Propagation of an attenuated virus by design: Engineering a novel receptor for a noninfectious foot-and-mouth disease virus

(picornavirus/receptor/intracellular adhesion molecule 1/antibody engineering)

E. Rieder*, A. Berinstein*, B. Baxt*, A. Kang†, and P. W. Mason‡

*Plum Island Animal Disease Center, North Atlantic Area, Agricultural Research Service, U.S. Department of Agriculture, Greenport, NY 11944; and
†Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037

Communicated by Harley W. Moon, Iowa State University, Ames, IA, July 5, 1996 (received for review March 20, 1996)

ABSTRACT To gain entry into cells, viruses utilize a variety of different cell-surface molecules. Foot-and-mouth disease virus (FMDV) binds to cell-surface integrin molecules via an arginine-glycine-aspartic acid (RGD) sequence in capsid protein VP1. Binding to this particular cell-surface molecule influences FMDV tropism, and virus/receptor interactions appear to be responsible, in part, for selection of antigenic variants. To study early events of virus-cell interaction, we engineered an alternative and novel receptor for FMDV. Specifically, we generated a new receptor by fusing a virus-binding, single-chain antibody (scAb) to intracellular adhesion molecule 1 (ICAM1). Cells that are normally not susceptible to FMDV infection became susceptible after being transfected with DNA encoding the scAb/ICAM1 protein. An escape mutant (B2PD.3), derived with the mAb used to generate the genetically engineered receptor, was restricted for growth on the scAb/ICAM1 cells, but a variant of B2PD.3 selected by propagation on scAb/ICAM1 cells grew well on these cells. This variant partially regained wild-type sequence in the epitope recognized by the mAb and also regained the ability to be neutralized by the mAb. Moreover, RGD-deleted virions that are noninfectious in animals and other cell types grew to high titers and were able to form plaques on scAb/ICAM1 cells. These studies demonstrate the first production of a totally synthetic cell-surface receptor for a virus. This novel approach will be useful for studying virus reception and for the development of safer vaccines against viral pathogens of animals and humans.

The first step in viral infection is the binding of virions to cell-surface receptors. Receptors for many different viruses have been identified, and although all of these molecules share a cell-surface topography, there does not appear to be any correlation between the natural function of a cell-surface molecule and the type of virus that uses it as a receptor. In some cases, closely related viruses can use widely divergent cell-surface molecules as cellular receptors. Receptor distribution can also play a role in tissues and/or host species that viruses infect (see ref. 1 for a review).

Receptor molecules have been identified for several members of the Picornaviridae. The poliovirus receptor (PVR) has been identified as a cellular protein of unknown function (2), and the receptor for the major group of human rhinoviruses has been identified as an intracellular adhesion molecule 1 (ICAM1; refs. 3–5). Both of these receptor molecules are members of the immunoglobulin (lg) superfamily. In contrast, the minor group of human rhinoviruses attach to cells via proteins in the low density lipoprotein receptor family (6) which are not members of the lg superfamily. The ubiquitous extracellular matrix binding proteins, the integrins, have been identified as receptors for several picornaviruses, including echovirus (7), coxsackievirus type A9 (CA9; ref. 8), and foot-and-mouth disease virus (FMDV; ref. 9). In the case of FMDV, type A12 virus is able to compete with CA9 for the same member of the integrin family (αβ; ref. 9). The finding that this arginine-glycine-aspartic acid (RGD)-specific integrin is a receptor for FMDV is consistent with our work showing that the RGD sequence found in the δβ–δH loop of VP1 of type A12 virus is absolutely required for infecting cells in culture (10). Recently, we have made use of the requirement of the RGD for infection to engineer “noninfectious,” receptor-binding site-deleted virions that can serve as vaccines for FMD (11).

