Abstract

The studies presented in this thesis demonstrate that foot-and-mouth disease virus (FMDV) infection mediated by the integrin αvβ6 takes place through clathrin-dependent endocytosis. Inhibition of clathrin-dependent endocytosis by sucrose treatment or transient expression of a dominant-negative version of AP180 inhibited virus entry and infection. Similarly, inhibition of endosomal acidification inhibited an early step in infection. Blocking endosomal acidification did not interfere with surface expression of αvβ6, virus binding to the cells, uptake of the virus into endosomes, or cytoplasmic virus replication. These observations suggest that the low pH within endosomes is required for a post-entry step of infection, most likely capsid uncoating and delivery of viral RNA into the cytosol. FMDV infection occurred in the absence of caveolae and inhibition of lipid-raft dependent endocytosis did not inhibit virus uptake or infection.

Using immunofluorescence confocal microscopy, FMDV colocalised with αvβ6 at the cell surface but not with the B subunit of cholera toxin, a marker for lipid rafts. At 37°C, virus was rapidly taken up into the cells and colocalised with markers for early and recycling endosomes but not with a marker for lysosomes, suggesting that infection occurs from within the early or recycling endosomal compartments. This conclusion was supported by the observation that FMDV infection is not inhibited by nocodazole, a reagent that inhibits vesicular trafficking between early and late endosomes (and hence trafficking to lysosomes).

Internalisation of αvβ6 and its accumulation in early and recycling endosomes was triggered by virus binding, suggesting that the integrin serves not only as an attachment receptor but also to deliver the virus to the acidic endosomes.
Acknowledgements

First I would like to thank my supervisor Dr. Terry Jackson for his help throughout the course of my PhD, in light of which I am prepared to overlook the fact that he is a Millwall fan. I would also like thank my co-supervisor Dr. Paul Monaghan, especially for advice on matters microscopical. I would also like to thank my Surrey supervisor, Dr. Lisa Roberts.

Thanks go also to those people that provided reagents used in this work, including Dr. Harvey McMahon (University of Cambridge) for providing dominant negative AP180, Dr. Dean Shepherd (University of California) for providing cell lines and the antibody 4B5, and Biogen Inc for the anti-integrin antibodies 6.8G6 and 6.3G9.

A special thank you goes to all friends, family, colleagues, and doctors, that have helped me to reach this point.

Lastly I would like to thank the pride of Kent, the mighty Gillingham FC.
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<th>Abbreviation</th>
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<td>aa</td>
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<td>Adaptor protein</td>
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<tr>
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<td>Adenosine triphosphate</td>
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<tr>
<td>CB4</td>
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<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
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<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin b subunit</td>
</tr>
<tr>
<td>cre</td>
<td>cis-acting replication element</td>
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<tr>
<td>DAF</td>
<td>Decay acceleration factor</td>
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<td>DMEM</td>
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<td>Early endosomal antigen-1</td>
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<td>E/F VP4</td>
<td>Chimeric version of VP4 consisting of the N-terminal domain of ERAV VP4 and the C-terminal domain of FMDV VP4</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERAV</td>
<td>Equine rhinitis A virus</td>
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<td>eIF</td>
<td>Eukaryotic initiation factor</td>
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<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
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<td>F/E VP4</td>
<td>Chimeric version of VP4 consisting of the N-terminal domain of FMDV VP4 and the C-terminal domain of ERAV VP4</td>
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<tr>
<td>FMDV</td>
<td>Foot-and-mouth disease virus</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N’-(2-ethane)sulphonic acid</td>
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<td>Human rhinovirus</td>
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<td>Internal ribosome entry site</td>
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<td>Kilobase pairs</td>
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<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LAMP-2</td>
<td>Lysosome-associated membrane protein-2</td>
</tr>
<tr>
<td>LAP-1</td>
<td>Latency associated protein-1</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MβCD</td>
<td>Methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>MDBK</td>
<td>Madin-Darby bovine kidney cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>m.o.i</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P40</td>
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<tr>
<td>PABP</td>
<td>Poly (A) binding protein</td>
</tr>
<tr>
<td>PCBP</td>
<td>Poly (C) binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PI-3-kinase</td>
<td>Phosphatidyl inositol-3-kinase</td>
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<tr>
<td>PKa</td>
<td>Dissociation constant for an acid</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
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<tr>
<td>RGE</td>
<td>Arginine-Glycine-Glutamic Acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris [hydroxymethyl] aminomethane</td>
</tr>
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<td>Simian virus 40</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very Low density lipoprotein</td>
</tr>
<tr>
<td>VP0*</td>
<td>Uncleavable version of VP0</td>
</tr>
<tr>
<td>VPg</td>
<td>Viral protein, genome linked</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
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Chapter One: Introduction

