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Efficient Infection of Cells in Culture by Type O Foot-and-Mouth Disease Virus Requires Binding to Cell Surface Heparan Sulfate

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Foot-and-mouth disease virus (FMDV) enters cells by attaching to cellular receptor molecules of the integrin family, one of which has been identified as the RGD-binding integrin αvβ3. Here we report that, in addition to an integrin binding site, type O strains of FMDV share with natural ligands of αvβ3 (i.e., vitronectin and fibronectin) a specific affinity for heparin and that binding to the cellular form of this sulfated glycan, heparan sulfate, is required for efficient infection of cells in culture. Binding of the virus to paraformaldehyde-fixed cells was powerfully inhibited by agents such as heparin, that compete with heparan sulfate or by agents that compete for heparan sulfate (platelet factor 4) or that inactivate it (heparinase). Neither chondroitin sulfate, a structurally related component of the extracellular matrix, nor dextran sulfate appreciably inhibited binding. The functional importance of heparan sulfate binding was demonstrated by the facts that (i) infection of live cells by FMDV could also be blocked specifically by heparin, albeit at a much higher concentration of inhibitor; (ii) pretreatment of cells with heparinase reduced the number of plaques formed compared with that for untreated cells; and (iii) mutant cell lines deficient in heparan sulfate expression were unable to support plaque formation by FMDV, even though they remained equally susceptible to another picornavirus, bovine enterovirus. The results show that entry of type O FMDV into cells is a complex process and suggest that the initial contact with the cell surface is made through heparan sulfate.

The seven serotypes of foot-and-mouth disease virus (FMDV) (types O, A, C, and Asia-1 and the South African Territories types 1, 2, and 3) constitute the *Aphthovirus* genus of the family *Picornaviridae*. FMDV are small, nonenveloped, icosahedral viruses with a single-stranded, positive-sense RNA genome of approximately 8,400 nucleotides (3). The virus capsid is made up from 60 copies each of four virus-encoded proteins, VP1 to VP4; VP1 to VP3 form most of the capsid shell, with VP4 lining the interior surface.

FMDV enters cells via a mechanism of receptor-mediated endocytosis in which the low pH of the endosomal compartment triggers uncoating of the viral genome (7, 10, 30). For FMDV A_{12} the receptor has been identified as the Arg-Gly-Asp (RGD)-binding integrin αvβ3 (4). The RGD to which the integrin binds is located on the G-H loop of VP1 and is highly conserved among all seven serotypes. Such conservation in a region that otherwise varies considerably in length and sequence suggests that all FMDV serotypes use RGD-binding integrins, including αvβ3, as receptors for virus internalization.

The G-H loop of VP1 was initially implicated in the entry of FMDV into cells by treatment of the virus with trypsin. Trypsin removes both the G-H loop and the C terminus of VP1 and results in virus particles with greatly reduced infectivity, an effect attributed to an inability to attach to cells (46, 49, 50). The involvement of the G-H loop was further implicated by competition for cell attachment sites by synthetic RGD-containing peptides, although high concentrations of peptide were required to achieve inhibition (2, 16). The RGD has now been shown to be essential for cell entry by the introduction of mutations into a cloned infectious copy cDNA of FMDV A_{12} (31). Viruses mutated in the RGD motif were noninfectious and failed to bind to cells. This work also demonstrated that the RGD was required only for cellular entry, as the mutated viruses replicated after entering cells by endocytosis as an antibody-virus complex (31). However, the cell entry processes of other FMDV serotypes and subtypes of serotype A appear to be more complex. Experiments involving competition between different FMDV serotypes for cell attachment sites have shown that serotypes O and A are good competitors of type C and the three South African Territories serotypes, whereas in reciprocal experiments the latter serotypes are poor competitors of types O and A (1, 41). To account for these observations, it has been suggested that serotypes O and A are able to use more than one receptor for cell attachment (41). Binding of FMDV Asia-1 to cells appears to occur via nonspecific adsorption and does not seem to require specific receptors, as virus attachment sites cannot be saturated (45). There is also evidence which suggests a role for the C terminus of VP1 in cell binding. Enzymatic digestion of FMDV A_{10} 61 with the lysine-specific endoproteinase Lys-C, which removes residues 203 to 213 from the C terminus of VP1 and leaves the G-H loop intact, resulted in viruses with reduced cell binding activity (16). Similar results were obtained with FMDV type O_1K (16).

