Role of the Cytoplasmic Domain of the β -Subunit of Integrin $\alpha v \beta 6$ in Infection by Foot-and-Mouth Disease Virus

LAURA C. MILLER,¹ WENDY BLAKEMORE,¹ DEAN SHEPPARD,² AMHA ATAKILIT,²
ANDREW M. Q. KING,¹ AND TERRY JACKSON^{1*}

*Pirbright Laboratory, Institute for Animal Health, Pirbright, Surrey GU24 0NF, United Kingdom,¹ and
Lung Biology Center, Cardiovascular Research Institute, Department of Medicine,
University of California, San Francisco, California 94143-0854²*

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Field isolates of foot-and-mouth disease virus (FMDV) are believed to use RGD-dependent integrins as cellular receptors in vivo. Using SW480 cell transfectants, we have recently established that one such integrin, $\alpha v \beta 6$, functions as a receptor for FMDV. This integrin was shown to function as a receptor for virus attachment. However, it was not known if the $\alpha v \beta 6$ receptor itself participated in the events that follow virus binding to the host cell. In the present study, we investigated the effects of various deletion mutations in the $\beta 6$ cytoplasmic domain on infection. Our results show that although loss of the $\beta 6$ cytoplasmic domain has little effect on virus binding, this domain is essential for infection, indicating a critical role in postattachment events. The importance of endosomal acidification in $\alpha v \beta 6$ -mediated infection was confirmed by experiments showing that infection could be blocked by concanamycin A, a specific inhibitor of the vacuolar ATPase.

Foot-and-mouth disease virus (FMDV) is one of the most infectious animal pathogens known. It causes a vesicular disease of cloven-hoofed animals, which spreads by aerosol, sometimes over long distances. The primary site of infection in cattle is the epithelium of the pharynx (47). It can be assumed that the pathology of foot-and-mouth disease and its ability to spread reflect processes at the cellular level concerning the choice of surface receptor and mechanism of infection.

FMDV is the type species of the *Aphthovirus* genus within the family *Picornaviridae*. The small nonenveloped virion consists of an 8.5-kb strand of RNA within an icosahedral capsid formed from 60 copies each of four proteins (VP1 to VP4). The virus infects cells by attaching to integrin receptors through a long surface loop, the G-H loop, of VP1 (28, 30, 31, 34, 35). The sequence of this loop contains the conserved tripeptide, Arg-Gly-Asp (RGD), which is characteristic of the ligands of several members of the integrin family (27). Some FMDVs can use heparan sulfate proteoglycans as alternative receptors, but this ability appears to be an adaptation to growth in cell culture, and there is no convincing evidence of a role for heparan sulfate in cell entry by field strains of FMDV (22, 29, 40, 46).

Integrins are a family of cell surface glycoproteins which function in a variety of adhesion and signaling phenomena (6, 16, 24, 27). Each molecule is composed of two large, type 1 transmembrane polypeptides, α and β , which, in most cases, have short cytoplasmic domains. To date, two species of RGD-binding integrin, $\alpha v \beta 3$ and $\alpha v \beta 6$, have been reported to mediate FMDV infection in cell culture (5, 31). Of the two, only $\alpha v \beta 6$ is expressed in epithelial tissues, and this species is currently the most plausible receptor in the animal host (31). We

recently reported that this integrin serves as an attachment receptor for FMDV, but we have no information on what part, if any, this integrin plays in subsequent events during infection (31). The work described in this paper was undertaken to elucidate the part played by $\alpha v \beta 6$ in the events that follow virus attachment to the host cell.

For an infecting virion, the entry pathway culminates in the uncoating of the RNA genome and its transfer across a membrane into the cytosol. These processes are poorly understood for nonenveloped viruses, although it is clear that picornaviruses have evolved a variety of strategies for gaining entry to cells. For enteroviruses and the ICAM-1 receptor group of rhinoviruses, binding to the receptor triggers a profound structural transformation to the so-called altered or A particle, and this change is thought to be a prerequisite for release of the RNA (3, 11, 18, 21, 26, 42). For other picornaviruses, such as coxsackievirus A21 and certain enteroviruses that bind decay-accelerating factor, virus binding to the primary attachment receptor does not lead to formation of A-particles; instead A-particle formation is initiated by virus binding to a secondary or coreceptor (42, 43, 48). For FMDV, by contrast, there is no evidence for A-particle formation. Such a transformation would not be needed by a virus, like FMDV, that dissociates into pentamers, RNA, and VP4 at pH values just below neutrality (10, 14, 20). Instead, the virus is thought to enter endosomes, leading to capsid uncoating in the acidic environment of this compartment. Some evidence for this mechanism comes from the inhibitory effect of monensin (4) which raises endosomal pH. Capsid dissociation by some picornaviruses, such as the minor receptor group rhinoviruses, appears to involve aspects of both the enterovirus and FMDV pathways, since A-particle formation is triggered not by virus binding to its receptor but by acidic pH following virus uptake into endosomes (44). FMDV can thus be considered a simple model for endocytic cell entry by a nonenveloped virus. In the present study, we set out to determine what role $\alpha v \beta 6$ plays during the postat-

