayers (Abu-Ghazaleh, 1996). To quantify this dependence, an infectious centre assay was used to compare productive infectious events in wild-type CHO cells and the derivative cell line, psgD-677, which specifically lacks HS (Esko et al., 1987). We previously have reported that the inability of psgD-677 cells to support plaque formation is caused by a defect in virus uptake, rather than in replication, and that this defect is directly attributable to the absence of HS rather than to any reduction in the expression of integrin receptors (Jackson et al., 1996). By assaying infectious centres, we can see from Figure 2 that the HS-deficient cells are infectable by FMDV, but at a rate ~100-fold lower than that of their HS-bearing progenitors. The mechanism of the residual infectivity is unknown.

HS-binding sites could not be quantified on live CHO or MDBK cells, as the Scatchard plots were anomalous (data not shown). On paraformaldehyde-fixed MDBK cells, however, the receptors could be resolved reproducibly into two distinct components (Figure 3A), a minor one (~5×10^3 sites/cell) having an apparent $K_d$ of ~2×10^{-11} M, and a major component present at ~0.5×10^6 copies per cell, apparent $K_d$ ~10^{-9} M. As the overwhelming majority of virus binding by MDBK cells is mediated through HS (Jackson et al., 1996), the major component of the FMDV receptor population can be attributed to HS. This confirms that HS is an abundant high-affinity ligand for FMDV O1BFS on fixed cells. The affinity of HS on live cells, while not directly measurable, is likely to be higher still. A clue to just how much higher can be gained by comparing fixed and live cells for susceptibility of virus binding to competition by heparin. We have reported previously that heparin competes with HS on fixed cells at extremely low concentrations, with an IC_{50} in the range of 10–100 ng/ml for all cell types tested, including BHK-21, MDBK and CHO (Jackson et al., 1996; data not shown). Figure 3B shows that, with live
cells by contrast, \(10^{-3}\)-to \(10^{4}\)-fold more heparin is required for 50% inhibition of virus binding, implying that the affinity of live cell HS for FMDV exceeds that of fixed cell HS by a comparable number of orders of magnitude. Competition was studied at both low and high virus/cell multiplicities, the latter to eliminate complications due to the presence of small numbers of high affinity integrins. The main difference between the two plots of Figure 3B is not, however, in IC50, which is relatively insensitive to the number of virus particles per cell, but in the enhancement in binding at intermediate heparin concentrations, presumably due to virus clumping; a smaller ‘kink’ was also seen reproducibly in the high specific activity virus plot at the same heparin concentration.

**Crystallographic analyses of O1BFS–HS receptor complexes**

We have determined the structures of complexes of FMDV O1BFS with HS by analyses of crystals of the virus soaked with each of two different molecular weight species of porcine heparin (approximate mean mol. wts 6 and 3 kDa) and with porcine HS. Data were collected at either 10 or 20 mg/ml concentration of sulfated oligosaccharide. Table I contains a summary of the various data collected. The centred lattice of the O1BFS crystals is 50 Å, ample to permit diffusion of a 6 kDa sugar (a linear chain of ~10 disaccharide units). Data were collected to 1.9 Å (6 kDa soaked crystals) and 2.5 Å (3 kDa and HS soaked crystals). Difference maps calculated against the native O1BFS data \(F_{o}(\text{soak}) - F_{o}(\text{native})\) showed clear electron density for three saccharide units and weaker density corresponding to a further saccharide at either end. The electron density was rather blurred, suggesting either mobility or conformational heterogeneity for the bound oligosaccharide. An additional feature of these unbiased maps was the improved order of the VP1 C-termini beyond the point of interaction with the sugars at residue His195. Difference maps calculated between the various sets of heparin and HS soaked data \(F_{o}(\text{soakA}) - F_{o}(\text{soakB})\) were essentially featureless, indicating that a common set of sulfated sugars was bound in similarly occupied conformations. Further heparin complexes were analysed for crystals soaked under differing conditions.