In addition to binding integrins through interactions involving an RGD motif, many of the natural ligands for αvβ3, such as vitronectin and fibronectin, have binding sites for several extracellular matrix components, including heparan sulfate (HS) (15, 36). HS is an example of a glycosaminoglycan (GAG). Other common types of GAGs are chondroitin sulfate A, dermatan sulfate (chondroitin sulfate B), and keratan sul-

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fate (23). GAGs are polymers of disaccharide repeats which are highly sulfated, and hence negatively charged, and occur as the carbohydrate component of a family of glycoproteins known as proteoglycans. Proteoglycans are widely distributed in animal tissues, where they are found on virtually all cell types both as parts of the extracellular matrix and as integral membrane components (23).

In this report we show that type O FMDV has a specific affinity for HS and that this binding most likely is the initial event in cellular entry and is required to establish an efficient infection in cells grown in culture.

MATERIALS AND METHODS

Reagents. Paraformaldehyde (PFM) was purchased from Merck BDH, and Indubiose was purchased from BioSepra. All other reagents, enzymes, and antibody-enzyme conjugates were purchased from Sigma.

Viruses and cells. Madin-Darby bovine kidney (MDBK) and baby hamster kidney (BHK) cells were grown in Dulbecco's modified Eagle's medium–5% fetal calf serum. Wild-type CHO cells, pgsD-667 (667), and pgsA-745 (745) were grown in Ham's F-12 medium supplemented with 10% fetal calf serum and glutamine. O_1BFS was purified on sucrose gradients as described by Curry et al. (10). The same procedure was used to purify bovine enterovirus (BEV) except that Nonidet P-40 was used in place of Triton X-100 and precipitation with ammonium sulfate was not performed. Working stocks of type O FMDV (O_1BFS and B64) and of BEV were prepared by using BHK cells.

PFM treatment. Subconfluent cell monolayers, in 96-well plates, were fixed for 45 min at room temperature with 4% PFM. The cells were then washed with BB (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.4], 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$) and blocked sequentially with 0.1 M glycine (pH 7.2) and BBB (BB with 1% bovine serum albumin [BSA]) for 30 min each.

Virus attachment and inhibition assay. (i) Attachment. Viruses were allowed to attach to PFM-fixed cells in BBB for 1 h. Unbound virus was removed by three rinses with BB. Bound virus was detected with a guinea pig anti-type O FMDV polyclonal antiserum and a rabbit anti-guinea pig alkaline phosphatase conjugate. After the addition of substrate, the optical densities (ODs) of the wells were read at 405 nm. The background OD was determined by omitting the virus from triplicate wells.

(ii) Inhibition assay. Potential inhibitory agents were incubated with virus for 30 to 45 min prior to the addition of the virus to fixed cells. Bound virus was detected as described above. The background OD was determined by adding the inhibitor (10 μg/ml) to the wells in the absence of the virus. All experiments were performed several times and gave consistent results.

Enzyme treatments. PFM-fixed cells were treated with enzymes (heparinase I or heparinase III) in 20 mM Tris (pH 7.5)–50 mM NaCl–4 mM $CaCl_2$–0.1% BSA for 1 h at 37°C. After enzyme treatment, the cells were washed with BB and virus attachment was performed as described above. Live cells were treated with heparinase III in phosphate-buffered saline (PBS)–0.1% BSA at 37°C for 1 h.

Plaque reduction and neutralization assays. (i) Neutralization assay. Virus was incubated with heparin or dextran sulfate in PBS at room temperature for 30 min. The virus was then allowed to attach to subconfluent cell monolayers at room temperature for 15 min. The cells were then washed with PBS and incubated at 37°C for a further 15 min to allow virus internalization. Virus which had not been internalized was removed from the cell surface by washing with PBS (pH 6.0). The cells were then overlaid with 4 ml of Eagle's overlay (0.6% Indubiose A37, 5% Tryptone phosphate broth, 1% fetal calf serum in Eagle's medium). At 48 h postinfection, the cells were fixed and stained with 4% formaldehyde and methylene blue.

