Foot-and-Mouth Disease Virus Receptors: Comparison of Bovine αV Integrin Utilization by Type A and O Viruses

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Foot-and-mouth disease virus (FMDV) is one of the most feared viral pathogens of livestock. Outbreaks can result in high morbidity and loss of production in infected animals, but the most devastating economic consequence to affected countries results from mass livestock culling and international trade restrictions imposed on animals and animal products. FMDV is the type species of the Aphthovirus genus of the Picornaviridae family and exists as many subtypes and variants within seven different serotypes.

Limited trypsin digestion of FMDV results in the generation of noninfectious virions that are unable to adsorb to susceptible cells (5) due to the cleavage of VP1 at the Arg (R) residue of a highly conserved Arg-Gly-Asp (RGD) motif (62) located within a flexible external loop between the βG and βH strands (G-H loop) (1, 36, 41). In addition, peptides containing the RGD sequence inhibited the adsorption of the virus to tissue culture cells (6, 21), and genetically engineered virions containing either mutations or deletions of the RGD sequence were unable to bind to cells or cause disease in susceptible animals (37, 45, 46). These observations have led to the conclusion that the virus utilizes cell surface integrin molecules as receptors via this RGD site. Subsequently, it has been demonstrated that at least three integrins, α5β1, α5β3, and α5β6, can serve as receptors for FMDV in vitro (10, 32, 33, 52, 53).

Integrins are heterodimeric type I membrane glycoproteins composed of two subunits (α and β) that interact noncovalently at the cell surface (30). They mediate cell-cell interactions and the binding of cells to the extracellular matrix, and in doing so, they play a crucial role in cell division, differentiation, migration, and survival (23). The eighteen α and eight β mammalian integrin subunits can assemble into 24 different heterodimers (29). While the consensus binding motif of some integrins is unknown, at least eight integrins recognize and bind to ligands via an RGD sequence (63). Interestingly, despite recognizing this tripeptide sequence, these integrins bind different extracellular matrix ligands (40, 69). While the reasons for this specificity are obscure, there is evidence that ligand sequences either flanking the RGD motif or located in other regions of the ligand are responsible for binding specificity (12, 54). One subgroup within the integrin family is the αV integrins, comprising five heterodimers (αVβ1, αVβ3, αVβ5, αVβ6, and αVβ8), all of which recognize the RGD motif on their natural ligands (30, 63).

While the aforementioned observations strongly suggest that RGD-dependent integrins probably direct FMDV to target tissues during a natural infection in susceptible hosts, the fact that the virus can utilize multiple αV integrins in vitro leaves open the question of how these different receptors may function in determining viral pathogenesis. To begin to answer this question, we molecularly cloned the bovine β1, β3, and β6 integrin subunits and, using previously cloned bovine αV and β3 subunits (52), compared αV integrin receptor utilizations among several different representatives of FMDV serotypes A and O. Surprisingly, we found that viruses of these two serotypes utilized these integrins with different efficiencies. We also...
examined the roles of the β subunit ligand-binding domains (LBDs) and the G-H loops of the different ligands in αv integrin utilization.

**MATERIALS AND METHODS**

**Sequencing of bovine integrin β6, β5, and β3 subunits.** Sequencing of the bovine integrin subunits was performed prior to molecular cloning in order to ascertain the exact sequences of the 5' and 3' ends. The primers used for cloning and sequencing of the integrin subunits are listed in Table 1. Total RNA was extracted from primary bovine tongue keratinocytes (55) (for the sequencing of the integrin subunits) and from secondary calf thyroid cells (for the amplification of the virus). All PCR amplifications were performed with Pfu polymerase (Stratagene).

For the amplification of the β5 subunit, the forward primers 34 and 35 (Table 1) were selected from alignments of the GenBank human and feline integrin genes. The primers used for cloning of the β5 subunit, forward primer 9 and reverse primer 11 (Table 1) were selected from consensus sequences (GenBank accession numbers NM000888 and AF115376, respectively), to use a 2.2-kbp fragment by PCR. The PCR products were inserted into the Zero Blunt TOPO vector (Invitrogen), as described by the manufacturer, and sequenced on an ABI 3700 DNA analyzer (Applied Biosystems) by using an ABI Prism Big Dye terminator cycle-sequencing ready reaction kit (Perkin-Elmer).

The assembled cDNA was transferred into pBovH9251 and pBovH9252 for expression of the complete integrin subunit cDNAs were resequenced and analyzed by coupled in vitro transcription-translation using the rabbit reticulocyte TNT Quick Coupled system (Promega) as described previously (52). Plasmids pBovH9251-ZEO and pBovH9252-ZEO have been described previously (52).