* Corresponding author. Mailing address: Pirbright Laboratory, Institute for Animal Health, Ash Rd., Pirbright, Surrey GU24 0NF, United Kingdom. Phone: 44-1483-232441. Fax: 44-1483-237161. E-mail: terry.jackson@bbsrc.ac.uk.

FIG. 1. Schematic diagram showing the amino acid sequences of the different $\beta 6$ cytoplasmic domains analyzed in these studies. The sequence of the wild-type $\beta 6$ cytoplasmic domain is shown using the single-letter amino acid code. The broken lines above the sequence indicate the regions important for $\alpha\beta 6$ localization to focal adhesions (13). The conserved NPXY (NPLY) motif is shown in bold type. The underlined sequence is unique to the integrin $\beta 6$ subunit. SW480- $\alpha\beta 6$ expresses wild-type $\alpha\beta 6$ as a functional heterodimer. SW480-T1 to SW480-T5 are transfectants expressing $\alpha\beta 6$ with deletions in the $\beta 6$ cytoplasmic domain. The white boxes represent the sequence retained in the $\beta 6$ subunits. In SW480-T5, the broken line represents an internal deletion.

tachment phases of infection by FMDV by investigating the effects of deletions in the cytoplasmic domain of the $\beta 6$ subunit. We chose this target because many of the functional properties of integrins, including the regulation of binding affinity, signal transduction, and integrin-mediated uptake of ligands, are dependent on the integrity of the cytoplasmic domain of the β -subunit (6, 8, 16, 24, 27, 41, 50, 55). Our data show that although the $\beta 6$ cytoplasmic domain is not required for virus binding, this domain is essential for infection, indicating a critical role in postattachment events. In addition, we show that $\alpha\beta 6$ -mediated infection is dependent on active endosomal acidification, implying that infection mediated by $\alpha\beta 6$ most likely proceeds through endosomes. Together, the results of these experiments are consistent with FMDV entering cells through receptor-mediated endocytosis and are discussed in relation to the normal cellular functions of the integrin β -subunit cytoplasmic domain.

MATERIALS AND METHODS

Cells and viruses. BHK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 20 mM glutamine, penicillin (100 Système International d'Unités [SI] units/ml), and streptomycin (100 μ g/ml). Construction of SW480 cell transfectants expressing wild-type $\alpha\beta 6$ or $\alpha\beta 6$ with deletions in the cytoplasmic domain of the $\beta 6$ -subunit has been described (1, 13, 38, 54). Transfectants were cultivated in DMEM supplemented with 10% FCS, 20 mM glutamine, penicillin (100 SI units/ml), streptomycin (100 μ g/ml), and 1 mg of Geneticin (Life Technologies) per ml. Stocks of the non-heparin-binding FMDV strains O1Kcad² and SAT-3 Zim4/81 (SAT-3) were propagated in primary bovine thyroid cells or BHK cells, respectively. The multiplicity of infection (MOI) was based on the virus titer on BHK cells. FMDV purification was done as described previously (15).

Antibodies, peptides, and reagents. The sequence of the RGD peptide used in these studies was derived from the GH loop of VP1 of type O FMDV (142-VPNLRGDLQVLA-153). The antiintegrin antibodies used in these studies were P1F6 (anti- $\alpha\beta 5$), E7P6 (anti- $\alpha\beta 6$) (54), and 10D5 (anti- $\alpha\beta 6$) (25) all from Chemicon. The anti-FMDV monoclonal antibodies (MAbs) B2 and D9 (36), which recognize type O virus, were purified using protein A (Pierce) according to the manufacturer's instructions. Concanamycin A was purchased from FLUKA and stored at -20°C as a 10 mM stock in dimethyl sulfoxide (DMSO).