(ii) Enzyme treatments. Enzyme-treated cells (see above) were washed with PBS. The virus was then allowed to attach for 15 min at room temperature in the presence of fresh enzyme. The cells were then treated as described above.

(iii) Infection of CHO cells and GAG-deficient mutants. Virus was added to subconfluent cell monolayers, and the cells were incubated at 37°C for 20 min. The cells were then overlaid as described above, without washing, and returned to 37°C.

RNA extraction and electroporation. Viral RNA was extracted from sucrose gradient-purified B64. Sucrose fractions containing virus were extracted sequentially with Tris-buffered phenol–chloroform (1:1, vol/vol) and chloroform, and the RNA was precipitated with ethanol at −70°C. RNA (5 μg) was introduced into the various cell lines by electroporation with the Bio-Rad Gene Pulser, using a cuvette gap of 0.4 cm and a final volume of 0.8 ml. Cells were electroporated with 280 V and a capacitance of 250 mF; two pulses were used. Subconfluent cell sheets were trypsinized, resuspended in PBS, and cooled on ice before being resuspended at 2×10^6 cells per ml in ice-cold buffer (21 mM HEPES buffer [pH 7.05], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM glucose) (8). Transfected and mock-transfected cells were divided into two aliquots. One aliquot was cultivated in cell growth medium. At 24 h posttransfection, cell media were assayed for the presence of virus by the ability to form plaques on BHK cells. The second aliquot was used to make a dilution series in Eagle's overlay and plated directly onto a monolayer of BHK cells in an infectious-center assay.

Electron microscopy. Virus samples (with and without heparin treatment) were adsorbed to Formvar-carbon-coated copper grids for 90 s, stained with 2% phosphotungstic acid (pH 7.0) for 15 s, and examined in a JEOL 1200ex microscope.

FACScan analysis. FACScan was performed with a Becton Dickinson analyzer. Monoclonal antibody PB1 (anti-hamster α5) was obtained from R. L. Juliano (University of North Carolina, Chapel Hill).

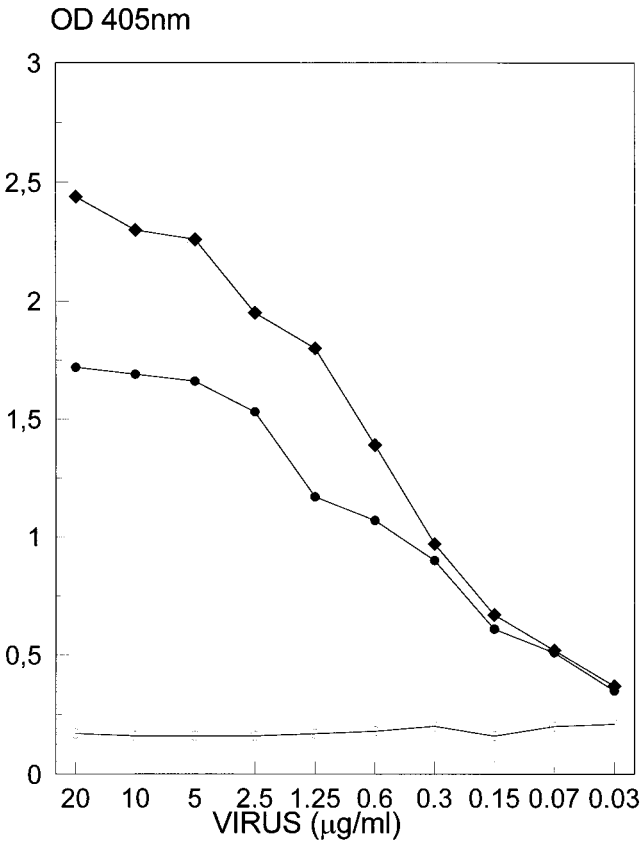

FIG. 1. Binding of O_1BFS to PFM-fixed cells. Virus was added to PFM-fixed cells in 96-well plates at the indicated concentrations and left for 45 min. Unbound virus was removed by washing, and bound virus was detected with a guinea pig anti-type O FMDV polyclonal antiserum followed by a rabbit anti-guinea pig alkaline phosphatase conjugate. After the addition of substrate, the ODs of the wells were read at 405 nm. The background OD was determined by omitting the virus from triplicate wells. MDBK (squares), CHO (closed circles), and 667 and 745 (open circles) cells were used. Each point on the graph represents the mean for duplicate wells.