**Viruses and cells.** FMDV type A19 strain 119ab (A19) was derived from the infectious cDNA clone pMRCV (61). The cDNA was assembled from a virus with an unknown high-passage history in both bovine kidney and BHK-21 cells and which, following recovery from transfected BHK-21 cells, has been passaged numerous times in this cell type. The virus exhibits mild virulence in cattle. An antigenic variant of type A19 (A19-SSP), harboring the VP1 sequence present in a bovine-tissue-derived virus, was assembled into a cDNA (vRM-SSP) as described previously (59). Following recovery from transfected BHK-21 cells, the virus was passaged twice in CHO cells expressing an engineered receptor con-
sitting of a single-chain anti-FMDV antibody fused to intercellular adhesion molecule 1 (scAb/ICAM1) (60). The virulence of this virus in cattle has not been tested. FMDV type A25Cruzio (A25Cru) was recovered from a foot lesion of a steer experimentally inoculated with virus intradermally into the tongue. The virus was used directly from the vesicular fluid and not passaged in tissue culture. The cattle-virulent variant of type O1Camp (O1Camp) was derived from the infectious cDNA clone pCRM8, which contains capsid sequences isolated from a vaccine seed stock and has been described previously (64). FMDV O/Taw/2/99 was isolated from the esophagopharyngeal fluid of a bovine with a subclinical FMDV infection (27). The virus was passaged three times in BHK-21 cells in Taiwan and then sent to the Institute for Animal Health, Pirbright, United Kingdom, where it was passaged once in primary bovine thyroid cells. It was then sent to the Plum Island Animal Disease Center, where it was passaged twice in BHK-21 cells. This virus did not cause clinical disease when it was experimentally inoculated into the species of cattle from which it was originally isolated (27, 28), nor did it cause clinical disease when inoculated into cattle at the Plum Island Animal Disease Center, but all animals seroconverted (P. W. Mason, personal communication). The genetically engineered type A25 virus chimera, where the G-H loop has been replaced with the homologous loop sequences from type O1 BFS (A/O) virus, has been described previously (58). A summary of the properties of these viruses is presented in Table 2.

BHK-21 cells were maintained in minimum essential medium containing 10% calf serum and 10% tryptose phosphate broth. COS-1 cells were maintained in Dulbecco’s minimum essential medium (Life Technologies) containing 10% fetal calf serum, an additional 2 mM concentration of L-glutamine, and 1 mM sodium pyruvate.

### Expression of integrin subunits in COS-1 cells and viral replication assays

Expression of integrin subunits in COS-1 cells and analysis of viral replication were performed as described previously (52). Brieﬂy, cells plated on six-well tissue culture plates were transfected with 2 μg each of pBovic/ZEO and the appropriate β subunit cDNA plasmid by using the transfection reagent FuGENE6 (Roche Molecular Biochemicals). Transfected cells were incubated overnight, followed by trypsinization and replating onto 24-well plates. After a further overnight incubation, transfected cultures were infected with FMDV type A12-A24 Cru, O1 Camp, or O/Taw/99 at a multiplicity of infection of 10 PFU/cell or A25-SSP at a multiplicity of infection of 1 PFU/cell. Infected cells were labeled with [35S]methionine between 4 and 18 h postinfection, and viral replication was determined on infected cell lysates by radioimmunoprecipitation (RIP) of equal amounts of trichloroacetic acid-precipitable counts per minute of lysate by using monoclonal antibody (MAb) 6EE2 directed against FMDV type A12 (8) or 10GA4 directed against type O1 (67) as described previously (52). Immuno precipitated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% polyacrylamide gel. Transfected cells in one of the wells were not infected but were analyzed for integrin expression by immunohistochemistry as described previously (52) by using MAbs LM609, 6S6, and CS6 directed to the human integrins αβ1, β1, and β6, respectively, or a polyclonal rabbit serum directed against the bovine β1 subunit.

### RESULTS

#### Cloning and sequence analysis of bovine integrin β subunits

At least three different α integrins, α1β1, α2β1, and α2β6, have been reported to function as FMDV receptors in vitro (10, 32, 33, 53), and Neff et al. have previously shown that the virus utilized bovine α1β2 more efficiently than its human homolog (52). In preliminary experiments using K562 cells stably transfected with human α1β2, we were unable to demonstrate receptor utilization of that integrin by FMDV type A12 (unpublished observation). In light of the aforementioned observations, we cloned and analyzed the cDNAs encoding bovine β1, β3, and β6 subunits. The β1 and β3 subunit-coding sequences were amplified from cDNA prepared from primary bovine tongue keratinocytes, while sequences coding for the β6 subunit were amplified from cDNA prepared from secondary bovine thyroid cell cultures (see Materials and Methods).