Infectious center assay. Cells were harvested using 20 mM EDTA in phosphate-buffered saline (PBS) (pH 7.5), washed, and resuspended in cell culture

media. One million cells were infected with SAT-3 (MOI = 1 PFU/cell) or with O1Kcad² (MOI = 0.5) in 100 μ l of DMEM supplemented with 1% FCS at 37°C for 1 h with continuous rotation. Following infection, virus that remained outside the cells was inactivated by the addition of 1 ml of 0.1 M citric acid buffer (pH 5.2) for 1 min. The cells were washed with PBS (pH 7.5) containing 2 mM CaCl_2 and 1 mM MgCl_2 and resuspended in 300 μ l of the same buffer supplemented with 0.5% FCS. Dilutions of the infected cells (100 μ l) were layered onto subconfluent monolayers of BHK cells as previously described (31), and the monolayers were incubated at 37°C for 40 to 48 h. Infectious centers were visualized as plaques by staining with methylene blue and 4% formaldehyde in PBS (pH 7.5). When antiintegrin antibodies were used to block infection, these reagents were added to the cells for 30 min at room temperature prior to infection.

Concanamycin A experiments. (i) **Pretreatment of cells.** Confluent monolayers of SW480 cells expressing wild-type $\alpha\beta 6$ in 35-mm-diameter dishes were treated with concanamycin A or DMSO (mock treatment) in DMEM with 5% FCS for 30 min at 37°C . The drug was removed, and the cells were infected with SAT-3 (MOI = 2 PFU/cell) for 1 h at 37°C in the presence of fresh drug. Virus that remained outside the cells was acid inactivated as described above. Cell monolayers were washed with PBS (pH 7.5) and incubated in DMEM with 5% FCS at 37°C . At 12 h postinfection, the titer of infectious virus in the cell supernatant was determined by plaque assay on BHK cells as previously described (31).

(ii) **Treatment with concanamycin A postinfection.** To confirm that concanamycin A was not acting on a postentry step of virus replication, cell monolayers were treated with the drug after infection. Cell monolayers were infected, and virus that remained outside the cells was acid inactivated as described above. At 1 h postinfection, the culture medium was replaced by fresh medium containing either concanamycin A or DMSO, and incubation at 37°C continued for 1.5 h. The cell monolayers were then washed and incubated in fresh cell culture media at 37°C . At 12 h postinfection, the virus titer in the cell supernatant was determined by plaque assay as described above.

Flow cytometry analysis. (i) **Integrin expression.** Cells were harvested using EDTA as described above, washed, and resuspended at $\sim 10^7$ cells per ml in buffer A (cold PBS [pH 7.5], 2 mM CaCl_2 , 1 mM MgCl_2 , 1% goat serum, 3% bovine serum albumin, 0.1% sodium azide). All subsequent steps were performed on ice. Cells (30 μ l) were incubated with primary antibodies (10 μ g/ml) for 30 min. The cells were washed and incubated with a goat anti-mouse immunoglobulin G IgG-biotin conjugate for 30 min. Following another washing step, the cells were incubated with a streptavidin-R-phycoerythrin conjugate (Southern Biotechnology Associates). The cells were then washed twice and resuspended in 1% paraformaldehyde in PBS. Fluorescence staining was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson) counting 10,000 cells per sample. Background fluorescence was determined by omitting the primary antibody from the assay.

FIG. 2. Flow cytometric analysis of FMDV (O1Kcad²) binding to SW480 cells expressing wild-type or mutated integrin. Control cell samples were processed in the absence of virus (broken line). Virus binding (continuous line) was detected on all cells expressing $\alpha v\beta 6$ (SW480- $\alpha v\beta 6$ [$\alpha v\beta 6$] and SW480-T1 to SW480-T5 [T1 to T5, respectively]). Virus binding was not detected with the mock-transfected cells.

(ii) **Virus binding assay.** Cells were prepared in buffer A as described above and incubated with O1Kcad² (10 μ g/ml) for 30 min on ice. The cells were washed twice and incubated sequentially with the anti-FMDV MAb D9 (10 μ g/ml), followed by a goat anti-mouse IgG2a-specific R-phycoerythrin conjugate. Background fluorescence was determined by omitting either FMDV or MAb D9 from the assay. Both control conditions gave near-identical results.

