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Point Mutations within the βG-βH Loop of Foot-and-Mouth Disease Virus O_1K Affect Virus Attachment to Target Cells

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The amino acid sequence Arg-Gly-Asp (RGD) is a highly conserved region located on the P1D protein of most sero- and subtypes of foot-and-mouth disease virus (FMDV) and participates in binding of FMDV to their target cells. In order to analyze the role of the RGD sequence in FMDV infection of cells in more detail, 13 mutations within or near the RGD sequence of virus type O_1Kaufbeuren were designed by using a full-length cDNA plasmid. Transfection of baby hamster kidney cells (BHK-21) with in vitro-transcribed cRNAs containing mutations bordering the RGD sequence led to the production of infectious virus in most cases. In contrast, almost all of the mutants containing changes within the RGD sequence produced noninfectious viral particles indistinguishable from wild-type virus by electron microscopy. In order to demonstrate that these noninfectious progeny from the RGD mutants were defective only in their cell adsorption, the respective cRNAs were cotransfected together with a cRNA expressing the wild-type P1 protein. The resulting virus particles were able to infect BHK-21 cells. These results demonstrate the important role of the RGD sequence in FMDV binding to cells but also emphasize the influence of other amino acids in the bordering region.

Foot-and-mouth disease virus (FMDV) belongs to the *Aphthovirus* genus of the family *Picornaviridae* and causes a severe disease of cloven-hooved animals. The viral genome, a single-stranded plus-strand RNA is encapsidated by four structural proteins, namely, P1A, P1B, P1C, and P1D, which are produced by posttranslational cleavage of a common precursor (4, 9, 41, 42). Among these proteins, the P1D protein is of particular interest, because it forms a loop between the β-strands G and H on the virus surface as shown by X-ray diffraction (1). Comparison of the amino acid sequences within the βG-βH loops of many FMDV serotypes and subtypes shows highly conserved amino acids located within the extremely variable loop region which also harbors the main antigenic determinant (5, 26, 29). As indicated in Fig. 1, the amino acids at positions 136 (tyrosine) and 145 to 147 (arginine-glycine-aspartic acid) are conserved with the exception of strain $A_{10}61$, in which a serine is inserted between R 144 and G 146 (10).

The tripeptide RGD plays a central role in binding of extracellular matrix proteins like fibronectin and vitronectin to various cell surface receptors called integrins (23, 39, 40, 47). Therefore, an integrin could function as a cellular receptor for FMDV. This suggestion was supported by blocking experiments, in which RGD-containing peptides (16) or antibodies against the vitronectin receptor α-chain (8) could reduce the infectiousness of FMDV to a significant level. Furthermore, Mason and coworkers (30) recently showed for FMDV A_{12} that mutations within the RGD sequence led to noninfectious viral particles. In addition, Roivainen and coworkers (37, 38) have demonstrated that coxsackievirus A9 can also use the RGD sequence for cell attachment and infection.

The existence of an infectious cDNA of FMDV O_1Kaufbeuren (O_1K) (48) provides a useful tool to investigate the role of the RGD sequence and the surrounding region in receptor binding and cell infection. Mutations within and close to the RGD sequence, based on the sequences of naturally occurring virus mutants, were introduced into the viral genome of serotype O_1K via the full-length cDNA. After in vitro transcription and transfection of BHK cells with the mutated cRNAs, the resulting viruses were examined for their infectious potential. Noninfectious viruses were analyzed in cells in which the protein precursor P1 is produced by using an expression vector system based on Semliki Forest virus (SFV) (7, 27, 28).

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MATERIALS AND METHODS

Cell line and plasmids. BHK-21 cells (ATCC CCL 10) were maintained in Dulbecco's modified Eagle's medium supplemented with 1 mM glutamine and 10% (vol/vol) fetal calf serum in a humidified atmosphere of 5% (vol/vol) CO_2 at 37°C. The full-length cDNA clone pSPffPolyC (48), which represents the complete genome of FMDV type O_1K (15), was obtained from E. Beck, Giessen, Germany. For mutagenesis, the P1-coding region was subcloned into a modified pEMBL-19 by using the single restriction sites *Afl*II and *Eco*RI.