RESULTS

FMDV binding to PFM-fixed cells. For our initial studies we used cells which had been treated with PFM. PFM treatment prevents virus internalization and conformational changes from occurring in membrane proteins, thereby allowing the initial interaction between the virus and the cell surface to be studied. Figure 1 shows binding of type O FMDV strain O_1BFS to PFM-fixed MDBK and CHO cells. Binding was found to be concentration dependent, but full saturation of virus attachment sites was not achieved with virus concentrations of up to 20 μg/ml. Similarly, virus attachment sites on BHK cells could not be saturated (data not shown).

Type O FMDV binds to cell surface HS. On the basis of the observation that natural ligands of αvβ3 bind HS, we investigated the role of GAGs in virus binding. Various species of

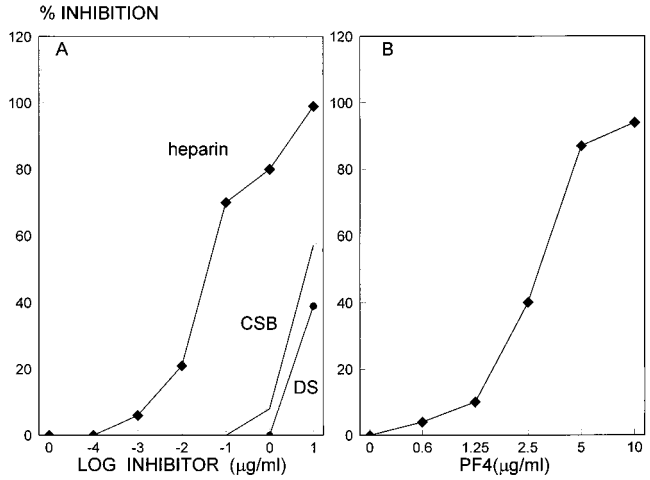

FIG. 2. Competitive inhibition of virus (O_1BFS) binding to PFM-fixed MDBK cells. (A) The virus (2 μg/ml) was treated with various concentrations of heparin, chondroitin sulfate B (CSB), or dextran sulfate (DS) for 45 min prior to the treatment of fixed cells with virus for 1 h. Unbound virus was removed by washing, and bound virus was detected as described for Fig. 1. The background OD was determined by adding an inhibitor (10 μg/ml) to fixed cells in the absence of virus and then adding the detecting antibodies. (B) Effect of platelet factor 4 (PF4) on virus (O_1BFS) binding to PFM-fixed MDBK cells. The virus (2 μg/ml) was allowed to attach to fixed cells in the presence of various concentrations of PF4 for 1 h. Unbound virus was removed by washing, and bound virus was detected as described for Fig. 1. Each point represents the mean of duplicate measurements.

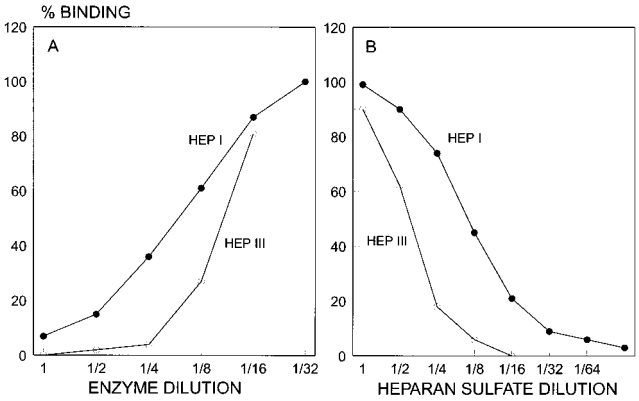

FIG. 3. (A) Effect of heparinases on virus (O_1BFS) binding to PFM-fixed MDBK cells. Fixed cells were digested with enzyme at various concentrations for 1 h. For heparinase I (HEP I) the dilution of 1 was 0.25 U/ml, and for heparinase III (HEP III) it was 0.06 U/ml. After removal of the enzyme by washing, the virus (2 μg/ml) was allowed to attach to cells for 1 h. Unbound virus was removed by washing, and bound virus was detected as described for Fig. 1. (B) PFM-fixed MDBK cells were digested with heparinase I (0.25 U/ml) or heparinase III (0.03 U/ml) for 1 h in the presence of various concentrations of soluble HS (HS dilution of 1 = 1 mg/ml). After digestion, the cells were washed and the virus (2 μg/ml) allowed to attach to the cells for 1 h. Unbound virus was removed by washing, and bound virus was detected as described for Fig. 1. Each point represents the mean of duplicate measurements.