The outbreak of foot-and-mouth disease (FMD) in the UK in 2001 graphically demonstrated the devastation that an epidemic of the disease in a non-endemic area can cause. FMD is one of the most contagious viral diseases known in mammals, and affects more than 33 cloven-hoofed species, including economically important livestock such as sheep, goats, cattle, and pigs. Infected animals typically suffer from a short fever, loss of appetite and dullness, followed by the appearance of vesicular lesions of the mouth, tongue, and feet, which give the disease its name. Although mortality rates in infected adult animals are relatively low the disease is economically devastating due to its severe impact on the productivity of infected animals. Milk yields in cattle and the growth rate of young infected animals, are particularly severely affected. In countries such as the UK, where the disease is not endemic, the slaughter of infected or at risk animals, and restriction on livestock movements, is the primary economic impact of the disease. The direct cost of the 2001 UK outbreak has been estimated to be 2.75 billion pounds (4).

1.1 Picornavirus Classification

Foot-and-mouth disease virus (FMDV) is a member of the family Picornaviridae, which includes a wide range of human and animal pathogens e.g. Poliovirus, the human, bovine, and equine Rhinoviruses, Hepatitis A virus, and FMDV. Picornaviruses have traditionally been defined in terms of serotypes grouped into genera on the basis of shared pathogenic characteristics and biophysical properties (300). However, recent sequence data, which reflects genetic relationships and evolution of viruses more accurately than previous empirical observations, has been
used to refine the taxonomic scheme. The species concept has now been applied to picornavirus classification, meaning that genera are divided into one or more virus species (300), which consist of several serotypes.

The current taxonomic structure of the picornavirus family is shown in figure 1.1 (N. J. Knowles, Personal Communication). There are nine genera: Enterovirus, Rhinovirus, Cardiovirus, Aphthovirus, Hepatovirus, Parechovirus, Erbovirus, Kobuvirus, and Teschovirus. There are also a number of viruses that have yet to be assigned to a genus. FMDV is the type species of the genus Aphthovirus, which also includes Equine Rhinitis A virus (ERAV). Members of the Aphthovirus genus have greater than 50% nucleotide identity in the region of the genome which encodes the structural proteins and greater than 70% identity in the non-structural proteins 2C and 3CD. There are seven serotypes of FMDV: types O, A, C, Asia-1, SAT 1, 2, and 3, each of which contains a number of subtypes (69, 161). Serotypes are antigenically but not clinically distinct from one another.

1.2 FMDV Structure

1.2.1 The FMDV Virion

The FMDV virion consists of a single copy of single-stranded positive-sense RNA, encased by an icosahedral capsid. The capsid is formed from 60 copies each of four structural proteins (VP1-4), and demonstrates icosahedral 5:3:2 symmetry (2). VP1-VP3 form the outer capsid shell whereas VP4 is located (with the vRNA) inside the capsid. The mature FMDV particles are 28-30nm in diameter, and appear spherical under the electron microscope.
<table>
<thead>
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<td>human enterovirus B1 to 36</td>
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<td>11</td>
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</tr>
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<td>Equine rhinitis A virus</td>
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<td>7</td>
<td>equine rhinitis A virus</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
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<td>hepatitis A virus</td>
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<td>avian encephalomyelitis virus</td>
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<td>Human parechovirus</td>
<td></td>
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<td>human parechovirus 1 &amp; 2</td>
</tr>
<tr>
<td>Equine rhinitis B virus</td>
<td></td>
<td>2</td>
<td>equine rhinitis B virus</td>
</tr>
<tr>
<td>Mouse virus</td>
<td></td>
<td>10</td>
<td>mouse virus</td>
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<tr>
<td>Porcine teschovirus</td>
<td></td>
<td>10</td>
<td>porcine teschovirus 1 to 11</td>
</tr>
</tbody>
</table>