Transient expression of the wild-type structural proteins in BHK-21 cells was carried out by using the SFV plasmid pSFV-P1. This plasmid was constructed by inserting the P1-coding region into the single *Sma*I site of pSFV1 (GIBCO/BRL) (7, 27, 28) as outlined in Fig. 2.

Construction of mutated full-length cDNAs. Point mutations were introduced into the P1D gene by site-directed mutagenesis as described previously (13, 49) by using the plasmid pEMBL-P1 or by PCR (14). Mutated fragments created by PCR were cloned into pEMBL-P1 using the restriction sites *Bam*HI and *Sma*I (Fig. 3) and sequenced through the entire amplified region by the method of Sanger et al. (43). All mutated fragments were then reintroduced into the full-length cDNA clone pSPffPolyC.

In vitro transcription and transfection of BHK-21 cells. The mutated full-length cDNA plasmids were linearized with *Hpa*I and in vitro transcribed by the method of Krieg and Melton (25). The resulting cRNAs were introduced into BHK-21 cells by using the mammalian transfection kit (Stratagene) or electroporation. Electroporation was performed as described by Liljeström and Garoff (28) with the following modifications: 2×10^6 cells/0.4 ml of phosphate-buffered saline without Ca^{2+} and Mg^{2+} were mixed with 5 to 10 μg of transcribed RNA in a cuvette (width, 0.2 cm) and pulsed once at 1.5 kV and 25 μF in a gene pulser (Bio-Rad). The cells were then transferred into growth medium and incubated overnight at 37°C.

For cotransfections of BHK-21 cells, pSFV-P1 was linearized with *Spe*I and transcribed in vitro following the recommendations of GIBCO/BRL. Both the pSFV-P1 transcript and the mutated full-length cDNA transcripts were mixed with BHK-21 cells and electroporated as described above.

Analysis of viral protein synthesis and RNA replication. In order to demonstrate intracellular viral protein synthesis, BHK-21 cells were examined 12 h after transfection by indirect immunofluorescence using a FMDV-specific monoclonal

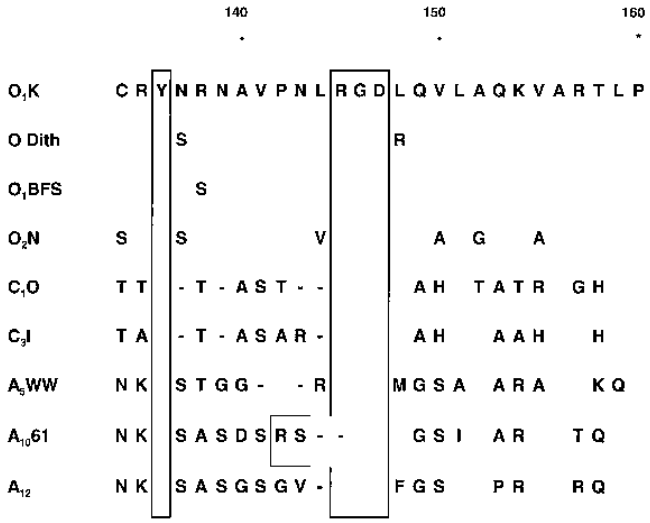

FIG. 1. Amino acid sequences of the βG-βH loops of different serotypes and subtypes. The amino acid sequence of the βG-βH loop is highly variable and is the main antigenic determinant of FMDV. The amino acid tyrosine (Y) in position 136 and the tripeptide Arg-Gly-Asp (RGD) within this region are conserved between the serotypes and subtypes (shown boxed) and are involved in cell attachment. One exception is the type $A_{10}61$ with the sequence RSGD. Dashes indicate gaps introduced to maximize alignment. FMDV type abbreviations: K, Kaufbeuren; Dith, Dithmarschen; BFS, British field strain; N, Normandie; O, Oberbayern; I, Indaial; WW, Westerwald.

antibody (MAb 37) (35) directed against the surface protein P1D. Immunofluorescence was performed by standard methods (18).