In addition to gaining access to the cytoplasm of target cells following binding to specific cell-surface proteins, some viruses can infect cells through the Fc receptor if they are complexed to IgG (12–14). In the case of dengue virus, this phenomenon, known as antibody-dependent enhancement of infection, has been proposed as the basis for a severe form of disease which accompanies dengue virus infection of hosts who have preexisting low levels of dengue antibodies (15). In the case of picornaviruses, we have demonstrated that FMDV can also enter cells in this manner (16, 17), although it is unclear if this route of infection is important in development of either the acute form of disease or the long-term carrier state that has been associated with infection (18).

In contrast to FMDV, poliovirus cannot productively infect cells by antibody-dependent enhancement of infection (16), suggesting that the PVR functions both to dock the virus to the cell surface and to induce structural changes to the virion that are necessary for uptake and uncoating. This dual role for some picornavirus receptors in infection is consistent with the finding that soluble forms of PVR or ICAM1 are able to bind to poliovirus or rhinovirus particles, respectively, and induce formation of altered virions that may be related to intermediates in virus uncoating (19, 20). The critical role of the virus-binding domain of PVR in conformational alteration of the virion is supported by the fact that virus-binding domains of PVR can be transferred to alternative cell-surface molecules such as ICAM1 (21, 22) or CD4 (23), producing chimeric receptor molecules that confer viral susceptibility on cells lacking PVR.

The well-characterized three-dimensional structure of the antigen-binding site of IgG make it particularly amenable to engineering (24). Recombinant DNA technology has been used to generate proteins, based on preexisting IgGs, that have enhanced affinity (25) or altered specificities (26). Using

Abbreviations: FMDV, foot-and-mouth disease virus; RGD, arginine-glycine-aspartic acid; ICAM1, intracellular adhesion molecule 1; BHK, baby hamster kidney; CHO, Chinese hamster ovary; PVR, poliovirus receptor; WT, wild type; scAb, single-chain antibody; moi, multiplicity of infection.

†To whom reprint requests should be addressed: Agricultural Research Service, U.S. Department of Agriculture, Plum Island Animal Disease Center, P.O. Box 848, Greenport, NY 11944, e-mail: petermas@asrr.arsusda.gov.
recombinant DNA approaches, it has also been possible to
generate libraries of IgG-derived molecules that can be used to
probe the specificity of antibody/antigen interactions at the
structural level (27). In some of these applications, the antigen-
binding domains of the two IgG chains are cloned independ-
ently and used in combination to increase the complexity of
the libraries (28), taking advantage of the natural system of
generating antibody diversity. It is also possible to engineer
single-chain molecules (29, 30) that contain the complement-
tarity determining regions that characterize the “variable”
antigen-combining sites of both heavy and light chains of IgG
molecules, producing a single gene that encodes a functional
antigen binding domain.

The existence of multiple types of integrins in cultured
cells, and the finding that some serotypes and subtypes of
FMDV may be able to utilize more than one integrin (S. Neff
and B.B., unpublished data), complicates study of the early
steps of FMDV interaction with cells in culture. To study
infection of FMDV in a simpler system, we have engineered a
novel receptor for this virus, which consists of the virus-
bounding fragments of an FMDV-specific monoclonal antibody
(mAb) fused to ICAM1, thus linking the virus-binding domain
of the mAb to the cell surface. Chinese hamster ovary (CHO)
cells, which cannot normally replicate type A12 FMDV, are
rendered susceptible to virus infections by transfection with
DNA encoding the single-chain antibody (scAb)/ICAM1 pro-
ten. Evaluation of infection of the scAb/ICAM1 cells by mAb
escape mutants and RGD-deleted viruses clearly demonstrate
that this novel receptor is mediating infection. This new
cell-surface molecule will be useful for studying the interaction
of FMDV with cells in culture, and for the large-scale culti-
vation of receptor binding site-deleted viruses for use as safer
vaccines for FMD.