The complete coding sequence for the bovine β1 subunit comprised 2,397 nucleotides coding for a 798-amino-acid-protein, consisting of a 23-residue signal peptide, a 708-residue ectodomain, a 29-residue transmembrane domain, and a 41-residue cytoplasmic domain. The coding sequence for the β3 subunit comprised 2,403 nucleotides coding for an 800-amino-acid- ectodomain, a 29-residue transmembrane domain, and a 51-residue cytoplasmic domain. A comparison with the human β6 sequence revealed that the bovine homolog contained one additional codon located within the ectodomain. The coding sequence for the β6 subunit comprised 2,367 nucleotides coding for a 788-amino-acid- residue protein consisting of a 26-residue putative signal peptide, a 681-residue ectodomain, a 29-residue transmembrane domain, and a 52-residue cytoplasmic domain.
The nucleotide and predicted amino acid sequence similarities within the different subunit functional regions among the human and bovine β subunits are shown in Table 3. Consistent with the previously analyzed bovine and human αV, β1, and β3 homologs (52), the greatest degree of amino acid sequence divergence of the mature β1 and β3 subunits occurred within the ectodomain. The β2 subunit exhibited the highest amino acid sequence divergence within the ectodomain of the three subunits and, surprisingly, had a higher degree of amino acid sequence divergence within the transmembrane domain (Table 3). The amino acid sequences of the cytoplasmic domain were totally conserved for the β1 and β2 subunits, but there was a single codon change in the β3 subunit. The cytoplasmic domain of the human β3 subunit has been shown to be required for the integrin to function as a receptor for FMDV (49), which is in contrast to the bovine β3 subunit, where deletion of almost the entire cytoplasmic domain does not affect the viral receptor function of the integrin (51). Characteristic of all integrin β subunits is the high content of cysteine residues and four tandem cysteine-rich epidermal growth factor (EGF)-like domains known as the cysteine-rich repeats (24). Alignments of the human and bovine predicted amino acid sequences of the β1, β2, and β3 subunits showed that all cysteines were conserved, with the exception of a C → Y change in the bovine β1 ectodomain at residue 671, just downstream of the last EGF-like domain. Neff et al. have previously found a C → R substitution in the bovine β3 subunit within the second EGF-like domain (52).

Replication of type A12 and O1Camp viruses in COS-1 cells expressing bovine integrins. COS-1 cells were cotransfected with pBovαV,ZEO and pBovβ1, pBovβ2,ZEO, pBovβ3, or pBovβ5 as described in Materials and Methods. Cells were monitored for integrin expression by immunohistochemistry (52). More than 80% of the cells in the cultures transfected with αVβ3, αVβ5, or αVβ5 cDNA stained positively for the appropriate integrin (data not shown). COS-1 cells, however, express the β3 subunit naturally, possibly as the αVβ3 heterodimer. Since the available anti-human αV MAb did not cross-react with the bovine homolog, we were unable to effectively monitor αVβ3 expression in these cultures. The integrin expression in the transfected cells was always tested in parallel cell culture preparations in the subsequent FMDV infection experiments, and only the results of experiments where at least 80% of the cells expressed the proper integrins are reported.

Transfected and nontransfected COS-1 cultures were infected with either FMDV type A12 or type O1Camp to evaluate integrin usage preference between these two serotypes. Both viruses have been demonstrated to utilize both bovine and human αVβ3 as receptors, although they utilized the bovine integrin more efficiently (52, 53). In addition, neither virus utilizes heparan sulfate as a receptor, as has been demonstrated for some tissue culture-adapted FMDV strains (31, 53, 64). Infected cells were labeled with [35S]methionine between 4 to 18 h after infection, and viral replication was analyzed by RIP as described in Materials and Methods. In this and subsequent experiments, only cells originating from the same transfected culture were infected with the different viruses. In addition, within each strain, equal numbers of trichloroacetic acid-precipitable counts per minute were immunoprecipitated, allowing direct comparison of viral replication between cultures expressing different integrins. The results of a typical transfection-infection assay are shown in Fig. 1a. Under these conditions, virus-specific proteins could not be detected in nontransfected cells infected with type A12 virus. In contrast, low levels of virus-specific proteins were present in nontransfected cells infected with type O1Camp virus. This finding, which has been observed previously (52), may be due to contaminating virus which can utilize heparan sulfate as a receptor. In cells transfected with cDNAs encoding the bovine integrins and infected with either type A12, or O1Camp virus, virus-specific proteins were detected in all the integrin-transfected cell cultures. However, the level of viral protein synthesis in type A12 virus-infected cells expressing either αVβ3 or αVβ5 was much greater than that in cells transfected with either αVβ1 or αVβ5. In contrast, type O1Camp virus appeared to utilize the αVβ5 integrin more efficiently than any of the other integrins and utilized αVβ1 more efficiently than either αVβ3 or αVβ5.