The existence of viral minus-strand RNA was proven by isolation of whole cellular RNA as described by Chomczynski and Sacchi (12) 8 h after transfection, and subsequent reverse transcription using a strand-specific primer. After RNase treatment, the respective cDNA was amplified by PCR.

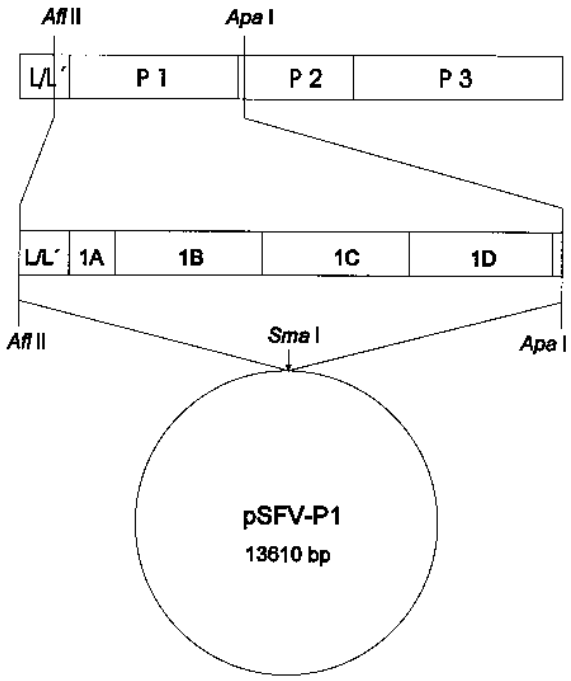

FIG. 2. Construction of the expression vector pSFV-P1. The *Afl*II-*Apa*I fragment, containing the structural genes (P1) was introduced into the single *Sma*I site of pSFV1 after the protruding ends were digested. Abbreviations: L/L′, L-protease; P1 (1A to 1D), structural genes; P2 and P3, nonstructural genes.

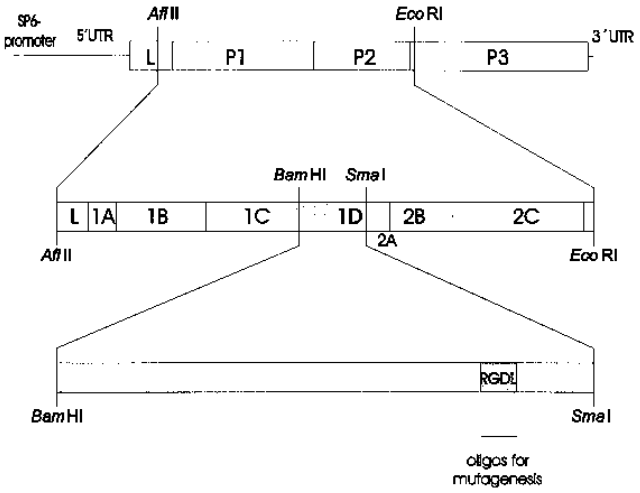

FIG. 3. Position of the mutated region within the FMDV genome. PCR-derived fragments were subcloned into the plasmid pEMBL-P1 and then reintroduced into the full-length cDNA clone. Abbreviations: UTR, untranslated region; L, L-protease; P1 (1A to 1D), structural genes; P2 and P3, nonstructural genes; oligos, oligonucleotides.

Sequence analysis of viral RNA. Infectious virus mutants were grown on BHK-21 cells. Cell lysates were centrifuged (3,000 × *g*, 20 min) to eliminate cellular debris. Virus suspensions were added to a solution consisting of 0.5% (wt/vol) sarcosyl, 50 mM Tris-HCl (pH 7.5), and 5 mM EDTA and stirred for 1 h at 4°C, and virus particles were pelleted overnight (110,000 × *g*, Beckman SW28 rotor, 4°C). The pellet was resuspended in NET buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl [pH 7.5]), and virus particles were isolated by sucrose density gradient centrifugation (10 to 30% [wt/vol] sucrose, 100,000 × *g*, 2 h, Beckman SW41 rotor, 4°C). Virus-containing fractions were treated with 2% (wt/vol) sodium dodecyl sulfate, and viral RNA was purified by phenol extraction. The RNA was directly sequenced by using the GemSeqK/RT System Reagenz Kit (Promega) following the manufacturer's instructions.