Firstly the crystal mother liquor (~18% ammonium sulfate) was exchanged for PEG, to avoid solvent competition for sulfate-binding sites; the structure of the heparin–FMDV complex was unaffected. Incorporating dithiothreitol (DTT) into the soak to break the disulfide bond which normally disorders the integrin-binding VP1 G–H loop (Parry et al., 1990, Logan et al., 1993) led to a structure showing the G–H loop in the expected position, demonstrating that sugar binding does not perturb the conformation of the integrin-binding loop in either conformational state. Conversely, ordering of the loop had no effect on sugar binding.

The 6 kDa heparin data set extending to Bragg spacings of 1.9 Å was used as the basis for determining the structure of the complex. We modelled (Jones, 1985) and refined (Brünger, 1992) two sugar conformations starting from X-ray coordinates for heparin (courtesy of D.Rees; Faham et al., 1996). In the absence of convincing evidence for deviations from non-crystallographic symmetry (NCS), we maintained strict NCS constraints throughout the structure determination process. The final \(R\)-factor is 16.2%, with r.m.s.d. (bonds) of 0.014 Å. The electron density for the sugar remained blurred compared with the protein electron density, and in places there was convincing evidence for two principal conformations of bound sugar. A heparin model consisting of two pentasaccharides (CONF1 and CONF2) bound simultaneously at half occupancy satisfactorily explained most of the features of the electron density map (Figure 4A).

**The conformation of the heparin receptor**

Despite the heterogeneity in the HS and the range of different experiments performed, the electron density maps clearly show that the O1BFS virus recognizes the same motif of sugars in each case. The virus selects a fully sulfated heparin motif (Figure 1) as does basic fibroblast growth factor (bFGF) (Faham et al., 1996). The oligosaccharide has a relatively extended conformation, forming a banana-like structure which projects the outer disordered sugars away from the protein. The GlcN-2, Idu-3 and GlcN-4 rings constitute the three central residues, with poorly defined electron density for an additional sugar ring at either end (Figure 4A). The GlcN sugar rings and one of the terminal Idu rings adopt the energetically

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**Table I. Data processing**

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\(a\) The figure in parentheses is for the highest resolution shell.

\(b\) \(R_{merge}=\left[\Sigma_{k}\left(\frac{F_{h_{k}}}{h_{k}}-\left<h_{k}\right>\right)^{2}\right]^{1/2}\times 100\), where \(h_{k}\) are unique reflection indices, \(I_{h_{k}}\) are intensities of symmetry-redundant reflections and \(\left<h_{k}\right>\) is the mean intensity.

\(c\) MFID is the mean fractional isomorphous difference in structure factor amplitudes, compared with data from crystals of the native virus.
Fig. 4. (A) A Bobscript (Esnouf, 1997) and RASTER3D (Merritt and Murphy, 1994) stereo depiction of the 1.9 Å (\(F_{\text{obs}} - F_{\text{calc}}\), \(\alpha_{\text{averaged}}\)) omit electron density map. Calculated structure factor amplitudes were generated from a model lacking heparin and the phases were derived from cyclic averaging (the starting phases for which were calculated from the same heparin minus model). The two heparin conformations are drawn, orientated with the sugars running from Idu-1 at the top to Idu-5 at the bottom, as ball-and-stick (CONF1, red and CONF2 green), with the outer sugars (Idu-1 and Idu-5) shown thinner commensurate with their lower accuracy. In CONF1, the oligosaccharide is stabilized by six internal hydrogen bonds, four via water molecules, and in CONF2 by eight internal hydrogen bonds, six via water molecules. (B) Stereo superimposition with bFGF. Coordinates for five sugar rings (CONF1) are shown in yellow using Bobscript (Esnouf, 1997) and RASTER3D (Merritt and Murphy, 1994) with a similar motif of sugars from the bFGF–heparin complex (Faham et al., 1996) (in green) having been superimposed using SHP (Stuart et al., 1979) only equivalencing the atoms that are reasonably similar. The orientation of the sugar chain is the same as in (A). The key sulfates that interact with Arg56 of VP3 are pinpointed along with the equivalent sulfates in the bFGF coordinates.