METHODS

Cell Lines, Plasmids, and Viruses. Baby hamster kidney
(BHK; clone 21) and CHO cell cultures were propagated as
described (16). A plasmid expressing the variable region of the
heavy chain and the \( \kappa \) chain (\( V_{H}K \)) of mAb 2PD11 (pvH \( \# \# \#6 \))
has been described (31). The eucaryotic expression plasmid,
pcDNA3 was obtained from Invitrogen. Plasmid pCB6-
ICAM1, containing the human ICAM1 cDNA (22), was
provided by E. Wimmer. Wild-type (WT) FMDV type A12 was
derived from infectious cDNA pRMCS (32), the 2PD11 mAb
escape mutant (B2PD.3), has been described (33), and RGD-
deleted FMDV type A12 was prepared by transfection of BHK
cell cultures with RNA derived from the infectious cDNA
plasmid pRM-RGD-\(^{-} \) (11). Viral growth curves and plaque
assays were performed by standard techniques (32).

Engineering \( V_{H}K \) Molecules for Expression in Eucaryotic
Cells. The cDNA encoding a 2PD11 \( V_{H}K \) was excised from the
prokaryotic expression plasmid, pvH \( \# \# \#6 \) (31), and ligated to
pcDNA3 DNA, behind the cytomegalovirus promoter. To
facilitate high-level expression and secretion from eucaryotic
cells, 60 bases of untranslated sequence and a 19-codon signal
sequence (MERCMCWVLVSSLKGVLC) from a cDNA
(N418) encoding a hamster IgG (A.K., unpublished data) were
added to the 5' end of the 2PD11 \( V_{H}K \) cDNA. This construc-
tion was made using an XhoI site added at the 5th and 6th
codons of the framework 1 domain of N418, using the poly-
merase chain reaction (PCR; ref. 34), which permitted in-
frame fusion to the same restriction endonuclease site in the
pvH \( \# \# \#6 \) cDNA (31), to produce plasmid pvH \( \# \# \#2 \) (Fig. 1A).

Engineering Molecules for Cell-Surface Expression of the
\( V_{H}K \). PCR was used to add a linker encoding a GG hinge and
an \( NheI \) site specifying L and A residues following the 14th
codon of the C\(_L\) domain of the 2PD11 \( V_{H}K \) cDNA (at the
V\(_{L}\)-C\(_L\) elbow). PCR was also used to add LA codons encoding
an \( NheI \) site in place of the NA codons found at the N terminus
of the mature ICAM molecule (35), and the two genes were
fused together at this site in pcDNA3, producing the recom-
binant plasmid pscAb/ICAM1\#11, shown schematically in
Fig. 1B. This fusion encoded an ICAM1 cDNA in which the
signal sequence had been replaced by the hamster IgG signal
sequence and \( V_{H}V_{L} \) portion of the cDNA encoded by
pvH \( \# \# \#2 \), linked by the junction sequence PS5GGLAQ (Fig.
1B).

Cell Transfection and Selection of CHO Cell Lines. DNA
preparations were introduced into CHO cell cultures using
Lipofectin (Life Technologies, Gaithersburg, MD), and trans-
fected cells were selected by growth in the presence of the
eucaryotic antibiotic G418 (600 \( \mu \)g/ml; Life Technologies).
Following several passages in the presence of this antibiotic,
single cell clones were selected from this population and
screened by immunofluorescence microscopy. Clones derived
from cells transfected with pvH \( \# \# \#2 \) DNA were tested using a
FITC-labeled goat anti-mouse IgG (Kirkegaard & Perry Lab-
oratories). pvH \( \# \# \#2 \) clones displaying a uniform high level of
mouse IgG antigen were expanded, and culture fluids col-

![Fig. 1. Schematic diagram of the \( V_{H}K \) and scAb/ICAM1 cassettes used to express 2PD11 derivatives in CHO cell cultures. (A) Plasmid pvH \( \# \# \#2 \)
cassette used for expression of a secreted \( V_{H}K \). (B) Plasmid pscAb/ICAM1\#11 cassette used for expression of the cell surface-bound scAb. CMV,
cytomegalovirus.](image-url)
lected from these cells were tested for the presence of functional antibodies using a radioimmunoprecipitation test. Clones derived from cells transfected with pscAb/ICAM1#11 DNA were infected with type A12 virus and screened by immunofluorescence microscopy for the presence of viral antigen by using serum from a bovine infected with FMDV and fluorescein isothiocyanate-labeled goat anti-bovine IgG (Kirkegaard & Perry Laboratories). pscAb/ICAM1#11 clones displaying a uniform high level of viral antigen staining were expanded and tested for virus susceptibility as described above.