**Figure 1.1.** The classification and nomenclature of the nine picornavirus genera (N. Knowles, Personal Communication)
The asymmetric unit of the mature FMDV capsid (termed a protomer) is made up of three proteins (VP1-3) of unique sequence but similar tertiary structure, and a fourth protein VP4, which does not form part of the outer surface of the virion. Capsid assembly from individual protomers is thought to be a two-stage process. The first stage of assembly is the formation of pentameric units, by the association of five protomers in a urea-sensitive fashion. The second stage involves assembly of twelve pentamers to form an icosahedral shell (the capsid). The mechanism by which the viral RNA is incorporated into the capsid remains unclear.

Protomers consist initially of one copy of VP0, VP1 and VP3, but on encapsidation of the viral RNA, VP0 is cleaved to yield VP4 (the N-terminal 85 residues of VP0) and VP2 (the remainder of VP0), in a process termed the maturation cleavage. VP0 cleavage has been shown to be essential for infectivity, since recombinant virions carrying mutations that prevent VP0 cleavage are non-infectious (160).

1.2.2 Atomic Structure of Mature FMDV Particles

The atomic structure of a number of picornaviruses, including poliovirus (128), several human rhinoviruses (272, 323), mengo virus (179) and foot-and-mouth disease virus (2, 59, 168, 169) have been determined by x-ray crystallography. The structure of type O FMDV is shown in figure 1.2. As dictated by the icosahedral arrangement of protomers, the capsid has 5-fold, 3-fold, and 2-fold centres of symmetry. There are twelve 5-fold centres of symmetry, one at the centre of each of the pentameric units. The VP1 proteins are located around the 5-fold symmetry axes, one copy being contributed by each of five protomers from a single pentamer. 3-fold centres of symmetry are formed at the intersection of three pentamers and are surrounded by three copies each of VP2 and VP3, arranged in an alternating pattern.
Figure 1.2. The all atom structure of reduced O serotype virus.

VP1 is coloured blue, VP2 green and VP3 red (VP4 is internal and not visible in these views). The VP1 GH loop residues 130/160 (GH loop) is shown as a 'worm' in cyan with the Arg/Gly/Asp residues in orange. Antigenic residues are colour-coded according to their classification into sites: site 1 and 5 (mid-blue), site 2 (pale yellow), site 3 (light blue), site 4 (magenta). The potential occupancy of the VP1 GH loop is modelled by a transparent sphere centred at the mid-point between the two ends of the loop. The heparin motif which has been visualized in complex with the virus is drawn in yellow ball-and-stick. A protomeric subunit (the smallest repeating unit, representing 1/60th of the capsid) is outlined and enlarged. The FMDV GH loop is further enlarged with residues that are conserved (or virtually conserved) across all strains drawn in full. In this depiction of the loop sequence variability between strains is proportional to the thickness of the tube. The residue numbers are shown for the conserved residues with the residue type being indicated for the proposed integrin binding RGD motif (orange).

Figure adapted from (141)
2-fold centres of symmetry are located at the centre of the interacting surfaces of adjacent pentamers, and are also formed from alternating copies of VP2 and VP3.

VP1, 2, and 3 have tertiary structures that are remarkably similar to one another (2). Each protein has a topologically conserved “core” consisting of an eight stranded β-barrel, with variable “elaborations” formed by the loops that connect the individual β-strands. The exterior surfaces of the virus are formed primarily by these variable elaborations.