Electron microscopy of noninfectious virus mutants. Supernatants from BHK-21 cells which had been transfected with mutated cRNAs and showed no cell lysis were collected 24 h after transfection. Virus particles were precipitated with polyethylene glycol as described by Wagner et al. (46). The virus suspension was placed on a sucrose density gradient (25, 42, and 60% [wt/vol] sucrose) and ultracentrifuged overnight (90,000 × *g*, 13 h, Beckman SW55Ti rotor, 20°C). Virus particles were removed from the top of the 60% (wt/vol) sucrose layer and analyzed by electron microscopy after negative staining with 1% (wt/vol) uranyl acetate.

RESULTS

Introduction of point mutations into the infectious cDNA of FMDV. In order to investigate the roles of the RGD sequence and the conserved tyrosine at position 136 in cell attachment of FMDV, several point mutations were introduced within and close to the RGD sequence. For the introduction of the point mutations into the viral genome, site-directed mutagenesis and PCR-based mutagenesis of a full-length cDNA clone of FMDV serotype O_1K were used. Table 1 shows all mutants which have been constructed. Conservative (RGDL → RGEL and RGDL → RADL) and nonconservative (RGDL → RGAL, RGDL → GGDL, and RGDL → SGDL) amino acid exchanges were chosen to demonstrate to what extent the amino acids of the conserved tripeptide could be changed. Furthermore, the amino acid leucine in the lesser-conserved first position after the RGD sequence was examined for its influence on virus adsorption to cells. Therefore, the mutants 148 His (RGDL → RGDH), 148 Arg (RGDL → RGDR), 137 Ser, and the double mutant 137 Ser/148 Arg were constructed. The amino acid exchanges of the latter three mutants had also been selected based on the sequence comparisons of the FMDV O Dithmarschen in which potential relationships be-

TABLE 1. Amino acid sequences of the wild-type virus and the mutants within the βG-βH loop and their infectivity in cell culture

Virus	Amino acid sequence[a]				CPE[b]
	130	140	150	160	
Wild-type virus	*	*	*	*	
O_1K	V	NGECRYNRNA	VPNLRGDLQV	LAQKVARTLP	+
Mutant viruses					
136 Ser	-	-----S----	----------	----------	−
145 Ser	-	----------	----S-----	----------	−
144 Arg/145 Ser	-	----------	---RS-----	----------	+
145 Gly	-	----------	----G-----	----------	−
146 Ala	-	----------	-----A----	----------	−
147 Ala	-	----------	------A---	----------	−
147 Glu	-	----------	------E---	----------	+
148 Arg	-	----------	-------R--	----------	−
137 Ser/148 Arg	-	------S---	-------R--	----------	+
137 Ser	-	------S---	----------	----------	+
148 His	-	----------	-------H--	----------	−
140 Val	-	---------V	----------	----------	+
142 Ser	-	----------	-S--------	----------	+

[a] Amino acids identical to those in the wild-type sequence are indicated (-).
[b] Symbols: −, no CPE was observed 96 h after transfection of cRNA; +, CPE and lysis of the whole cell culture occurred 24 to 48 h after transfection.

tween amino acids involved in virus attachment had been shown. Another field strain, FMDV $A_{10}61$, possesses a different sequence, RSGDL, within the loop, whereas the RGD sequence is interrupted by a serine. In order to simulate this case, the double mutant 144 Arg/145 Ser (LRGDL → RSGDL) was constructed. A third set of mutations was produced based on sequence data from monoclonal antibody-selected escape mutants (140 Val and 142 Ser) (34). In addition, the effect of the conserved tyrosine in position 136 on the infectivity of the resultant virus was investigated by an exchange to serine, another hydroxy amino acid.