RESULTS

Expression of the 2PD11 V_{H}K in Eucaryotic Cells. The cDNA corresponding to the mature portion of the 2PD11 V_{H}K found in the bacterial expression plasmid, pV_{H}K#6 (31), was transferred to the eucaryotic expression plasmid, pcDNA3, and a hamster IgG signal sequence was added to the 5' end of the V_{H}K cDNA using standard techniques (see Methods) to produce clone pV_{H}K#2 (shown schematically in Fig. 1A). Clones of CHO cells grown in the presence of G418 from populations of cells transfected with pV_{H}K#2 DNA were tested for their ability to express recombinant antibody as described in Methods. Preliminary immunoprecipitation assays revealed that all the selected clones (n = 3) released proteins of the expected size that reacted with anti-mouse sera, and one (designated 2.9) was used in subsequent studies. The autoradiogram shown in Fig. 2 demonstrates that the culture fluid harvested from radiolabeled CHO 2.9 cells produced a protein of approximately Mr 40,000 that was precipitated with goat anti-mouse IgGs. Furthermore, Fig. 2 shows that the V_{H}K present in the culture fluid harvested from CHO 2.9 cells was able to precipitate radiolabeled FMDV in the presence of anti-mouse IgGs and fixed Staphylococcus aureus bacteria, demonstrating that the substitution of the hamster IgG signal sequence for the pelB leader signal sequence found in pV_{H}K#6 (31) permitted efficient eucaryotic export of the 2PD11 V_{H}K.

Production of a Functional 2PD11 scAb/ICAM1 Fusion Protein. The DNA fragment corresponding to the hamster IgG signal sequence and the V_{H} and V_{L} domains of the 2PD11 V_{H}K molecule was amplified using PCR and fused in-frame to the 5' end of the cDNA encoding the entire ICAM1 molecule (exclusive of the ICAM1 signal sequence; ref. 34) as shown in Fig. 1B. CHO cell cultures transfected with the resulting plasmid, scAb/ICAM1#11, were passaged two times in the presence of G418 and tested for their ability to grow FMDV-type A12. In contrast to WT CHO cell cultures or cells transfected with pV_{H}K#2 (Table 1), which only contained residual input virus following an overnight growth experiment, cultures transfected with pscAb/ICAM1#11 appeared to replicate the virus (Table 1). Although these results provided the first evidence that the scAb/ICAM1#11 molecule could serve as an FMDV receptor, the titers of virus recovered from the pscAb/ICAM1#11-transfected cultures were much lower than those obtained in previous studies of CHO cells expressing the Fc receptor and infected with antibody-complexed virus (16). The lower-than-expected titer of virus harvested from these cultures was consistent with the lack of obvious virus-induced cytopathic effect, suggesting that not all of the cells in the culture were expressing functional receptors. This latter possibility was confirmed by immunofluorescent analyses which demonstrated that following infection at a high moi, only a portion of the cells in these transfected cultures contained viral antigen (results not shown). To further evaluate the cells transfected with pscAb/ICAM1#11 DNA, 10 single-cell clones were prepared from these transfected cultures (following two passages in G418; see Methods) and tested for their ability to replicate virus. These experiments identified three clones (designated 11.1, 11.8, and 11.9) that showed significant cytopathic effect when infected with virus, and immunofluorescent staining showed that large percentages of cells in all three cloned cultures were positive for virus antigen 5 h after infection at a moi of greater than 10. One clone, 11.1, was expanded and further tested.

Growth of WT and 2PD11-Resistant Viruses on the Cell Line Expressing the scAb/ICAM1 Protein. One-step growth curves confirmed that the CHO 11.1 cell line produced high titers of virus, whereas WT CHO cells were unable to replicate virus (Fig. 3). The amount of virus recovered from this cell line is similar to that recovered from infected BHK cell cultures.