The C-termini of VP1-3 are located on the outer surface of the virion and make limited contacts with each other. The N-termini of VP1-4 are all located on the inner surface of the capsid, and make extensive contacts with each other that help to stabilise the formation of pentameric assembly intermediates. Such contacts also help to hold the 2-fold related pentamers together. VP4 is covalently modified at its N-terminus by a hydrophobic myristate fatty acid chain (25, 50). By analogy with other picornavirus structures (117) it is likely that the association of myristate groups of VP4 at the 5-fold symmetry centre creates a hydrophobic globule which contributes to pentamer stability, and partly explains why these associations are urea-sensitive.

Further stability is provided by a ring of disulphide bonds which link the N-termini of VP3 around the 5-fold symmetry axes towards the exterior surface of the virus particle. These interactions ensure that pentamer formation is essentially irreversible.

Adjacent pentamers are held together by hydrogen-bonding interactions between VP2 and VP3. The FMDV particle is extremely sensitive to acid, dissociating into its constituent pentamers, VP4, and genomic RNA at pH values below neutrality (40). The extraordinary acid lability of FMDV is in part explained by the participation of a large number of histidine residues (pK 6.5-7.0) in inter-pentamer interactions (see section 1.6.5.2).
Sequence and structural variation at the N- and C-termini and interconnecting loops of the major structural proteins VP1-3 mean that the surface topography of different picornaviruses can vary considerably. Human Rhinoviruses for example contain large insertions in VP1, 2, and 3, which form protrusions on the surface of the virion, creating a deep cleft or “canyon” on the virion surface (272). The canyon forms a “moat” 25 Å deep and 12-30 Å wide, which separates the bulk of the VP1 subunits clustered at the 5-fold symmetry axis from the surrounding VP2 and VP3 subunits. A similar structure is observed for poliovirus (also termed a canyon) (128) and cardioviruses (termed a pit) (179). The canyon is thought to be wide enough to allow virus receptors to bind in its base, but not wide enough to allow the binding of neutralising antibodies. This is known as the canyon hypothesis (271).

The structural proteins of FMDV (in particular VP1) are significantly smaller than the equivalent proteins found in other picornaviruses. Since the core domains of these proteins are well conserved, this is primarily due to reductions in the size of variable surface loops. As a result the capsid of FMDV is smoother and thinner than that of other picornaviruses. The 17 C-terminal residues of VP1 form an arm, unique among picornaviruses, which occupies and fills the region which would be analogous to the canyon or pit. Consequently FMDV lacks a canyon or pit around the five-fold symmetry axis (2).

A major structural feature of the surface of FMDV is the long surface-exposed, conformationally flexible loop (the GH loop of VP1) shown in figure 1.2. This loop corresponds to residues 141-161 in serotype O viruses (174). In contrast to other picornaviruses such as HRV-14, where the GH loop is hidden from immune surveillance at the base of the canyon (271, 272), the VP1 GH loop of FMDV forms a highly antigenic surface protrusion (2). Synthetic peptides corresponding in sequence
to this loop induce high levels of protective antibodies (35), indicating that this site is highly immunogenic. In all serotypes of FMDV studied the VP1 GH loop is structurally disordered and so is virtually invisible in the crystal structures of unperturbed virus particles. However, analysis of the crystal structures of both native particles and neutralising antibody escape mutants, suggested that the GH loop could adopt two predominant conformations (2, 239). These conformations were termed “down” and “up” according to the orientation of the loop relative to the surface of the particle. It is thought that conformational restrictions are imposed by the tethering of one end of the loop by a disulphide bond which links cys134 of VP1 to cys130 of VP2 in FMDV strain O1BFS. This disulphide bond drives the loop towards the less intrinsically stable up conformation (239). Consistent with this theory the GH loop of VP1 was visualised in a well ordered down conformation by determination of the structure of O1BFS virus following chemical reduction using DTT (174). In this structure the loop lies in a small depression on the surface of the particle and forms a β-strand adjacent to strand C of VP2, which is followed by the integrin binding RGD motif (see section 1.6.1) presented in an extended conformation prior to a $\alpha_10$ helix. This loop structure was conserved in a complex of an FMDV loop peptide (serotype C) with the Fab fragment of two different neutralising antibodies (325, 326). This indicates that the internal structure of the loop observed in the reduced type O virus is conserved and therefore most likely biologically relevant. Direct evidence for the up conformation of the loop was obtained by analysis of the cryostructure of a type C virus complexed with the Fab fragment from a neutralising antibody, since this loop conformation best explained the observed location of the Fab fragment (124). Taken together this evidence supports a model whereby the VP1 GH loop exists as a self-contained unit which acts as a domain hinged relative to the surface of the particle.
that can adopt at least two radically different positions relative to the particle surface. Under reducing conditions, such as those found in the cytoplasm, the loop takes on the more ordered "down" conformation (174). The down conformation may allow the virus to be more efficiently released from infected cells. However under oxidising conditions, such as those found outside the cell, the loop adopts the more disordered up conformation. The mobility of the GH loop relative to the particle surface in the up conformation is likely to aid virus binding to its integrin receptor (144, 192) (see section 1.6.1).