Favourable \(4\text{C}_1\) or \(3\text{C}_4\) chair conformations (the other terminal Idu ring appears to adopt a \(2\text{B}^2\) conformation). The central Idu ring is seen in a skew boat \(2\text{S}_0\) conformation (CONF1) and also a \(3\text{C}_4\) chair conformation (CONF2) with the sulfate rotating about the C2–O2 bond by some 120° between these conformations, becoming adjacent to GlcN-2 in CONF2. The mobility of the sugar is facilitated by the flexibility of this Idu ring [Idu rings are generally...
assumed to be more flexible than the GlcN rings (Casu et al., 1988; Forster and Mulloy, 1993). The GlcN and Idu sugar rings on either side apparently adopt a single conformation. The \( B \)-factors for the heparin motif are mostly lower in CONF1 than in CONF2 (average \( B \)-factors for the central Idu ring of 60 and 110 Å\(^2\), respectively).

We find that the two more constrained rings, GlcN-2 and GlcN-4, provide key points of attachment to the virus. Flexibility at either end of the trisaccharide recognition motif provided by the adjacent Idu moieties may facilitate docking to the virus. The heparin conformations selected allow two sulfate groups from both GlcN-2 and GlcN-4 to interact with the same ligand, Arg56 of VP3. The r.m.s.d. for all atoms between CONF1 and CONF2 is 1.7 Å. Superposition of the central trisaccharide with the analogous fully sulfated central trisaccharide bound to bFGF (Faham et al., 1996) gives an r.m.s.d. of 2.9 Å for CONF1 and 3.1 Å for CONF2. The similarity in the overall disposition of the sugars between the FMDV and bFGF complexes extends to the less well defined outer sugars (as shown in Figure 4B). Of the sulfates that form the key interactions in FMDV, that of GlcN-2 is in a similar relative position in bFGF whereas that of GlcN-4 is radically different (Figure 4B). Thus, significant conformational differences within the sugar rings allow the oligosaccharide to adapt to the markedly different binding sites of FMDV and bFGF whilst maintaining a similar overall shape.

**The receptor attachment site**

Heparin is bound centrally to the biological protomer of FMDV O\(_1\)BFS (Figure 5) within a slight depression of positive electrostatic charge (Figure 6) and makes contacts with all three major capsid proteins (Figure 5D and E). The three sides of the depression (Figure 5C) are formed by (i) strand B1 of VP3 (residues 55–58) and the subsequent loop (residues 58–60, analogous to the ‘knob’ which forms the south side of the canyon in HRV14; Rossmann et al., 1985), (ii) the C-termini of VP1, residues 195–197, and (iii) the \( \alpha \)B helix of VP2 (residues 134–138), running perpendicular to the sugar. The base of the depression is formed by a \( \beta \)4 helix (residues 84–87 of VP3) running in the same direction as the sugar. This helix is homologous to helix \( \alpha \)A\(_0\) of Mengo virus VP3 which forms part of the putative receptor-binding site (Luo et al., 1987). The sequence 200-RHKQ1205 near the C-termini of VP1, previously noted for its similarity to a region of the heparin-binding site of vitronectin (KKQKR; Fox et al., 1989; Kost et al., 1992; Jackson et al., 1996), does not appear to be involved directly in binding.