Production of the mutant viruses and their infectivity in cell culture. The mutations were introduced into the infectious cDNA clone as described in Materials and Methods. The different cDNAs were transcribed in vitro, and the resulting full-length cRNAs were transfected into BHK-21 cells by using calcium phosphate. The transfected cells were analyzed for cytopathic effect (CPE) and plaque formation. Table 1 shows the results of all mutants tested so far. Cells transfected with the wild-type cRNA showed a CPE after 24 h and total lysis after further incubation. Identical observations were made when the cells were transfected with cRNAs representing the mutations 147 Glu, 137 Ser, 140 Val, and 142 Ser. In order to confirm the conservation of the mutations in the progeny from infections by these recombinant viruses, the viral RNAs were isolated and sequenced. The original mutations which had been introduced in the full-length cDNAs could be confirmed in all cases, demonstrating that these mutations had no influence on the viability of the resulting recombinant viruses. For example, in the case of the mutant 147 Glu, the supernatants containing the recombinant viruses were passaged two times on BHK cells. After each passage, whole cellular RNA was isolated and the original mutation (D → E) could be confirmed by sequencing after reverse transcription-PCR. In contrast, cells transfected with cRNAs representing the mutations 136 Ser, 145 Ser, 145 Gly, 146 Ala, 147 Ala, 148 Arg, and 148 His showed no CPE or cell lysis, even after 96 h (Table 1). All of the mutants unable to grow in cell culture had mutations in the highly conserved amino acids.

Detection of intracellular viral RNA replication and protein synthesis after transfection. In order to exclude the possibility that these mutations inhibit viral protein synthesis or viral RNA replication, both processes were investigated after transfection of the mutated cRNAs. Viral protein synthesis was tested in an indirect immunofluorescence assay by using a P1D-specific monoclonal antibody. Coat protein P1D was found 12 h after cRNA transfection with all mutants (data not shown). RNA replication was examined by detection of minus-strand viral RNA which is synthesized during RNA replication. These replicative intermediates could be detected with all mutants by a minus-strand-specific PCR. For example, Fig. 4 shows the existence of minus-strand RNA after transfection of cRNA of the mutant 148 Arg which is unable to grow in cell culture. For controls, RNA preparations of cells transfected with wild-type FMDV cRNA and RNA from untransfected BHK cells were used in the PCR.

Characterization of adsorption-deficient virus mutants. The transfection efficiency was increased by electroporation in order to prove the existence of viral particles resulting from transfection by those mutated cRNAs which produced no cell lysis. In this experiment, more than 80% of the BHK-21 cells were transfected and a CPE could be observed 10 to 15 h later. Recombinant viral particles could be isolated from the supernatants and analyzed by electron microscopy. Figure 5A shows FMDV particles produced after transfection by the wild-type cRNA; Fig. 5B and C show the viral particles produced by the mutants 136 Ser and 148 Arg, respectively.

The infectivity of the recombinant viruses was investigated by transferring the supernatants of the transfected and lysed cells to BHK-21 cells. After 3 h, cells were fixed and viral protein synthesis was detected by using a P1D-specific monoclonal antibody. Only the mutants 137 Ser, 140 Val, 142 Ser, 147 Glu, and the two double mutants showed evidence of virus infection. Recombinant viruses derived from cRNAs with mutations in the RGDL sequence (145 Ser, 145 Gly, 146 Ala, 147 Ala, 148 Arg, and 148 His) and in position 136 (136 Ser) were not able to infect BHK cells.

These findings confirm the supposition that these highly conserved amino acids are necessary for the initial step in the viral life cycle, the cell attachment of the virus. However, inhibition of cell attachment is not based solely on the primary structure of the βG-βH loop and the respective binding site. Experiments with two double mutants showed that the viral

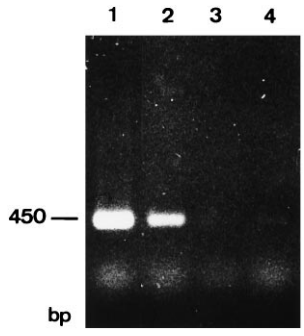

FIG. 4. Minus-strand-specific PCR. A specific product (450 bp) was detected after transfection of the cRNA of pSPffPolyC (lane 1) and of the mutant 148 Arg (lane 2). Lane 3, mock control. Lane 4, control (water). For details, see Materials and Methods.