Table 1. Recovery of FMDV from WT and transfected CHO cell cultures

<table>
<thead>
<tr>
<th>CHO cell culture*</th>
<th>Virus recovered,† plaque-forming units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>pscAb/ICAM1#11-transfected</td>
<td>9.5 x 10^3</td>
</tr>
<tr>
<td>pV_{H}K#2-transfected</td>
<td>2.0 x 10^3</td>
</tr>
<tr>
<td>WT cells</td>
<td>1.6 x 10^3</td>
</tr>
</tbody>
</table>

*Cells were second-passage, G418-selected cells or WT cells.
†Cells were grown in 35-mm diameter dishes, infected at a multiplicity of infection (moi) of 20 for 1 h at 37°C, rinsed in Mes-buffered saline (pH 6.0) to remove input virus (32), incubated overnight at 37°C, and lysed by freeze-thawing; virus titers were determined by plaque assay (see Methods).
Table 2. Plaque-forming ability of WT FMDV, RGD-deleted FMDV, B2PD.3, and B2PD.3.9 on CHO 11.1 and BHK cell cultures

<table>
<thead>
<tr>
<th>Virus*</th>
<th>CHO 11.1</th>
<th>BHK</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT FMDV</td>
<td>2.7 x 10^7</td>
<td>9.2 x 10^8</td>
</tr>
<tr>
<td>RGD-deleted</td>
<td>2.1 x 10^7</td>
<td>0</td>
</tr>
<tr>
<td>B2PD.3</td>
<td>9 x 10^6</td>
<td>8.7 x 10^6</td>
</tr>
<tr>
<td>B2PD.3.9</td>
<td>4.8 x 10^6</td>
<td>1.0 x 10^7</td>
</tr>
</tbody>
</table>

*Stock of virus tested. WT FMDV and B2PD.3 stocks were grown in BHK cell cultures, and RGD-deleted virus and B2PD.3.9 were grown in the CHO 11.1 cell line.
†Titers determined by staining monolayers at 48 h (BHK) or 72 h postinfection (CHO 11.1).
‡2PD11 mAb escape mutant (33).
§Variant of 2PD11 escape mutant selected by passage on the CHO 11.1 cell line (see text).

Furthermore, WT virus was able to form plaques on the CHO 11.1 cell lines (Table 2).

Specificity of this novel receptor for the epitope recognized by 2PD11 was demonstrated by analyzing the 2PD11 mAb escape mutant B2PD.3 (33). As shown in Table 2, B2PD.3 produced approximately 100,000-fold fewer plaques on the CHO 11.1 cell line relative to WT virus, although both viruses produced comparable plaque titers on susceptible BHK cell cultures. We derived a variant of B2PD.3, designated B2PD.3.9, by two blind passages on CHO 11.1 cells followed by plaque picking and growth on CHO 11.1 cells. B2PD.3.9 efficiently formed plaques on CHO 11.1 cells (Table 2) and had regained a partial ability to be neutralized by 2PD11 (the concentration of antibody neutralizing 50% of plaques, PRN_{50}, was 625 ng/ml for B2PD.3.9 versus >10,000 ng/ml for B2PD.3, and 20 ng/ml for WT virus). Based on data showing that the 2PD11 epitope includes residues found in the G–H loop of VP3 (33), we sequenced this region of B2PD.3.9. These analyses revealed that B2PD.3.9 differed by a single nucleotide from B2PD.3, regenerating a WT Thr codon at position 178 of VP3 of B2PD.3.9, a position that was an Ala codon in all previously characterized 2PD11 mAb escape variants (33).

**DISCUSSION**

Virus recognition of specific cell-surface receptors represents a key determinant in tissue and host cell tropism. Despite the accumulation of information on attachment of viruses to cells, the subsequent steps, including uptake and uncoating, are not well understood (for reviews, see refs. 1 and 36). In addition, little information exists on the participation of distinct cell-surface molecules in internalization. Studies in our laboratory have demonstrated that in addition to infecting cells via binding to its natural receptor, FMDV can also infect cells through the immunoglobulin Fc receptor in the presence of virus-specific antibodies (16). This observation suggests that, in the case of FMDV, the natural receptor may only serve in docking the virus to cells and that productive uptake by susceptible cells can be dissociated from binding.