(iii) **Competition experiments.** The anti- $\alpha v\beta 6$ MAb 10D5 (IgG2a) or peptides were added to the cells for 30 min before the addition of virus for a further 30 min. The cells were then washed, and cell-bound virus was detected using an anti-FMDV MAb, B2 (IgG1), followed by a goat anti-mouse IgG1-specific R-phycoerythrin conjugate.

RESULTS

The $\beta 6$ cytoplasmic domain is not required for FMDV binding. Previously, we showed that the RGD-dependent integrin $\alpha v\beta 6$ functions as a receptor for FMDV when expressed on transfected SW480 cells (SW480- $\alpha v\beta 6$) (31). To determine whether the cytoplasmic domain of the $\beta 6$ subunit is required for FMDV infection, we used a series of stably transfected cell lines expressing five different deletion mutations in the cytoplasmic domain of the $\beta 6$ subunit (SW480-T1 to SW480-T5 [Fig. 1]). These cells have been reported to show similar $\alpha v\beta 6$ expression at the cell surface (13). Initially, we confirmed this observation by flow cytometry using a MAb specific for the extracellular domain of $\alpha v\beta 6$. We found that on transfectants

SW480-T2 and SW480-T3, $\alpha v\beta 6$ expression was virtually identical to that of the wild-type integrin expressed on SW480- $\alpha v\beta 6$, whereas integrin expression on transfectants SW480-T1, SW480-T4, and SW480-T5 was slightly reduced (see Fig. 4).

We next assessed whether the mutated integrins support FMDV binding. Figure 2 shows that FMDV binds to cells expressing wild-type $\alpha v\beta 6$ (SW480- $\alpha v\beta 6$) but not to the mock-transfected cells, thus confirming our previous observation (31). In addition, Fig. 2 shows that cells expressing the mutant integrin also bind FMDV. However, a small proportion of the cells in the SW480-T5 clone did not appear to bind virus. From the experiments shown below (see Fig. 4), we estimate the proportion of nonbinding cells to be 10 to 15%. To verify that virus binding to the cells was mediated by an authentic RGD-dependent interaction with $\alpha v\beta 6$, we performed competition experiments using the anti- $\alpha v\beta 6$ MAb 10D5 and an RGD-containing peptide. Previously we have shown that these reagents inhibit FMDV binding to $\alpha v\beta 6$, whereas a functional blocking antibody to $\alpha v\beta 5$ or the RGE version of the peptide have no effect on virus binding (31). Pretreatment of cells with MAb 10D5 or the RGD peptide inhibited virus binding, confirming that as for cells expressing wild-type integrin, $\alpha v\beta 6$ serves as the major receptor for FMDV attachment on the cells

FIG. 3. FMDV binds to mutated $\alpha\beta 6$ through an RGD-dependent interaction. Cells were pretreated with the anti- $\alpha\beta 6$ MAb 10D5 (100 $\mu\text{g/ml}$) or peptides (1 mM) for 30 min prior to the addition of virus (O1Kcad²) (10 $\mu\text{g/ml}$). Cell-bound virus was detected by flow cytometry as described in Materials and Methods. Binding (mean fluorescence intensity) is expressed as a percentage of virus bound to cells pretreated with assay buffer alone (control).

expressing mutated integrins (Fig. 3). Furthermore, these results show that the $\beta 6$ cytoplasmic domain is not required for FMDV binding to $\alpha\beta 6$.

In general, a good correlation between virus binding and $\alpha\beta 6$ expression was observed with the transfected cells with the exception of SW480-T2, as these cells supported less virus binding relative to the level of integrin expression (Fig. 4). $\alpha\beta 6$ receptors expressed on SW480-T2 have been shown to be deficient in binding to latency-associated protein of TGF β 1, the natural ligand for $\alpha\beta 6$ (38). Taken together, these data

FIG. 4. The $\beta 6$ cytoplasmic domain is not required for FMDV binding but is essential for $\alpha\beta 6$ -dependent infection. Expression of $\alpha\beta 6$ (white bars), FMDV binding (hatched bars), and the production of infectious centers generated with O1Kcad² (black bars) in SW480 cells expressing either wild-type $\alpha\beta 6$ ($\alpha\beta 6$) or integrins with deletions in the $\beta 6$ cytoplasmic domain (SW480-T1 to SW480-T5 [Fig. 1]). The data are expressed as percentages of the value for the control (SW480- $\alpha\beta 6$). Integrin expression was determined by flow cytometry using MAb E7P6, and the means $\pm$ standard errors of the mean (SEM) of four independent experiments are shown. Virus binding was determined as described in the legend to Fig. 2, and the means $\pm$ SEM of five independent experiments are shown. The infectious center assay was performed as described in Materials and Methods, and the means $\pm$ SEM of three independent experiments are shown.