The heparin makes ionic interactions with the protein using two sulfates, GlcN-2-2-\( \text{O-SO}_3\) and GlcN-4-6-\( \text{O-SO}_3\). These sulfates are 7.7 Å apart and are less mobile than those exposed to the solvent. That they make a substantial contribution to the affinity of the heparin is suggested by the observation that sulfate ions are bound at similar positions in the unliganded virus crystallized from 0.8 M ammonium sulfate (Acharya et al., 1989). The 2-\( \text{N-Sulfate}\) of GlcN-2 is particularly well matched (Figure 5D). It is interesting that the corresponding binding site is similarly occupied in crystallized unliganded FGF. Arg56 of VP3 plays a central role in organizing the sulfate groups, making interactions with both. The use in this way of the fixed geometry of the guanidinium group may be a mechanism for conferring specificity despite the inherent flexibility of the side chain. The role of this residue was investigated by crystal soaking experiments (at 3 mM) with a closely related O\(_1\) isolate, O\(_1\)Kcar2, which differs from O\(_1\)BFS in just six residues (Lea et al., 1995), including a histidine in place of the arginine at residue 56 of VP3 (Figure 6). No evidence of bound heparin was seen in these crystals, nor could binding by this virus to HS be detected by enzyme-linked immunosorbent assay (ELISA) using paraformaldehyde-fixed BHK or RS2 cells (Figure 6). These results clearly confirm that histidine at VP3 56 drastically reduces the affinity of O\(_1\) FMDV for heparin and HS. In contrast, O\(_1\)KB64, which possesses an arginine at position 56 of VP3, is a strong HS binder (Figure 3; see also Jackson et al., 1996).

A further arginine residue, Arg135 of VP2, plays a subsidiary role in heparin binding by interacting via bridging water molecules with GlcN-4 and Idu-5 in CONF2, and also probably polarizes Asn88 of VP3, increasing its affinity for GlcN-4-6-\( \text{O-SO}_3\). There do not appear to be any protein interactions with Idu-5 in CONF1 and there are no interactions with Idu-1 in either CONF1 or CONF2. That the sugar, at a concentration of ~3 mM, fully displaces the sulfates (present at a concentration of 0.8 M) suggests that the interaction with the sugar is not purely ionic (although entropy and avidity may also favour the binding of the larger molecule). Non-ionic features include the interactions between His195 of VP1 and the central Idu ring, including a van der Waals stacking interaction (~5 Å separation) between the apolar patch of GlcN-4 and the imidazole ring of the histidine (which rotates by 36° about \( C_p \) to fulfil this role). Although there does not appear to be a similar stacking interaction in the FGF–heparin complex, such interactions have been observed frequently in monosaccharide complexes such as sucrose with lentil lectin (Casset et al., 1995). The ligands are residues 134, 135 and 138 of VP2, residues 56, 59, 60, 87 and 88 of VP3, and residue 195 of VP1 (Figure 5D and E), with bridging water molecules playing an important part. There are virtually no conformational changes in the protein to accommodate the sugar, except for the side chain of His195 of VP1 (in which mobility previously has been noted; Lea et al., 1995). Nevertheless, beyond residue 195, the C-terminus of VP1 becomes better ordered (average \( B \)-factor of 42 Å\(^2\) in the native virus and 34 Å\(^2\) in the complex, whereas the overall mean \( B \)-factors for the main chain atoms of the proteins are 16 Å\(^2\) in both cases). It is possible that the C-terminal residues of VP1 may stabilize His195 in a position suitable for heparin binding. This would explain the reduction in cell binding for serotypes O\(_1\)BFS and A10 when the C-terminal residues are cleaved by trypsin (Fox et al., 1989).

**Relationship with the integrin-binding site**

The RGD integrin recognition motif in the ordered VP1 G–H loop of the reduced virus (Logan et al., 1993) is ~15 Å from the closest sugar moiety, Idu-5 (Figure 5A), and our structural data demonstrate that it is unaffected by heparin binding. Electron density maps calculated at lower resolution (4 Å) (to filter out high frequency noise
which might mask a weak signal from a disordered part of the structure) were calculated for the more disordered oxidized state (i.e. crystallized without DTT), which showed no differences compared with the non-bound structure (although with the latest phases we see traces of density for the loop). The two binding sites therefore
appear independent; nevertheless, we would expect that the very bulky integrin heterodimer would compete with HS binding at the adjacent sites.