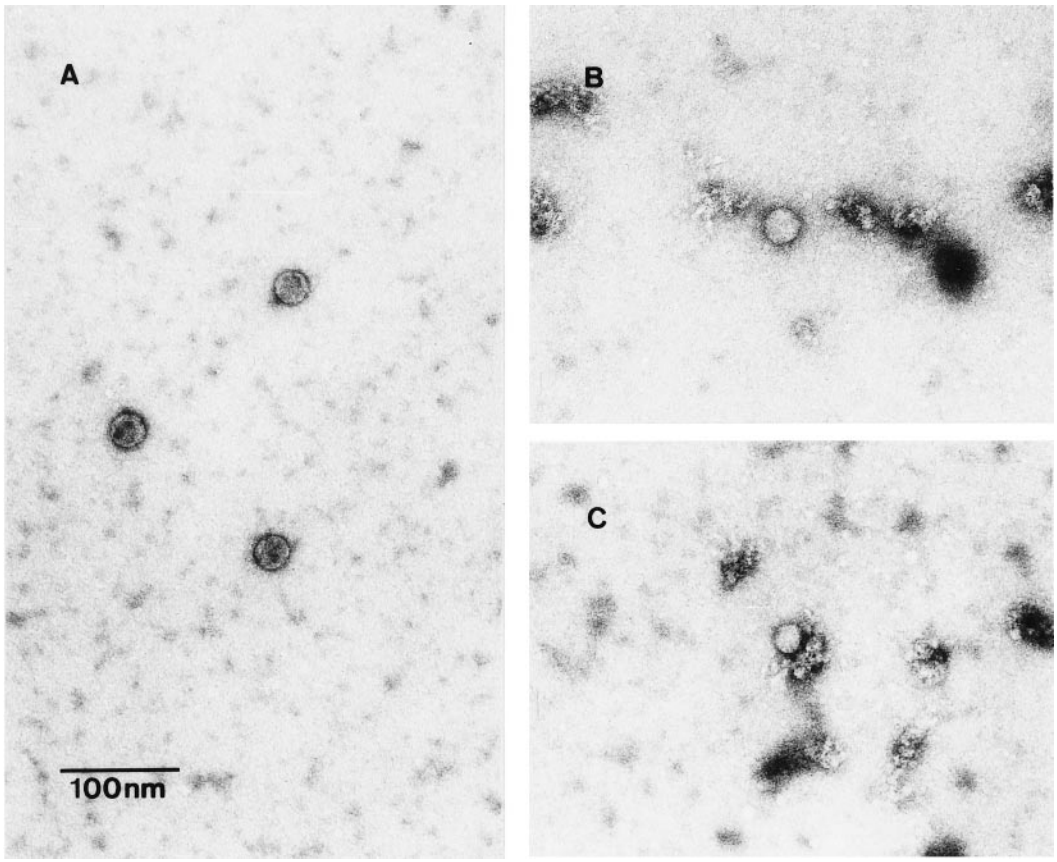

FIG. 5. Electron microscopy. Attachment-deficient virus particles were isolated by sucrose density gradient centrifugation and stained with uranyl acetate. (A) Wild-type virus; (B) mutant 136 Ser; (C) mutant 148 Arg.

attachment to the cell surface could be reconstituted. Transfections with cRNAs from the double mutants 144 Arg/145 Ser and 137 Ser/148 Arg once again produced cell lysis. Sequence analysis of the resulting viral RNAs isolated from the recombinant viruses confirmed the conservation of the two mutations. In these cases, infectious virus could be rescued by the second mutation.

Recovery of the infectious phenotype by complementation. A complementation experiment was performed in order to show that noninfectious virus mutants are able to proliferate normally under certain conditions. Partial replacement of the defect coat protein in the virus capsid by the wild-type protein led to virus mutants with an infectious phenotype, while the genome still contains the original mutation. These infectious virus mutants can undergo only one infection cycle on normal BHK cells, because the progeny viruses express only the mutated P1.