1.3 FMDV Genome Organisation

The organisation and main features of the genome of FMDV are shown in figure 1.3. The genome consists of a single molecule of single-stranded, positive-sense RNA, which is over 8300 nucleotides in length. This RNA molecule contains a single long open reading frame which encodes the viral proteins. The open reading frame is flanked by a long 5' untranslated region (5' UTR) of over 1300 nucleotides, and a relatively short 3' untranslated region (3' UTR) of 100 nucleotides excluding the poly(A) tail (191). A small virally encoded peptide termed VPg (also known as 3B) is covalently linked to the 5' end of the genome (83, 104).

The single open reading frame has two alternative initiation sites, and encodes a single viral polyprotein which is processed by viral proteases to give a total of fifteen mature polypeptide products and a variety of partial cleavage intermediates (106). As shown in figure 1.3 the polyprotein is divided into four regions. These are from 5' to 3': L (the leader protease), P1 (the structural proteins 1A-1D, also known as VP4, VP2, VP3 and VP1), P2 (non-structural proteins 2A-2C), and P3 (non-structural
Figure 1.3. Schematic diagram of the FMDV genome showing the position of the genetic elements.

(A) Overview: open boxes indicate protein-encoding regions, lines indicate RNA structures. Thick lines below the genome diagram indicate prominent partial cleavage products.

(B) Expanded view of the 5'Untranslated region: S-fragment, short fragment of the genome, poly(C), poly cytosine tract, PK, pseudoknot, cre, cis replication element (showing the conserved 'AAACA' sequence), IRES, internal ribosome entry site.

Figure adapted from (188).
proteins 3A-3D). The non-structural proteins are involved in the replication of the FMDV genome (see section 1.4.4) and processing of the viral polyprotein (see section 1.4.3). The poly(A) tail of FMDV differs from those of cellular messenger RNA's in that it is genome encoded rather than added as a post-translational modification.

1.3.1 The 5' UTR

The structures present in the 5' UTR of the FMDV genome are shown in figure 1.3b). The 5' UTR includes a number of elements involved in RNA translation and replication, including the S-fragment, poly C tract, pseudoknots, and cis replication element (cre) and internal ribosome entry site (IRES).

1.3.1.1 The S Fragment

The S-fragment is approximately 360 nucleotides long and is predicted to form a large hairpin structure in silico (52, 81). Little data exists about the role of the S-fragment in the life cycle of FMDV or any proteins which interact with it; however, deletion of the S-fragment renders FMDV non-viable. The equivalent structure found in poliovirus is the "cloverleaf". This structure is only around 80 nucleotides in length and has been shown to interact with the cellular protein poly(rC) binding protein 2 (PCBP2) and the poliovirus 3CD protein (via sequences located within 3C) to form a ternary complex (10, 120, 219, 240). The binding of PCBP2 to the cloverleaf is required to maintain the stability of poliovirus RNA (219), whereas the binding of 3CD is required for the initiation of synthesis of negative strand copies of the poliovirus genome and also helps to stabilise the viral RNA (15). A ternary complex is formed by the 5' cloverleaf, PCBP2, and 3CD. This complex interacts with poly A binding protein (PABP) bound to the 3' poly(A) tail resulting in the circularisation of
the poliovirus genome, an event thought to be required for the initiation of negative strand RNA synthesis (15, 120, 180, 309)