TABLE 1. FMDV infection of cells expressing $\alpha\beta 6$ with deletions in the $\beta 6$ cytoplasmic domain

Virus and cells ^a	No. of infectious centers ^b	% of control ^c
O1Kcad ²		
SW480- $\alpha\beta 6$	14,430 $\pm$ 458	100
SW480-T1	87 $\pm$ 7.9	0.6
SW480-T2	55 $\pm$ 21.3	0.4
SW480-T3	9,300 $\pm$ 1,081	65
SW480-T4	4,700 $\pm$ 1,135	33
SW480-T5	510 $\pm$ 156	3.5
Mock	28 $\pm$ 9.1	0.19
SAT-3		
SW480- $\alpha\beta 6$	23,000 $\pm$ 4,582	100
SW480-T1	330 $\pm$ 30	1.5
SW480-T2	80 $\pm$ 34.6	0.5
SW480-T3	13,000 $\pm$ 1,732	56
SW480-T4	6,700 $\pm$ 4,592	29
SW480-T5	490 $\pm$ 96	2.0
Mock	14 $\pm$ 6.2	0.10

^a Cells (10⁶), either mock-transfected cells (mock) or cells expressing wild-type $\alpha\beta 6$ (SW480- $\alpha\beta 6$) or $\alpha\beta 6$ with deletions in the $\beta 6$ cytoplasmic domain (SW480-T1 to -T5 [Fig. 1]), were infected with FMDV O1Kcad² or SAT-3 at MOIs of ~ 0.5 and 1 PFU/ml, respectively, as described in Materials and Methods.

^b Mean number of infectious centers $\pm$ standard deviation for one experiment carried out in triplicate.

^c Mean number of infectious centers expressed as a percentage of the control (SW480- $\alpha\beta 6$) value.

indicate that the majority of the $\alpha\beta 6$ on SW480-T2 is expressed in a conformation that is unable to bind RGD ligands.

The $\beta 6$ cytoplasmic domain is essential for $\alpha\beta 6$ -mediated infection by FMDV. The above data show that the cytoplasmic domain of the $\beta 6$ -subunit is not required for FMDV binding. We next sought to determine whether this domain is required for $\alpha\beta 6$ -mediated infection. Truncations in the cytoplasmic domain of the $\beta 6$ subunit have been shown to change the surface distribution of $\alpha\beta 6$ from sites of tight contact with the underlying substratum (focal contacts) and that free in the plasma membrane (13). Since the amount of $\alpha\beta 6$ localized to focal contacts could potentially influence the number of integrin receptors available for virus binding and therefore infection, we performed infectivity studies using an infectious center assay with dispersed cells in suspension, thereby permitting a direct comparison to be made between virus binding and infection.

Virus binding to transfectants SW480-T3 and SW480-T4 generated a significant number of infectious centers (Table 1 and Fig. 4) indicating that the deleted regions of the $\beta 6$ cytoplasmic domain are not essential for infection. Consistent with the observation that virus binding to SW480-T3 and SW480-T4 is inhibited (>98%) by pretreatment of cells with this antibody (100 $\mu\text{g/ml}$), whereas the anti- $\alpha\beta 3$ MAb P1F6 had no effect on infection (data not shown). By contrast, virus binding to SW480-T1, SW480-T2, and SW480-T5 resulted in little or no infection (Table 1 and Fig. 4). The small number of infectious centers generated with SW480-T5 was also completely inhibited by MAb 10D5 (data not shown). Taken together, the results of these experiments show that although the cytoplasmic domain of $\beta 6$ subunit is not required for FMDV

FIG. 5. $\alpha\beta 6$ -dependent infection by FMDV requires active endosomal acidification. Cells were treated with either concanamycin A (10 μ M) or DMSO before (black bars) or after (hatched bars) infection by SAT-3 as described in Materials and Methods. At 12 h postinfection, the virus titer in cell culture media was determined by plaque assay on BHK cells. The virus titer (mean $\pm$ SEM of three independent experiments) is expressed as a percentage of cells treated with DMSO. The virus titer at time 0 h postinfection was less than 30 PFU/ml.

binding, this domain has a critical role in regulating postattachment steps in $\alpha\beta 6$ -dependent infection.