To quantitate the level of viral replication in the transfected cells, we digitized the autoradiogram and quantitated the intensities of the VP1 bands relative to the intensity determined for nontransfected cells. αVβ3 and αVβ5 were equally efficient in mediating infection by type A12 virus, while αVβ1 and αVβ5 were poor receptors for this virus (Fig. 1b). In contrast, replication of type O1Camp virus was mediated by αVβ5 and αVβ1 to a greater degree than by either αVβ3 or αVβ5 (Fig. 1c).

To determine whether the transfected cells became susceptible to FMDV as a specific consequence of integrin expression, we pretreated transfected cells with function-blocking anti-integrin MAbs against human αVβ3, αVβ5, or αVβ1, followed by infection with either type A12 or O1Camp virus in the presence of the antibodies. Viral replication in either αVβ1- or αVβ5-transfected cells was inhibited in the presence of the appropriate antibody (data not shown). Treatment with the available anti-αVβ5 blocking MAb resulted in a low degree of inhibition of viral replication (data not shown). While this antibody has been demonstrated to inhibit the replication of a type O1 virus in cells transfected with human αVβ5 (33), we found by using flow cytometry that it reacts poorly with the bovine integrin (data not shown). The available function-blocking antibodies against human αVβ5 did not cross-react
with the bovine integrin, so we were unable to perform this study with cells transfected with this integrin.

Role of the $\beta_2$ subunit LBD in mediating virus infection. The LBD of the intact integrin consists of discrete regions of both the $\alpha$ and $\beta$ subunits (20, 74). To analyze what role, if any, the $\beta_2$ subunit LBD plays in distinguishing different virus strains, we exchanged homologous regions corresponding to the putative LBDs of the individual subunits, which resulted in a series of $\beta_2$ subunit chimeras, as described in Materials and Methods. These chimeras were cotransfected with the bovine $\alpha_2$ subunit into COS-1 cells, followed by infection with either type $A_{12}$ or $O_1$Camp virus. Viral replication mediated by the chimeras was analyzed as described in Materials and Methods and compared to replication mediated by the wild-type integrins, and a representative experiment is shown in Fig. 2.

In transfected cells infected with type $A_{12}$ virus, replacement of the LBD of the $\beta_3$ subunit with the LBD of either the $\beta_1$ or $\beta_5$ subunit reduced the efficiency of the $\alpha_2\beta_3$ integrin as a receptor. Conversely, replacing the $\beta_3$ LBD with the $\beta_1$ LBD did not increase the efficiency of the $\alpha_2\beta_1$ integrin as a receptor for this virus; however, replacing the $\beta_3$ LBD with the $\beta_5$ LBD resulted in a small increase in the efficiency of the $\alpha_2\beta_5$ integrin. Exchanging LBDs of the $\beta_1$ and $\beta_3$ subunits appeared to lower the efficiencies of both of these integrins as viral re-
ceptors for both types A12 and O1Camp, type A12 virus utilized the $\beta_4[\beta_3]$ subunit and type O1Camp virus utilized the $\beta_4[\beta_3]$ subunit slightly more efficiently than the subunits with the reciprocal exchanges.

**Analysis of integrin utilization by other type A and O viruses.** In order to ascertain whether the preference for different integrins was unique to types A12 and O1Camp or was common to the individual serotypes, we analyzed viral replication in cells transfected with each of the cloned integrin cDNAs of two additional type A viruses and one additional type O virus. These viruses and their passage histories are described in Materials and Methods and summarized in Table 2. The results of this experiment are shown in the autoradiogram in Fig. 3a, and the quantitation is shown in Fig. 3b and c. All of the type A viruses efficiently utilized the $\alpha_v\beta_3$ integrin as a receptor. Types A12 and A24Cru also utilized the $\alpha_v\beta_3$ integrin efficiently as a receptor, while type A12-SSP does so to a lesser extent. Surprisingly, A24Cru also utilized the $\alpha_v\beta_1$ integrin as a receptor with relatively high efficiency. In contrast, the two type O viruses utilized only the $\alpha_v\beta_6$ integrin with high efficiency and utilized $\alpha_v\beta_1$ slightly better than $\alpha_v\beta_3$.