In a first step, the gene for the coat proteins of FMDV was inserted into the expression plasmid pSFV1. After in vitro transcription, the resultant cRNA was transfected into BHK-21 cells by electroporation. Synthesis of FMDV P1 protein was detected both by indirect immunofluorescence and Western blotting. Figure 6 shows the 92-kDa P1 protein of FMDV, which has not been processed into the three mature coat proteins P1AB, P1C, and P1D, because the specific viral protease was absent. Complementation was then achieved by cotransfection of mutated cRNAs together with the cRNA able to synthesize wild-type FMDV P1 protein. A CPE was observed 10 to 12 h after transfection with the mutants 136 Ser, 145 Ser,

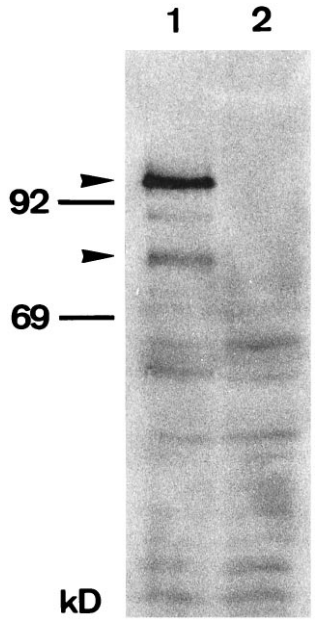

FIG. 6. Western blot analysis of P1-expressing BHK cells. Cells were harvested 24 h after electroporation with pSFV-P1. The cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Lane 1, structural protein precursor P1 with a molecular mass of 92 kDa and a cleavage product (indicated by arrowheads); lane 2, mock control.

TABLE 2. Complementation of the defect coat protein in P1-expressing BHK cells

Virus mutant	Amino acid sequence[a]	CPE[b]	
		Without complementation	With complementation
pSPffPolyC	RGDL	+	+
136 Ser		−	+
145 Ser	SGDL	−	+
145 Gly	GGDL	−	+
146 Ala	RADL	−	+
147 Ala	RGAL	−	+
148 Arg	RGDR	−	+
148 His	RGDH	−	+

[a] Amino acid sequences are shown from positions 145 to 148.
[b] Symbols: −, no CPE could be observed 96 h after transfection of cRNAs; +, CPE and lysis of the whole cell culture occurred 24 to 48 h after transfection.

145 Gly, 146 Ala, 148 Arg, and 148 His, as outlined in Table 2. Transfer of supernatants to BHK-21 cells led now in all cases to the infection of these cells. The existence of viral proteins in the infected cells was proved by indirect immunofluorescence. These results demonstrate that the deficiency in cell attachment of the latter mutants could be overcome by incorporation of wild-type coat proteins into the viral particles.

DISCUSSION

Virus attachment to specific cell surface molecules is the initial step of the infectious process and contributes to the understanding of FMDV infection cycle. In this work, the viral component involved in attachment of FMDV to its target cells was analyzed by using single or double amino acid substitutions within the highly conserved Arg-Gly-Asp sequence (RGD) and the adjacent region. The RGD sequence is located on the βG-βH loop of the structural protein P1D and seems to be involved in binding to the cellular receptor (16, 19, 34, 44). This amino acid sequence plays a central role in binding of adhesive glycoproteins to their cell surface receptors (2, 39, 45). Therefore, it was expected that amino acid substitutions within this tripeptide would prevent virus attachment to the target cells. As described previously (31, 36), uncoating and membrane penetration of FMDV do not require the presence of the putative FMDV receptor or the RGD sequence. Therefore, FMDV can infect cells also by an antibody-dependent pathway, and in contrast to poliovirus (17, 20, 24), only attachment of virus to the cell surface is sufficient to initiate infection (30).