sulfated polyanions were tested for their abilities to interfere with virus binding to PFM-fixed cells. Figure 2A shows that virus attachment to PFM-fixed cells can be completely inhibited by including heparin during the virus attachment period. The inhibitory effect of heparin was concentration dependent and specific. Other sulfated GAGs and the synthetic polyanion dextran sulfate did not appreciably inhibit virus attachment. To achieve 50% inhibition with chondroitin sulfate B required a concentration approximately 250-fold higher than that with heparin (Fig. 2A). Dextran sulfate inhibited attachment to a lesser extent, and chondroitin sulfate A and chondroitin sulfate C, at up to 10 μg/ml, had no effect on virus binding (data not shown). Similarly, de-N-sulfated heparin did not inhibit binding (data not shown). The inhibitory effect was specific for FMDV, as cell attachment of another picornavirus, BEV, was not affected by heparin at 10 μg/ml (data not shown). Heparin inhibition of O_1BFS binding could be reproduced with different cell lines (CHO, HeLa, and BHK) (data not shown). That the structure of the virus was not disrupted by heparin treatment was demonstrated by examination of heparin-treated virus under an electron microscope (data not shown).

The foregoing results show that type O FMDV has a specific affinity for a cellular ligand which can be displaced by heparin. That the ligand is HS was confirmed in three ways. First, virus attachment to PFM-fixed cells could also be inhibited by platelet factor 4 (Fig. 2B). Platelet factor 4 is a small basic growth factor which binds to cell surface heparin and HS. Second, PFM-fixed cells were treated with enzymes that digest glycosidic linkages present in HS. Fixed cells treated with either heparinase I, which degrades heparin and HS, or heparinase III, which degrades only HS, were no longer capable of supporting virus attachment (Fig. 3A). The possibility that the effect seen after enzyme digestion was due to a contaminating enzyme activity was discounted by adding increasing amounts of an alternative source of substrate (in the form of soluble HS) during digestion with a fixed concentration of enzyme. Figure 3B shows that HS at 1 mg/ml prevented either enzyme from rendering the cells incapable of supporting virus attachment. The third approach was to examine the attachment of O_1BFS to CHO cells and mutant CHO cell lines which are deficient in GAG biosynthesis. The line 667 is deficient in HS but has normal amounts of chondroitin sulfate, whereas 745 makes little if any GAG (12, 13, 26). The wild-type CHO cells which had been fixed with PFM bound O_1BFS with a profile similar to that of MDBK cells, as shown in Fig. 1, whereas virus binding to PFM-fixed GAG mutant cell lines above background could not be detected at up to 20 μg/ml.

The reversibility of virus binding to PFM-fixed cells was examined. Figure 4 shows that virtually all of the bound virus could be eluted from the cell surface by washing for 15 min with either heparin (1 mg/ml) or NaCl (0.25 M), whereas washing with 10 mM EDTA or binding buffer (BBB) had no effect.

These results suggest that the initial event in cell attachment of O_1BFS is binding to surface HS, most likely in the form of HS proteoglycan.

Binding to HS is required for efficient infection. Experiments were performed to establish whether binding to HS is necessary for type O FMDV to infect cells. Figure 5A shows that addition of heparin during the period of virus attachment reduced plaque formation in a concentration-dependent manner, reaching a maximum reduction of greater than 95%, although the concentration of heparin required to obtain a 50% reduction in plaque number was 10^4 times greater than that required to achieve a 50% inhibition of virus binding to PFM-fixed cells. The effect was specific for heparin, as dextran sulfate had no effect on plaque number over the same concentration range and neither heparin nor dextran sulfate had any effect on plaque formation by BEV (data not shown). The possibility that heparin could be acting directly on the cells to suppress O_1BFS plaque formation was discounted by pretreating cells with heparin for 30 min prior to the addition of virus.