**Influence of the G-H loop on $\alpha_v$ integrin utilization.** The above-mentioned results indicate that the viral serotype, not the tissue culture history or the relative bovine virulence of the individual isolates, appeared to determine the efficiency of integrin utilization. While there could be many factors which determine the integrin specificity of the individual serotypes, we focused on the G-H loop. A comparison of the sequences of the loops for all of the viruses utilized in this study (Fig. 4) showed that the sequences are more conserved within the serotype than between the serotypes. All of the loops have a conserved Tyr near the N terminus of the loop and a conserved Leu-Ala at the RGD + 4 position. The two type O viruses have a Leu at the RGD + 1 position, and the DLXXL sequence has been suggested to be an $\alpha_v\beta_6$ recognition sequence (35). The most striking difference between the serotypes is the presence of a Cys at the base of the loop in the type O viruses which forms a disulfide bond with a Cys residue in VP2 (41, 56). The type A viruses do not have this disulfide bond, allowing the G-H loop to assume a conformation, relative to the rest of the capsid, that is different from what is seen in the type O viruses (16). Reduction of the disulfide bond in the type O G-H loop results in the rearrangement of the loop (41) into a conformation similar to that seen in the type A viruses (16). To examine the role of the loop in integrin differentiation, we transfected COS-1 cells with cDNA encoding either $\alpha_v\beta_3$ or $\alpha_v\beta_3$, followed by infection with type A12 or O1Camp virus that was treated with 10 mM dithiothreitol (DTT) or a type A12 virus chimera where the G-H loop was replaced with sequences representing the G-H loop of type O1BFS (58). Treatment of either type A12 or O1Camp virus with DTT did not reduce the infectivity of the virus, as determined by plaque assay in BHK-21 cells (data not shown), confirming previously reported results (41). Our results, shown in Fig. 5, indicate that neither treatment with DTT nor replacement of the type A12 G-H loop

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**FIG. 2.** Viral replication in COS-1 cells expressing $\beta$ subunit LBD chimeras. Cells were cotransfected with cDNA plasmids encoding the bovine $\alpha_v$ subunit and either wild-type $\beta$ subunits or $\beta$ subunit LBD chimeras as indicated in the figure and infected with either type A12 or O1Camp virus. Viral replication was analyzed by RIP and SDS-PAGE as described in Materials and Methods. Lanes: nontx, immunoprecipitated proteins from nontransfected infected cell lysates; M, the locations of the viral structural proteins from lysates prepared from FMDV-infected BHK-21 cells are indicated.
with a type O₁ loop had any dramatic effects on the differentiation of the two receptors by either type A₁₂ or O₁ Camp virus.

**DISCUSSION**

In addition to integrins, FMDV has been shown to utilize various cell surface molecules as receptors in vitro. These include Fc receptors (7, 43, 45), heparan sulfate (4, 31, 42, 53), and a single-chain antibody fused to intercellular adhesion molecule 1 (60). Since the original identification of the α₅β₃ integrin as a receptor for FMDV (10, 52, 53), two additional integrins, α₅β₆ (33) and α₅β₁ (32), have been shown to function as FMDV receptors in cell culture. The present report expands on these earlier studies by using molecularly cloned

![Image of viral replication in FMDV-infected COS-1 cells expressing bovine integrins.](image)

**FIG. 3.** Viral replication in FMDV-infected COS-1 cells expressing bovine integrins. COS-1 cells were cotransfected with cDNA plasmids encoding the bovine integrin α₅ subunit and the β₁, β₃, β₅, or β₆ subunit. (a) Transfected cells were infected with different FMDV types as noted in the figure and labeled with [³⁵S]methionine, and viral protein synthesis was analyzed by RIP and SDS-PAGE as described in Materials and Methods. Lanes: nontx, immunoprecipitated proteins from nontransfected infected cell lysates; M, the locations of the viral structural proteins from lysates prepared from FMDV-infected BHK-21 cells are indicated. (b and c) The autoradiogram shown in panel a was digitized in a MultiImage Light Cabinet, and the intensities for VP1 bands in cells infected with type A (b) or type O (c) viruses were quantitated with the spot density utility of AlphaEase software, version 4.0. The bars represent the intensities of the VP1 band in transfected cells relative to that of the same region in nontransfected cells.