Discussion

Basis of receptor specificity and affinity

The relatively large pores extending through the virus crystals have permitted us to visualize an FMDV–GAG receptor complex by soaking. The GAG binds in a groove (Figure 5C) in the centre of the biological protomer constructed from structural components analogous to those that form the so-called ‘pit’ in Mengo virus (Luo et al., 1987) (Figure 5D). The binding site recognizes the fully sulfated form of heparin, as does bFGF (Faham et al., 1996). For FMDV, the minimal or tightest binding sequence appears to be a trisaccharide, as found for the complex of polyomavirus with sialic acid (Stehle et al., 1994) and of bFGF with HS (Faham et al., 1996). Whilst the HS motifs recognized by bFGF and FMDV are in a similar conformation, selectivity is achieved differently. In FMDV, there are hydrogen bonds to the carboxyl and ring oxygen of the Idu, and to the sulfate groups of the GlcN rings (a 6-O-sulfate and 2-N-sulfate), whilst in bFGF, the interactions are with two N-linked GlcN sulfates and the O-linked Idu sulfate. Arg56 of VP3 is crucial to the interaction with FMDV, and bridging waters appear to be important in stabilizing the FMDV complex. The FMDV heparin-binding site is recessed [akin to the sialic acid-binding groove in polyoma virus (Stehle and Harrison, 1996) and influenza hemagglutinin (Weis et al., 1988)], suggesting that shape complementarity contributes to receptor specificity. Indeed the protein conformation remains essentially unchanged; however, conformational flexibility of the Idu ring (Casu et al., 1988) may facilitate binding.

A single point of attachment is unlikely to be sufficient to explain the observed affinity of $10^{-9}$ M for FMDV bound to fixed cell HS (Jackson et al., 1996) since the central trisaccharide occludes only ~280 Å$^2$ of solvent-accessible surface on the virus and is heavily dependent on a single amino acid for affinity. HS chains contain discrete patches, each comprising 4–8 heavily sulfated sugars suitable for binding virus, separated by ~50 Å (J.Gallagher, personal communication). This spacing approximately matches the distance (a little less than 70 Å) between neighbouring HS attachment sites on the virus (Figure 5A). The high affinity is probably achieved by the flexible sugar chain wrapping around the virus, taking in several binding sites on the virus particle or even multiple particles, as suggested by the inhibition data of Figure 3B, which shows clear evidence of virus cross-linking by heparin.

Receptor adaptation: a driving force in virus evolution?

In subtype O$_1$ viruses, Arg56 of VP3 confers high affinity HS binding on O$_1$BFS and O$_1$KB64, which require HS to infect CHO cells efficiently. These two strains were both adapted to growth in BHK-21 cells (O$_1$KB64 was passaged 64 times), whereas the HS-independent O$_1$Kcar2, although from the same cattle outbreak as O$_1$KB64, was produced with minimal exposure to BHK cell culture (Xie et al., 1987). We have determined the coat protein sequences of 20 independent O$_1$ FMDVs isolated from outbreaks over the past 25 years without recourse to passage in BHK-21 or other established cell lines. Every one has a histidine at VP3 56 (data not shown), confirming that the H56R substitution in VP3 is an adaptation to cell culture, which agrees with the data of Sa-Carvalho et al. (1997) and Neff et al. (1998).

The dominant hypothesis, until now, has been that the receptor ‘footprint’ on the capsid surface will be conserved whilst antigenic variation occurs at other surface residues (Rossmann et al., 1985). This does not fit with our observations; thus the HS and integrin receptor attachment sites, between them, overlap three of the five antigenic sites of FMDV. Furthermore, some residues central to the antigenic sites are also key points of attachment to the receptors, notably Arg56 of VP3 (Figure 7). Many viral receptors belong to families of related cell surface molecules, such that receptor switching may be relatively facile and indeed has been reported recently for HIV (Wu et al., 1996), Theiler’s virus (Jarouse et al., 1994, 1996; Zhou et al., 1997) and polyoma virus (Stehle et al., 1994; Stehle and Harrison, 1996). Although there is no evidence for positive Darwinian selection on the bulk of the accessible surface of FMDV (Haydon et al., 1998), we suggest that at key points on the virus surface there is interplay between receptor selection and antigenic variation. In this situation, the host defences might provide a motor for receptor switching by selection of a virus with an alternative receptor.