Infection of $\alpha\beta 6$ -expressing cells requires active endosomal acidification. Previous studies have demonstrated that infection of BHK cells by FMDV is inhibited by treatment of the cells with the ionophore monensin, one effect of which is to raise the pH within endosomes, implying that infection proceeds through this compartment (4). To determine whether it is also true of $\alpha\beta 6$ -mediated infection, we investigated the effect on infection of concanamycin A, a specific inhibitor of the vacuolar proton-ATPase (19). Preliminary studies, using one-step growth curve analysis, showed that pretreatment of cells with concanamycin A reduced the virus titer in cell supernatants compared to that of mock-treated controls over time in a concentration-dependent manner (data not shown). Figure 5 shows that treatment of cells with concanamycin A (10 μ M) prior to infection reduced the virus titer in the cell supernatant compared to that of mock-treated controls. Treatment of cells with concanamycin A at 1 h after infection had a minimal effect on the virus titer (Fig. 5) showing that the drug was not inhibiting a postentry step necessary for virus replication. These data show that active endosomal acidification is required for an early event in $\alpha\beta 6$ -mediated infection and imply that infection proceeds through endosomes.

The concentration of concanamycin A used in the experiment shown in Fig. 5 is greater (1,000 times) than that required to inhibit infection by some other viruses, such as influenza virus where infection is known to be dependent on the acidic pH within endosomes (23). An important difference between these viruses is that the conformational change required for influenza virus internalization is triggered at a pH much lower (pH 5.0 to 6.0) than is uncoating of FMDV (9, 14). Therefore, the comparative difficulty in inhibiting FMDV infection with concanamycin A may reflect the extreme acid sensitivity of the FMDV capsid, as the endosomal pH would necessarily have to

be raised to ~ 7 to achieve complete inhibition of capsid disassembly and infection.

DISCUSSION

Entry of picornavirus into host cells is a complex process initiated by virus binding to a specific cell surface receptor. Although receptors for FMDV have been identified, there is little information on the precise mechanisms by which these receptors promote entry. Recently, we showed that the RGD-dependent integrin $\alpha\beta 6$ serves as a receptor for FMDV attachment and that virus binding to the integrin results in an increased rate of cell entry (31). In the present study, we have focused on the role of the $\beta 6$ cytoplasmic domain in FMDV infection. Our results show that complete truncation of the $\beta 6$ cytoplasmic domain resulted in receptors that although expressed on the cell surface and able to bind FMDV, were defective in mediating infection.

The obvious question that arises from these observations is what is the role of the $\beta 6$ cytoplasmic domain in infection. The need for $\beta 6$ cytoplasmic domains implies that $\alpha\beta 6$ does not merely serve to pass virus onto a second receptor for internalization but plays an active role in events subsequent to attachment. The sensitivity of infection to concanamycin A confirms that $\alpha\beta 6$ -mediated infection involves an acidification step and is consistent with virus entry proceeding through endosomes. This observation suggests that the regions deleted from the $\alpha\beta 6$ expressed on SW480-T1, SW480-T2, and SW480-T5 may contain important signals for virus localization to endosomes. Interestingly, all of these receptors lack the $\beta 6$ cytoplasmic domain NPXY motif (Fig. 1). This motif is known to function as a signal that directs various membrane proteins into clathrin-coated vesicles (12). However, the role of the β -subunit NPXY sequence in integrin endocytosis is not well defined (50, 51, 55), and further experimentation will be necessary to more precisely define the role of this motif in FMDV infection.

While infection by FMDV depended completely on the presence of the $\beta 6$ cytoplasmic domain, smaller inhibitory effects were also seen with $\beta 6$ chains C terminally truncated in such a way as to leave the NPXY motif intact. Loss of the C-terminal 11 residues, for example, caused approximately 80% reduction in infection (Fig. 4, mutant T4). Interestingly, a similar observation was reported for the T4 cell line by Agrez et al. (2) when studying infection by another picornavirus, coxsackievirus B1. The reason for this impairment is unclear, however, as we found that deleting a further 7 residues (T3) partially restored receptor function to $\alpha\beta 6$.