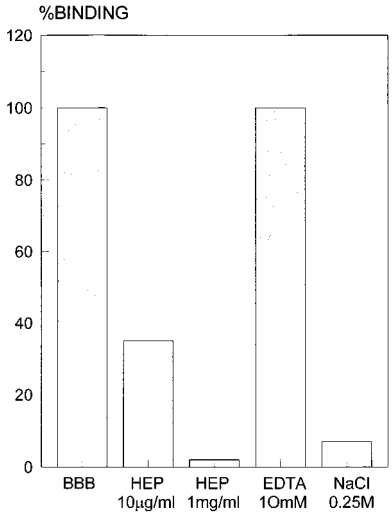

FIG. 4. Binding of virus (O_1BFS) to HS is reversible. The virus (2 μg/ml) was allowed to attach to PFM-fixed MDBK cells for 1 h. Unbound virus was removed by washing. Bound virus was then subjected to further washing with the indicated agent for 15 min. Heparin (HEP) and NaCl were added in BBB, whereas EDTA was added in BBB without divalent cations. Virus that remained bound was detected as described for Fig. 1. Virus that remained bound after washing with BBB = 100%. The bars show the means of duplicate measurements.

The heparin-treated cells supported O_1BFS plaque formation as well as untreated cells (data not shown).

We also examined the effect on plaque formation of pretreatment of cells with heparinase III. Enzyme treatment of cells had the effect of reducing the O_1BFS plaque number by 75% (Fig. 5B). The effect was not due to enzyme degradation of O_1BFS, as enzyme-treated virus remained as infectious as untreated virus (data not shown). The effect was specific for FMDV, as plaque formation by BEV was not affected by enzyme treatment at 4 U/ml.

The results described above suggested that binding to HS may be required for type O FMDV to infect cells. To clarify the role of HS binding in infection, we attempted to infect the GAG mutant cells (667 and 745) with type O FMDV (O_1 BFS

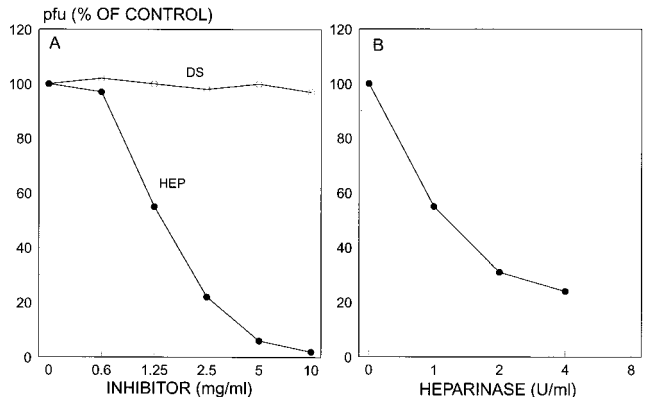

FIG. 5. (A) Effect of treatment of virus (O_1BFS) with heparin (HEP) or dextran sulfate (DS) on plaque formation on BHK cells. The virus was pretreated with HEP or DS prior to addition to cell monolayers (100% PFU = 190 plaques). (B) Effect of heparinase III treatment of BHK cells on plaque formation by O_1BFS. Cells were digested with the enzyme at the indicated concentration for 1 h prior to the addition of the virus in the presence of fresh enzyme (100% PFU = 183 plaques). Each point represents the mean for duplicate plates.

TABLE 1. Relative plaquing efficiencies of viruses on CHO cells and GAG-deficient mutant 667 and 745 cells

Virus dilution	No. of plaques								
	O1BFS[a]			B64[a]			BEV		
	CHO	667	745	CHO	667	745	CHO	667	745
10^{-1}	CPE[b]	0	0	CPE	0	0			
10^{-2}	CPE	0	0	CPE	0	0			
10^{-3}	NC[c]			NC					
10^{-4}	46			65			NC	NC	NC
10^{-5}							15	43	46
0	0			0			0	0	0

[a] Type O FMDV.
[b] CPE, cytopathic effect.
[c] NC, plaques not counted.

and B64) (Table 1). Type O FMDV strain B64 is a cell culture-adapted virus that is characterized by rapid replication and plaque formation. In a plaque assay, both type O viruses (O_1BFS and B64) gave rise to cytopathic effect on the wild-type CHO cells. However, neither virus gave rise to plaques on either of the GAG mutant cell lines (Table 1).