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**Fig. 5.** (A) A space-filling representation of the reduced O$_1$BFS virus (structure determined in the presence of DTT). The surface is shaded according to radius using Bobscript (Esnouf, 1997) and RASTER3D (Merritt and Murphy, 1994). The FMDV VP1 G–H loop is highlighted in cyan, with the RGD integrin-binding tripeptide (residues 145–147) picked out in a brighter blue. The heparin motif is shown in white. The distance from one heparin attachment site to both its 5-fold neighbour and its 3-fold neighbour is a little less than 70 Å, whilst the nearest 2-fold-related neighbour is ~90 Å away. (B) Ribbon cartoon of the reduced O$_1$BFS biological protomer with proteins coloured as in Figure 1. The heparin coordinates for five sugars (CONF1), running left to right, are shown in grey. (C) A Grasp (Nicholls et al., 1991) accessible surface representation (grey) showing the binding depression occupied by the five sugar rings with bonds drawn as rods and coloured using the standard convention. (D) A ball-and-stick representation of the central three heparin sugars (with conventional atom colours). The protein backbones are drawn with the colouring scheme defined in Figure 1; side chains interacting with the heparin are in ball-and-stick and coloured as the protein backbone; His195 of VP1, Lys134, Arg135 and Tyr138 of VP2, and Arg56, Ser87 and Asn88 of VP3. Bridging water molecules have been excluded for clarity. Yellow spheres mark the positions at which sulfates bind in the native virus. (E) Sugar–protein interactions depicted using LIGPLOT (Wallace et al., 1995) for each of the bound conformations, CONF1 (left) and CONF2 (right). Note that only the protein side chains that interact directly are shown and that these are rearranged to clarify the hydrogen-bonding pattern. They are labelled with the residue number and the chain ID as the least significant digit, e.g. 563 is residue 56 of VP3. Ligand bonds are drawn in black and non-ligand bonds in green. Hydrogen bonds are depicted by olive green dashed lines. Non-ligand residues involved in hydrophobic contacts are shown as a red, fringed semi-circle. Water molecules are coloured red.
Fig. 6. (A) Grasp (Nicholls et al., 1991) electrostatic, accessible surface representations for the biological protomer of FMDVs for which three-dimensional structures have been determined: O₁BFS, O₁Kcar2, A22, Cs8c1 (Acharya et al., 1989; Lea et al., 1994, 1995; Curry et al., 1996), A10/61 (E.E.Fry, S.Curry, J.W.I.Newman, T.Jackson, W.E.Blakemore, S.M.Lea, A.M.Q.King and D.I.Stuart, unpublished results). Note that the VP1 G–H loop is absent in all these representations. The scale on which the electrostatic potential was coloured was similar for each subtype, with positive charge in blue and negative in red. The position of the heparin-binding site in O₁BFS is delineated in yellow and shown approximately on the other structures. (B) A comparison of virus binding to fixed CHO and GAG mutant cells carried out as described in Materials and methods. Binding of FMDV strains O₁BFS, A10/61 and Cs8c1 was significantly higher to CHO than to the GAG mutant cells (677 and 745). Virus binding for strains O₁Kcar2 and A22 was virtually identical for all three cell lines and was never greater than background. Each point on the graph represents the mean of duplicate wells. (C) A structural-based sequence alignment of the O₁BFS heparin ligands in other aphthoviruses for which the three-dimensional structure is known. The row highlighted corresponds to the serotype in the complex reported here. The column highlighted is the key ligand. The passage history refers to the number of passes in BHK cells (H = highly passaged, L = low passage number). It should be noted that all these viruses were processed for crystal growing and were thus plaque purified. From initial plaquing to crystals requires a minimum of six passes in BHK cells.
Possible mechanisms of HS-mediated cell entry