Indeed, with one exception, amino acid substitutions within the RGD sequence (positions 145 to 147) led to adsorption-defective virus mutants. Moreover, a virus mutant with an amino acid exchange from tyrosine to serine in the conserved position 136 is no longer capable of infecting cells. This confirmed the presumption that other regions of the virus capsid are also involved in virus attachment, as demonstrated by Fox and coworkers (16). Incubation with antibodies directed against the last 13 amino acids of P1D reduce adsorption rates of virus particles. The participation of the P1D C terminus in virus attachment can be explained by an influence of this region on the conformation of the βG-βH loop, as shown in X-ray studies (1, 33). In contrast to other serotypes, the conformation of the βG-βH loop of FMDV type O differs remarkably due to the formation of a disulfide bond. This finding correlates with the existence of type O-specific antibodies which have a predilection for binding to conformational epitopes (33). Furthermore, competition experiments of Baxt and Bachrach (6) with FMDV serotypes O, A, and C showed differences in attachment to BHK cells. These experimental results lead to the presumption that FMDV can infect cells by using different cell surface molecules which bind RGD-containing proteins. This is also supported recently by Carvalho and coworkers (11) who showed that FMDV type O_1 but not A_{12} can infect $\alpha_5\beta_1$-expressing cells. In addition, genetically engineered serotype A_{12} containing the FMDV loop of type O_1 cannot infect these cells either. Therefore, results by Mateu and coworkers (32) concerning mutations in position 136 of serotype C using peptide inhibition could not simply be transferred to other serotypes.

Amino acid substitutions in position 148 from leucine to arginine or histidine also caused adsorption-deficient virus particles. Unlike FMDV type A_{12} (31), amino acid variation in this position seems to be important for the adsorption of FMDV type O_1K. As shown for coxsackievirus A9 (21), a change in the amino acid position located directly after the RGD sequence can also influence virus growth.

The complexity of the receptor binding site is demonstrated by the analysis of the double mutants 144 Arg/145 Ser and 137 Ser/148 Arg. These mutants were created based on the amino acid sequences of type $A_{10}61$ (RSGDL) and type O Dithmarschen. Whereas the single mutations led to adsorption-deficient virus mutants (145 Ser and 148 Arg), a second amino acid substitution could rescue the infectious phenotype. Consequently, critical amino acid exchanges in O_1K virus capsids can be compensated for by changes in adjacent regions. In addition, our experiments show the importance of the amino acids arginine and glycine in virus attachment, because variation of these amino acids led to adsorption-defective virus particles. On the other hand, Hughes and coworkers (21) showed for coxsackievirus A9 that deletion of the RGD tripeptide did not lead to a loss of infectivity but to a reduced virus growth.

In contrast to the results of Mason and coworkers (31), the mutant 147 Glu with the exchange from aspartic acid to glutamic acid showed no difference in infectivity compared to the wild-type virus. This difference may be explained by the amino acid variations between the serotypes A and O and possibly by different attachment mechanisms mentioned above. Furthermore our result was supported by Humphries and coworkers (22) who showed that a change in the fibronectin recognition site G**RGD**S to G**RGE**S did not destroy the binding of fibronectin to its receptor.

In order to gain more insight into the mechanisms underlying FMDV attachment to and infection of cells, further studies will have to be performed. As an initial approach towards this end, we established a method for propagation of adsorption-deficient virus mutants. For this purpose, the wild-type capsid protein P1 was transiently expressed in BHK-21 cells. After cotransfection of mutated cRNAs, RNA replication, protein synthesis, and virus assembly could be demonstrated with the resultant virus mutants. These results confirm that amino acid exchanges at certain positions in the βG-βH loop are responsible for the attachment deficiency.

FMDV with its RGD sequence is likely to bind to an integrin. Due to the observation that a number of ligands may be recognized by more than one integrin (23), it is also possible that FMDV can use more than one cell surface receptor. Indeed, studies by Berinstein and coworkers (8) demonstrated that FMDV infection of monkey cells, but not of other cells commonly used for FMDV growth, could be blocked using antibodies against the α-chain of the vitronectin receptor. Furthermore, Amadori and coworkers (3) described the influence

of the fibronectin receptor on viral infection. Further effort is needed to establish a stable P1-expressing cell line for proliferation analyses of virus mutants. By using this cell line, amino acid exchanges in other regions of the capsid proteins could be investigated with respect to their function in cell attachment. Such work could lead to the identification of a cellular receptor molecule and to a better understanding of FMDV infection.

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