**FIG. 4.** Sequences of the G-H loops of type A and O viruses.

<table>
<thead>
<tr>
<th>Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁₂</td>
<td>tkYtas-gsgvRGDfpsLAprva</td>
</tr>
<tr>
<td>A₂₄Cru</td>
<td>tskYavg-gsgvRGDMgsLArvv</td>
</tr>
<tr>
<td>A₁₂-SSP</td>
<td>tkYtas-gsgvRGDgsLAprva</td>
</tr>
<tr>
<td>O₁ Camp</td>
<td>ecrYsrnavpvnvRGDLqvLAqkva</td>
</tr>
<tr>
<td>O/Taw/299</td>
<td>nckYgespvtvRNGDlqvLAgkaa</td>
</tr>
</tbody>
</table>

**FIG. 4.** Sequences of the G-H loops of type A and O viruses.
bovine integrin subunits and demonstrates differences in αv integrin receptor utilizations by different FMDV strains.

We employed a transient integrin expression assay system in COS-1 cells (51, 52) to compare the efficiencies of each of the αv integrins in mediating infection by strains representing two different FMDV serotypes. In each experiment, the transfected cells used for infection originated from the same transfected culture and all transfections and infections were done at the same time. Thus, we minimized the degree of variation that may result from differences in the expressions of the different integrins in the transient expression assay system. Our results demonstrated that the efficiencies by which these four bovine integrins are able to mediate infection differed between the two virus serotypes. While the type O viruses utilized αvβ3 with the highest efficiency, followed in descending order by αvβ6, αvβ1, and αvβ3, all of the type A viruses utilized αvβ3 with high efficiency and utilized αvβ6 with equal or lesser efficiency. Only one type A virus (A24Cru) utilized αvβ1 with moderate efficiency, and all utilized αvβ3 very poorly. These results, obtained by using integrins from a species susceptible to FMDV, confirmed the finding of Jackson et al. (32, 33) that type O1 FMDV utilizes αvβ6 and αvβ3 as receptors in vitro.

While interactions between the α and β subunits contribute to the LBD of integrins, specific regions of both subunits have been shown to interact directly with ligands (20). Since the same α subunit was present in all of the integrins in this study, we concentrated on differences in the β subunits as possible explanations for differences in receptor specificities between the two viruses. To further define the reasons for the observed specificities among the β subunits, we exchanged regions of the subunits’ putative LBDs. The regions we chose to exchange have been identified functionally as LBDs by various biochemical criteria, and exchanging these regions among β subunits resulted in changes in the ligand specificities of the integrins (11, 18, 40, 69, 70). The recently solved crystal structure of the αvβ3 integrin, which has led to the structural definition of the integrin’s LBD, showed that the region exchanged in this study interacts with the α subunit within the ligand-binding cleft and contains the RGD binding site, but the entire LBD of the β subunit appears to encompass a larger region of the ectodomain than that exchanged in this study (74, 75). In all cases, however, removing even this portion of the LBD from its natural context and replacing it with an LBD of another β subunit lowered the efficiency of the integrin as a receptor for FMDV. The only exception appeared to be in the β3 subunit, where replacing the β5 LBD with the β3 LBD resulted in a slight increase in the efficiency of the αvβ3 receptor. When the LBDs for the β3 and β6 subunits were exchanged, we observed
that the type A12 virus utilized the β3 subunit containing the β3 LBD with higher efficiency than the β3 subunit with the β6 LBD. Conversely, the O1Camp virus utilized the β3 subunit with the β6 LBD with slightly higher efficiency than the β6 subunit with the β3 LBD. These results suggest that the β subunit LBD plays a role in the recognition of the different viral serotypes; however, with some of the chimeric constructs, we were unable to directly measure integrin expression since reactivity with the anti-integrin MAb s appeared to be either reduced or abolished. Thus, some of the differences in viral replication could be related to differences in the expression of the chimeric integrins on the cell surface. Neff et al. have previously shown that the increased efficiency of the bovine β3 subunit compared with that of the human homolog as a receptor for FMDV appeared to map to the EGF-like cysteine-rich repeat region downstream of the LBD (52), raising the possibility that differences in receptor specificities for FMDV among the integrins may be related to either the LBD or the LBD and other regions of the subunit ectodomain.