O1 BFS virus is almost completely dependent on HS for the ability both to bind to and infect CHO cells, suggesting that increased binding somehow enhances susceptibility; but how? HS might function as an accessory molecule, as it does for several herpes viruses (Spear, 1993), enhancing the efficiency of a second, internalization, receptor. The affinity of O1 BFS for HS is considerably less than for integrins [either in the form of immobilized α3β3 (Jackson et al., 1997) or as found on fixed MDBK cells (Figure 3A)], facilitating transfer of the virus from the HS to the integrin. An abundant surface molecule like HS might accelerate virus uptake by concentrating the virus at the cell surface, thereby improving its chance of encountering a slower binding integrin receptor. This mechanism of enhancing the infection rate would not require especially tight binding. A crude calculation suggests that, at a density of $5 \times 10^6$ sites/cell (Figure 3A), a receptor would operate with an effective concentration at the cell surface of $\sim 10^{-4} \text{ M}$, enough to concentrate even a weakly binding virus, especially since a virion can bind multiple proteoglycan receptors.

Virus uptake may be accelerated further through direct interactions between proteoglycan and integrin receptors. Several adhesion molecules, including vitronectin and fibronectin, share with FMDV a dual affinity for integrin and HS (Fielding-Harbermann and Cheresh, 1993; Potts and Campbell, 1994), and it is likely that the two types of receptor are capable of interacting with each other to facilitate ligand transfer between them and/or as a mechanism of integrin activation. Whilst we did not observe any conformational change in the integrin-binding loop of the virus on binding heparin, this does not preclude the possibility of integrins and HS proteoglycans interacting with each when bound close together on the virus surface; indeed some contact between such bulky ligands would be almost unavoidable. Recent studies of fibronectin have shown HS- and integrin-binding sites close in space but on opposed molecular faces, suggesting that the two receptors may bind simultaneously (A.Sharma, personal communication).

For high affinity strains like O1 BFS, there are several reasons for thinking that HS proteoglycans may be able, by themselves, to internalize FMDV, without the mediation of integrins. Mason et al. (1994) have shown that integrin receptors can be bypassed readily by binding FMDV to an alternative (in their case, Fe) receptor, and Neff et al. (1998) have shown that O1 BFS can replicate in human cells that do not express α3β3. Secondly, some membrane proteoglycans undergo endosomal cycling (Yanagishta and Hascall, 1984; Iozzo, 1987) and, therefore, are potential internalizing receptors. Thirdly, multiple passage in cell culture was found recently to select a variant type C FMDV which, when exposed to antibody against the integrin-binding loop, became able to dispense with its RGD motif (Martinez et al., 1997), implying that it had acquired an increased affinity for an alternative receptor, such as HS.

GAG receptors in the infected animal

If strong HS binding by FMDV is an adaptation to tissue culture, what role do GAGs play as FMDV receptors in nature? Preliminary measurements of HS-specific binding to paraformaldehyde-fixed CHO cells reveal a wide spectrum of affinities among the seven FMDV serotypes, demonstrating that a predisposition for binding HS is not confined to members of the O1 subtype (Figure 6B). Given the variability in HS contact residues (Figure 6C), it is not immediately clear if there is an underlying structural conservation in the different GAG-binding sites, or if each site was created de novo. This question, which has wide implications for the way proteins in general evolve polysaccharide-binding sites, has not been answered definitively. However, several lines of evidence suggest that FMDVs possess a structurally conserved depression which is predisposed to acquire by mutation a high affinity for HS, the site being positively charged in the high affinity state and observed in all strains of the serotypes (O, A and C) solved to date (Figure 6A).