The $\beta 6$ cytoplasmic domain could also be needed to complete the final uncoating step in which the viral genome is translocated into the cytosol. Such a requirement has recently been demonstrated for $\alpha\beta 5$ -mediated infection by adenovirus (52), but it seems less likely in the FMDV system, given that FMDV has been shown to infect cells through at least three integrin-independent mechanisms (29, 35, 45), thereby implying that the function of membrane permeabilization is an inherent property of the virus and not the receptor.

Many of the intracellular signaling pathways activated by integrins are dependent on the β -subunit cytoplasmic domain, which points to a further possible function of the $\beta 6$ cytoplasmic domain in facilitating virus entry. For example, signal

transduction through $\alpha\beta 6$ could be required to activate a coreceptor necessary for virus internalization. Signal transduction through integrins is known to regulate the ligand-binding affinity of other integrin species expressed on the same cell by a mechanism termed integrin cross-talk (7). SW480 cells normally express two RGD-dependent integrins, $\alpha\beta 5$ and $\alpha 5\beta 1$, and either of these integrins could conceivably be activated following virus binding to $\alpha\beta 6$. However, as we showed previously that neither of these integrins appear to be involved in infection of SW480 cells expressing wild-type $\alpha\beta 6$, this mechanism may be discounted (31). Although we consider it unlikely, we cannot rule out the possibility that signaling through the $\beta 6$ cytoplasmic domain is necessary for activation of a nonintegrin coreceptor. Alternatively, virus binding to $\alpha\beta 6$ could initiate intracellular signaling pathways that are advantageous for a postentry step in virus replication and/or assembly. However, since mock-transfected SW480 cells in the absence of $\alpha\beta 6$ are permissive for a heparin-binding strain of FMDV (31), such a role for $\alpha\beta 6$ in infection would similarly appear unlikely. However, on the basis of our data, we cannot rule out the possibility that signaling through $\alpha\beta 6$ may be required to promote infection by FMDV by stimulating endocytosis of $\alpha\beta 6$ itself. For example, endosomal uptake of the integrin $\alpha\beta 5$ has been shown to be stimulated by ligand binding (37), and more recently, $\alpha\beta 5$ -mediated endocytosis of adenovirus has been shown to require activation of phosphoinositide-3-OH kinase (PI3K), an event dependent on the $\beta 5$ cytoplasmic domain (33). Using one-step growth curve analysis, we have observed that treatment of cells expressing wild-type $\alpha\beta 6$ with wortmannin (50 to 200 nM), a specific inhibitor of PI3K, did not affect virus replication, suggesting that PI3K activity is not required for $\alpha\beta 6$ -dependent infection by FMDV (data not shown).

Our findings contrast with those obtained with some other picornaviruses, namely, poliovirus, coxsackievirus B3, and human rhinovirus type 14 (HRV-14), which show that the single cytoplasmic domain of their receptors are not needed for infection (32, 49, 53). Since such modifications might be expected to prevent receptor uptake into endosomes, these differences tend to support the view that for these latter viruses infection is not dependent on classical receptor-mediated endocytosis or at least not totally dependent on that route. For poliovirus, this view is supported by the observation that infection is not dependent on dynamic-mediated endocytic pathways (17). However, it should be noted that the same study (17) concluded the opposite for HRV-14, i.e., infection requires dynamin-mediated endocytosis to be functional. An additional difference between these viruses and FMDV is that unlike FMDV, they form A-particles on receptor binding and for poliovirus and HRV-14, conversion to A-particles has been shown to occur on binding soluble receptors which lack cytoplasmic domains (32, 49).

While this study was in progress, Neff and Baxt (39) reported findings with $\alpha\beta 3$ -expressing cells which would also lead to the opposite conclusion, namely, that infection of COS cells by FMDV does not require the cytoplasmic domain of either the α - or β -subunit. However, the role played by $\alpha\beta 3$ in this system remains unclear, since although this integrin was shown to be required for infection, studies with $\alpha\beta 3$ -expressing cells

have never clearly established that FMDV binds to this integrin (5, 39).

While the precise mechanisms involved in $\alpha\beta 6$ -mediated infection require further investigation, the finding reported herein helps in the understanding of postattachment events in FMDV infection.

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