One possibility to account for the failure of the GAG-deficient cells to support plaque formation is that these cells carry additional mutations that prevent virus replication. However, all three cell lines (wild-type CHO, 667, and 745) gave rise to plaques after infection with another picornavirus, BEV (Table 1), suggesting that this was unlikely. It was confirmed that the GAG mutant cells could support FMDV replication by electroporation of viral RNA, from B64, into the cells. Virus was detected in supernatants from all three cell lines (wild-type CHO, 667, and 745) at 16 h posttransfection by the ability to form plaques on BHK cells (data not shown). Virus was not detected in supernatants from cells transfected in the absence of viral RNA. Similarly, no plaques were seen after viral RNA was added directly to the BHK monolayer, showing that the infectivity was not due to virus contamination of the RNA. Transfected cells (wild-type CHO, 667, and 745) were also plated directly onto a monolayer of BHK cells in an infectious-center assay. Again, plaques appeared only on BHK monolayers overlaid with cells that had been transfected with viral RNA. For the three cell lines the numbers of infectious centers formed per 10^6 cells transfected were similar (3.3×10^3, 1.6×10^3, and 4.9×10^3 for CHO, 667, and 745 cells, respectively). These data show that the GAG mutant cells support virus replication equally well as wild-type CHO cells after delivery of viral RNA inside the cells and that failure of the GAG mutant cells to support plaque formation results from a defect in the cell entry process and not from intracellular deficiencies in virus replication.

Failure of the GAG mutant cells to support plaque formation could have also resulted from defective expression of integrin molecules at the cell surface. FACScan analysis with a monoclonal antibody (PB1) specific for hamster α5 demonstrated that the mutant cell lines (667 and 745) express at least as much of this integrin chain at the cell surface (data not shown). As both α and β chains are required for correct assembly of an integrin molecule at the cell surface (20), it follows that there is no general down regulation of integrin expression at the surface of the GAG mutant cells. In addition, heparinase treatment of BHK cells resulted in a reduction in plaque formation after infection with type O FMDV, presumably in the presence of integrin molecules (Fig. 5B).

The results described above demonstrate that for cells grown

in culture, binding to HS is necessary to establish an efficient infection and, furthermore, that HS binding is involved in the cell entry process. That other GAGs were not involved in these processes was demonstrated by using the mutant CHO cell line 667, as this cell line is deficient only in HS biosynthesis and makes normal amounts of other GAGs, including chondroitin sulfate B (12, 13, 26).

DISCUSSION

The data reported here provide several lines of evidence showing that type O FMDV has a specific affinity for HS and that this binding most likely is the initial event in cell attachment and is important for infection. HS proteoglycans are abundant cell surface proteins, and adhesion phenomena involving them are typified by relatively low affinities. Although precise measurements of these parameters for the binding of FMDV to GAG receptors have yet to be made, estimates of greater than 10^5 particles bound per cell have been obtained from experiments with radiolabelled virus under the conditions used for Fig. 1. The sensitivity of FMDV-HS binding to NaCl and the inability of undersulfated heparin (de-N-sulfated) to inhibit binding of O_1BFS to fixed cells confirm that the interaction has an important electrostatic component.

The fact that bound virus could be eluted by heparin but not by 10 mM EDTA suggests that although integrins are required for internalization (4), they do not participate significantly in the interaction of O_1BFS with PFM-fixed cells. Binding of RGD ligands to integrins is dependent on divalent cations, and RGD ligands are eluted from integrin affinity columns with 10 mM EDTA (37).