The five viruses we analyzed in this study have different degrees of virulence in the bovine (Table 2). The A24Cru and O1Camp viruses are virulent to bovines and cause a rapid and severe clinical disease upon exposure, while the A12 and O/Taw/2.99 viruses cause either a mild or subclinical disease. The A12-SSP virus has not been tested directly in animals. This suggests that differences in receptor recognition do not appear to be related to the virus’ ability to cause disease but rather to the serotype of the virus. Eight integrins recognize RGD as a binding motif sequence on their natural ligands (63). Since each of these integrins displays unique ligand specificities, other regions of the ligands are likely to influence recognition by the receptor. It is interesting that the G-H loops of the type A viruses used in this study contain a conserved leucine at the RGD + 1 position but lack a leucine at the RGD + 4 position (Fig. 4). The DLXXL motif has been shown to be involved in recognition of the αvβ6 integrin (35). These viruses, however, utilize the αvβ6 integrin quite efficiently. Thus, the differences in receptor specificities displayed by the type A and O viruses in vitro may be the result of the amino acid sequence divergence within the G-H loop surrounding the RGD sequence (Fig. 4), differences in loop structure, or interactions with other capsid regions outside of the G-H loop. To examine the first possibility, we made use of an engineered chimeric type A virus in which the G-H loop of a type O virus (O1BFS) was substituted for the type A loop (58). The sequence of the substituted loop was identical to that of the G-H loop from type O1Camp virus, with the exception of a Val → Leu change at the RGD + 1 position. This chimeric virus appears to utilize αvβ6 and αvβ3 to the same extent as does the wild-type A12 virus (Fig. 5). This result indicates that the amino acid residues immediately surrounding the RGD sequence in the G-H loop do not appear to greatly influence the receptor utilization by the virus, although we have not generated and tested a type O virus with a type A loop.

To analyze the influence that the conformation of the G-H loop has on receptor utilization, we infected cells expressing either αvβ3 or αvβ6 with type O1Camp virus that was pretreated with DTT. In type O viruses, the base of the G-H loop is linked via a disulfide bond, between a Cys at residue 134 and a Cys at residue 130 of VP2 (56). Reduction of this disulfide bond by DTT results in a rearrangement of the G-H loop within the viral particle, allowing for the determination of the loop structure (41). For the type A viruses, only the structure of the type A22 virus has been determined, and within the crystal, the G-H loop was disordered (16). Type A viruses, however, do not contain the Cys residue at the base of the G-H loop in VP1, and the structure of the residues at the base of the loop was very similar to that seen in the structure of DTT-treated, but not native, type O1 virus (16). In addition, DTT treatment of type O1 virus resulted in a shift of the G-H loop in VP3 (41) into a conformation almost identical to that seen in the type A22 particle (16). These results suggested that there are differences in the dispositions of the G-H loop between type A and O viruses, and these differences are eliminated by reduction of the disulfide bond in type O viruses. It has also been shown that the disulfide bond is reduced in newly released virus, suggesting that the reduced loop conformation may be biologically relevant (41). We therefore reasoned that by placing the G-H loop of type O1 virus into a more type A-like conformation, the virus would resemble the type A viruses in its receptor utilization. The results in Fig. 5 indicate, however, that treatment of the type O1 virus with DTT did not affect receptor usage. In fact, the reduced virus utilized αvβ3, to a lesser extent than did the native virus. Treatment of type A12 virus with DTT did not affect receptor utilization. In this experiment, we did not further modify the type O virus to prevent reformation of the disulfide bond; however, DTT was present during the entire adsorption period. Experiments examining the reoxidation of the disulfide bond in vitro have estimated that the half time for reformation of the bond at room temperature is 4 days (41). Thus, it appears that, since neither the primary sequence nor the structural conformation of the loop play a role in the relative utilization of the αvβ3 and αvβ6 integrins by types A and O, other regions of the viral capsid must be involved in this interaction.