The difference between fixed and live cells and the evidence for oligovalent interactions with soluble forms of HS indicate that effective receptor density is a key determinant of binding. This enables us to make predictions about the role of this site in field strains, although whether field strains have even a low affinity for HS remains to be established. Selective forces in cell culture are likely to be very different from those in the animal; thus, strains not passaged extensively in BHK cells (where selection is for tight binding rather than for a balance of binding and effective spread) all have VP3 His56, rather than arginine (which confers high affinity and presumably poor spread). Similarly for two other viruses that interact with oligosaccharide receptors on the cell surface, influenza virus and polyoma virus, full virulence requires either limited receptor affinity (Steble and Harrison, 1996) or a reduction in their number through the action of the viral neuraminidase (Palese et al., 1974; Gubareva et al., 1995).
For polyoma, selection in the wild is for moderate to tight binders since weak binders are so virulent (Freund et al., 1991; Bauer et al., 1995). It seems that the virulence ‘set point’ in the wild will vary with the nature of the infection and mechanism of spread. For FMDV, one possibility discussed earlier is that weak binding to HS proteoglycans serves to increase the rate of integrin-mediated virus uptake. Alternatively, the site may be specific for some other, as yet unidentified, ligand in the natural host, presumably structurally related to HS, and involved in cell entry. It should also be considered that the potential to be a strong GAG binder is itself selectively advantageous. In the infected animal, the virus might routinely switch into, and out of HS-binding mode to pervade particular tissues and, commensurate with the reduced virulence observed (Neff et al., 1998), may provide a mechanism of persistent infection—a longstanding puzzle to virologists.

Materials and methods

Cells and viruses

Cells were cultured as described (Jackson et al., 1996). Preparation of virus stocks and 35S labelling and purification on sucrose gradients of FMDV strain O1KB64 have been described (Jackson et al., 1997). The specific activity of radiolabelled virus was estimated by ELISA, μ for 2 min. The final cell pellet was resuspended in 500 l of PBS pH 5.0 for 2 min, removed from the dish with trypsin (2 min at 37°C) treated as above.

Binding of viruses to GAG mutant cells

Wild-type CHO cells and the GAG-deficient mutant CHO cell lines (677 and 745) were cultured as described (Jackson et al., 1996). The line 677 is deficient in HS but makes a greater amount of chondroitin sulfate than CHO cells, whereas 745 cells make little if any GAG (Esko et al., 1987). Binding of viruses to parafomaldehyde-fixed cells was determined by incubating cell monolayers in 96-well plates at 37°C for 60 min with BBB [20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM MnCl2, 1% bovine serum albumin (BSA)], and incubated with 150 l of virus in BBB at 4°C. The supernatant was removed, and the cell sheet washed twice with PBS. Bound virus was measured by resuspending the cell fraction in 0.25 ml of 0.5% SDS, and mixing, together with 2×0.25 ml H2O washings, with 1.5 ml of Optiscint liquid scintillant for radioactivity estimation. For Scatchard analyses (Figure 3A), radioactivity in the unbound virus was also measured by counting the pooled supernatant fractions in the same way. Binding to parafomaldehyde-fixed cells was determined by incubating cell monolayers in 96-well plates with 50 l of virus solution at room temperature for 3 h. Bound and unbound fractions were separated and counted as above, except that the former were solubilized by treatment with 2×100 l of 2 M NaOH at 80°C for 10 min.

X-ray crystallographic analyses

Crystals of FMDV O1BFS (space group I23, a = 552 Å) were grown using ammonium sulfate as precipitant (Fox et al., 1987). Crystals were soaked in 3 kDa/6 kDa heparin or HS (in the form of a sodium salt from Sigma) to permit ordering of the integrin receptor attachment site (the V1 G–H loop). Crystals of O1K also grown from ammonium sulfate (Curry et al., 1992) were soaked with 20 mg/ml 6 kDa heparin. Diffraction images were collected as 0.3° or 0.5° oscillations, at room temperature on a 30 cm MARRESEARCH imaging plate at the Central Laboratory of the
Acknowledgements


Role of VP2 amino acid 141 in tropism of Theiler’s virus within the central nervous system. J. Virol., 70, 8213–8217.