Entry of viruses into cells is a complex, multistep process, and for several viruses cell attachment and internalization have been shown to be distinct steps requiring different cell surface receptors (42, 44, 48, 52). For example, attachment of adenovirus type 2 to cells is mediated by the fiber, whereas internalization requires a second viral protein, the penton base, and is dependent on RGD-binding integrins, αvβ3 and αvβ5 (29, 48). It therefore appears that, like adenovirus type 2, FMDV attaches to the cell surface via interactions with a nonintegrin component of the plasma membrane or extracellular matrix before integrin-dependent internalization (4, 48). Herpesviruses also provide an example of a complex cell entry process. The initial contact of these viruses to cells is through HS in the form of HS proteoglycans on the cell surface (9, 22, 33–35, 39, 47, 53, 54). Secondary receptors are required for the subsequent steps of viral envelope fusion with the plasma membrane and entry of the nucleocapsid into the cell (6, 18, 21, 27).

What is the role in cell entry for virus binding to a highly abundant, low-affinity receptor, such as HS? Several possible roles have been proposed for HS when it is acting as a primary receptor for various growth factors and the attachment of herpesviruses to cells (40, 44). For monomeric basic fibroblast growth factor molecules to elicit their biological effects, they must cross-link and activate the basic fibroblast growth factor receptor. It has been proposed that several basic fibroblast growth factor molecules bind to a single HS chain, thereby forming a multivalent complex which is the effective ligand for receptor cross-linking (40). As FMDV is multivalent for the integrin binding site, it seems unlikely that binding to HS serves to facilitate integrin cross-linking. In the case of herpesviruses, it has been proposed that attachment to HS serves to concentrate the virus at the cell surface, by restricting movement to two dimensions, thereby improving the probability of binding to a secondary receptor(s) used for internalization (19, 44). This effect may be important for internalization of FMDV, since it has been shown that binding of protein ligands to purified RGD-binding integrins (including αvβ3) occurs at low rates (43). In biological systems which are not at equilibrium, the rate of formation of a complex can be more important than affinity (51). It has been suggested that this effect may be particularly relevant to viruses, such as FMDV, which flow in fluids such as blood and respiratory secretions (19). In the cellular entry of type O FMDV, HS could fulfill this role by acting as a primary receptor that allows for rapid cell binding, thereby allowing for more time for virus binding to integrins. In this respect, binding of type O FMDV to cells may be analogous to neutrophil adhesion to the vascular endothelium (25) and to platelet arrest on thrombogenic surfaces (38), since in both of these examples, transient contacts between the interacting surfaces are required to prolong the time available for stable adhesion to occur through secondary interactions involving integrins. Integrins, including several that bind to RGD, exist in low-affinity binding states that require activation to effect ligand binding (11, 14, 17, 20, 28). Further studies are required to determine whether binding of FMDV to cellular HS activates a high-affinity integrin interaction or whether it operates only to provide a high rate of virus binding to cells.

Although the initial attachment of herpesviruses to cells is sensitive to competition with heparin, there follows a conversion to binding that is insensitive to heparin competition, an event which presumably coincides with binding to the second receptor(s) (9, 32, 44). A similar transfer of virus from HS to the integrin during internalization of type O FMDV could explain why a higher concentration of heparin is required to neutralize FMDV infectivity than to inhibit its attachment to fixed cells. This difference in heparin sensitivities is extremely large, and this explanation, if true, provides an intriguing insight into the close functional synergy between the primary (HS) and secondary (integrin) receptors.

Binding to an abundant, charged molecule (HS) explains the inability of type O FMDV to saturate binding sites on fixed cells. Similarly, it may be that the inhibitory activity that small RGD peptides exert at high concentrations (2, 16) is due, in part, to nonspecific competition with HS. Although the location of the heparin binding site on the virus remains to be identified, we have observed that a region near the C terminus of VP1 of type O FMDV includes a highly conserved sequence motif (200-RHKQKI-205) which is similar to a region of the heparin binding site of vitronectin (KKQRF) (24). What of other FMDV serotypes? The C termini of VP1 proteins of all FMDV serotypes contain a high proportion of basic residues, which could be involved in ionic interactions at the cell surface. This observation suggests that in other serotypes of FMDV the C terminus of VP1 may be involved in cell attachment by binding to negatively charged moieties at the cell surface. Indeed, there is evidence for this. Attachment sites on BHK cells for Asia-1 FMDV cannot be saturated (45), suggesting that Asia-1, like type O FMDV, may attach to cells via an abundant receptor that is not used for internalization. Similarly, the South African Territories type 2 FMDV is known to cause haemagglutination, which suggests that this virus can attach to cell via sialic acid (5).

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