Based on this supposition, it seemed likely that a functional FMDV receptor could be obtained by joining the antigen-binding domain of an FMDV-specific antibody molecule to a cell-surface molecule. We chose mAb 2PD11 for the virus-binding portion of this synthetic receptor because 2PD11 was able to efficiently enhance infection of FMDV on cells expressing the Fc receptor at antibody-to-virion ratios of one-to-one (16). ICAM1 was selected for the cell-anchoring portion of the receptor for three reasons: (i) ICAM1 serves as the receptor for the major group of human rhinoviruses (3–5), (ii) the rhinovirus-binding domain is positioned at the most distal

![Fig. 3. One-step growth curves of WT FMDV released from infected BHK, WT CHO, and CHO 11.1 cell cultures. The low pH rinse (see Table 1) was performed immediately before collecting the 1-h sample. The time zero titer was obtained from the inoculum used to infect all three cultures, and the titers shown for the 1, 5, 8, and 24 h time points were obtained from cell-free culture fluid harvested at these times postinfection. All titrations were performed on BHK cells, and expressed in terms of the number of cells contained in parallel cultures harvested and counted at the zero time point. pfu, Plaque-forming units.](image1)

![Fig. 4. Plaques formed by two different dilutions of RGD-deleted FMDV on monolayers of CHO 11.1 cell cultures following 72-hr incubation (see Methods).](image2)
end (N terminus) of ICAM1 (37), and (iii) chimeric proteins created by fusing the virus-binding domain of PVR to the N terminus of intact ICAM1 are efficient receptors for poliovirus (22).

Our ability to genetically engineer a scAb/ICAM1 molecule that can function as an FMDV receptor indicates that both the virus-binding site of the scAb and the internalization activity of the ICAM1 molecule were preserved in our engineered receptor. In addition, dramatic differences between the infectivity of WT virus and 2PD11 mAb escape mutant, B2PD.3, on the CHO 11.1 cell line, demonstrate the specific interaction between the antigen-binding site expressed in the scAb/ICAM1 fusion protein and the epitope recognized by the antibody in VP3 of FMDV A12.

The rapid derivation of a CHO 11.1-binding variant of B2PD.3 is consistent with extensive antigenic variation and ability to escape from neutralization that has been documented for FMDV both in vitro and in field isolations (38, 39). The ready isolation of a virus that is capable of binding to this mAb receptor mimics the ability to isolate FMDV variants with enhanced affinity for the natural receptor on cells in culture from animal-derived viruses (40) and is consistent with the ease of isolation of poliovirus variants that are able to replicate on cell lines expressing mutant PVRs (41).

Our ability to derive a synthetic receptor for FMDV that permits infection following binding in an RGD-independent fashion has provided a method to propagate a previously characterized RGD-deleted virus that has shown usefulness as a safe and effective vaccine candidate (11). In a preliminary experiment, a bovine inoculated by the intradermal lingual route with 6 μg of the RGD-deleted virus did not show any signs of disease, including either vesicular lesions or fever (E.R. and P.W.M., unpublished data). This mass of virus corresponds to approximately 500 million bovine-infectious doses of the parental virus, derived from PRMC5 (T. S. C. McKenna and P.W.M., unpublished data). This novel approach to the generation of cell lines that can specifically complement receptor mutations introduced into viral genomes may have applications in the derivation of rationally attenuated vaccine candidates for other viruses of humans and domestic animals.

We thank Dr. Eckard Wimmer, Department of Microbiology, State University of New York, Stony Brook, for supplying plasmid pCB6-ICAM1, containing the human ICAM1 cDNA, and A. J. Franke and T. Kniepe for technical assistance. A.K. is a recipient of a Cancer Research Institute/Partridge Foundation Investigator Award.