Little information pertaining to the viral receptors important in the pathogenesis of FMDV in susceptible species is available. If the virus uses only one of the RGD-dependent integrins in vivo, then a correlation should exist between the sites where the virus replicates and causes the lesions and the tissue distribution of that particular integrin. The other possibility is that more than one integrin is used during different stages of the disease. In this scenario, the initial stages of infection of an aerosol-infected animal could involve the utilization of receptors in the upper respiratory tract, whereas later replication cycles and amplification in epithelial sites in the mouth and feet could involve the utilization of alternate receptors. While it appears that the disease process in susceptible animals is mediated by virus-integrin interactions (46, 53, 64), a type C virus containing an RGGD sequence has been isolated from a bovine which was not protected from virus challenge following immunization with an experimental peptide vaccine (68). In addition, a tissue culture-adapted type C virus with a genetically engineered RGG sequence, which was unable to bind to heparin, replicated in BHK-21 cells which express both heparan sulfate and FMDV integrin receptors and in heparan sulfate-negative CHO cell mutants which do not express FMDV integrin receptors (2, 3). The ability of these viruses to cause disease in susceptible animals, however, has not been demonstrated. More recently, a tissue culture-
adapted derivative of a Cathay topotype O1 virus isolated in China has also been shown to replicate in tissue culture in both a heparan sulfate- and integrin-independent manner and cause mild disease in swine (76). However, it should be stressed that, with the exception of the virus isolated from a bovine (68), the other viruses were obtained through either tissue culture adaption or genetic engineering. Thus, the possible role of non-integrin receptors in FMDV pathogenicity needs to be more closely examined.

While there are many reports on the distribution of some of the \( \alpha \) integrins in human tissues, little information on the tissue expression of the integrins in FMDV-susceptible animals is available. In human tissues, the \( \alpha_6 \beta_4 \) integrin is found on vascular endothelium and smooth muscle (15, 19, 39) but is not found on bronchiolar epithelium (47). This integrin has been shown to be expressed in an estrous cycle-dependent manner in bovine endometrial epithelium (34) and is expressed weakly in bovine and porcine airway epithelium (65). In contrast, the \( \alpha_5 \beta_1 \) integrin is restricted to epithelial cells (14) but is rarely found in normal tissues in humans (13, 25, 72, 73). High levels of \( \alpha_5 \beta_1 \) have also been found in the macula densa of the kidneys and the endometrial epithelium of secretory phase uterus (14). In general, the expression of \( \alpha_5 \beta_1 \) is highly regulated and is found during development, healing processes, and neoplasia (13). There is very little information available on the expression or function of the \( \alpha_6 \beta_4 \) integrin in specific tissues or organs, although this integrin is expressed on malignant cells (22, 48), in smooth muscle (17), and in the central nervous system (50). It has also been demonstrated to be a receptor for human parvovirus 1 (57, 71) and a coreceptor for human adenovirus (38). The \( \beta_1 \) subunit forms dimers with at least 10 different \( \alpha \) subunits, making the \( \beta_1 \) integrins the largest sub-group within the integrin family (30). However, only three of the \( \beta_1 \) integrins, \( \alpha_\text{V}\beta_1 \), \( \alpha_\text{V}\beta_3 \), and \( \alpha_\text{V}\beta_5 \), utilize the RGD sequence as a binding recognition motif (63).

Obtainment of information on integrin expression in susceptible animals would allow some correlation to be made between integrin use by the virus in cell culture and sites of virus replication in vivo. The tissues we utilized for integrin cloning gave us some indication of integrin expression in cattle. cDNAs for the \( \beta_1 \) and \( \beta_5 \) subunits were easily amplified from RNA extracted from primary bovine tongue keratinocytes. We have also been able to amplify both \( \alpha_\text{V}\beta_1 \) and \( \alpha_\text{V}\beta_5 \) cDNAs from these keratinocytes by PCR (unpublished data). In contrast, we were unable to amplify sequences coding for the \( \beta_5 \) subunit from cDNAs prepared from a number of bovine tissues collected at necropsy, including tongue epithelium, tongue keratinocytes, lung, and kidney. This was surprising given that some of these organs are targets for the virus during natural infection. We were able, however, to amplify cDNA encoding this integrin subunit from RNA prepared from secondary cultures of bovine thyroid. These cells are considered to be highly sensitive to FMDV infection and are often used for primary isolation of virus from field samples (26, 66). The role of the thyroid, if any, in FMDV pathogenesis in vivo is not known.

While this report has concentrated on the role of the viral receptor in pathogenesis, other viral and cellular factors may also affect both host range and virulence (44). In particular, it has recently been demonstrated that attenuation of virulence in bovines and reduced ability to replicate in bovine cells are associated with deletions in the nonstructural protein 3A (9, 55). At this time, it is difficult to reconcile why this virus would utilize at least three different receptors to cause disease. While infection with different FMDV strains may result in different degrees of disease severity, in most cases there are no apparent differences in clinical symptoms within a species. There are, however, differences in the clinical courses of disease among the different species which are susceptible to FMDV. Thus, it is possible that these may be related to differences in either expression patterns or degrees of expression of the known integrin receptors for FMDV among different species. Analysis of the distribution of the integrin receptors in susceptible species may be necessary to explain viral pathogenesis within different species.

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