1.3.1.2 Poly(C) Tract and Pseudoknots

On the 3' side of the S-fragment of FMDV RNA lies an RNase-T1 resistant tract containing approximately 90% C-residues, which is known as the poly(C) tract (41). A poly(C) tract is also found in the genomes of most cardioviruses but is absent from other picornaviruses such as Theiler's murine encephalitis virus. The poly(C) tract varies in length between strains of FMDV, from around 80 to 420 nucleotides. Field strains of FMDV contain a poly(C) tract of around 200 nucleotides on average, whereas shorter poly(C) tracts are generally found in tissue culture adapted strains. These observations indicate that there is strong selection pressure to maintain the poly(C) tract within FMDV RNA. The poly(C) tract may play a role in replication of the FMDV genome as the predicted binding of PCBP to the poly(C) tract could mediate the circularisation of the FMDV genome via an interaction with PABP bound to the 3' UTR.

The FMDV poly(C) tract is followed by a stretch of some 250 nucleotides in length that is thought to contain between 2 and 4 pseudoknots, depending on the virus strain in question (52, 80). The role of the pseudoknots found within FMDV RNA is as yet undefined, but it may be that these structures are involved in a joint function with the poly(C) tract. Cardiovirus RNA's are also predicted to contain multiple pseudoknots, but these structures are located on the 5' side of the poly(C) tract rather than the 3' side (73, 185). The pseudoknots found in EMCV have been suggested to play an undefined role in genome replication, since in vitro translation of viral RNA containing pseudoknot deletions occurs normally, whereas accumulation of viral RNA upon infection of Hela cells is impaired (185).
1.3.1.3 *Cis Replication Element (cre)*

Following the RNA pseudoknots found in the FMDV genome is a predicted hairpin loop, known as the cis replication element (cre), which is required for replication of the viral RNA. The first such element identified in Picornaviruses was a stem loop, located within the coding region for VP1 (1D) of HRV-14 RNA, that was required for RNA replication (200). This structure was termed a "cre", and subsequently similar structures were identified in the VP2 (1B) region of two cardioviruses (173), the 2C region of poliovirus (97), and the 2A region of HRV-2 (92). The role of this element in viral RNA replication is independent of its location within the genome (97) and is discussed further in see section 1.4.4.2.

1.3.1.4 *Internal Ribosome Entry Site (IRES)*

The IRES is a highly structured RNA element which mediates internal, non cap-dependent, initiation of translation of the FMDV genome. The role of this element in viral replication will be discussed in see section 1.4.2.

1.3.2 *The 3' UTR*

The 3' untranslated region of FMDV consists of two components, a region of about 100 nucleotides of heterogenous sequence and the poly(A) tail. Little is known about the role of the 3' UTR in infection, but deletion of the heterogenous sequence within this region blocks infection (282). It has also recently been shown that the FMDV 3' UTR can stimulate the activity of the FMDV IRES (178).

The poly(A) binding protein (PABP) binds to the poly(A) tail of the FMDV genome, but can also bind simultaneously to the translation initiation factor eIF4G (which binds the FMDV IRES) and PCBP (which binds to the FMDV IRES (301) and
presumably the poly(C) tract). Interaction of PABP with either eIF4G or PCBP could serve to circularise the FMDV genome, an event proposed to be required for RNA replication in poliovirus (see above) (120).

1.4 The FMDV Replication Cycle

Figure 1.4 shows the replication cycle of a picornavirus from cell attachment to particle assembly and release. The stages of the replication cycle are discussed below.

1.4.1 Attachment and entry

FMDV replication takes place within the cellular cytoplasm, therefore the viral RNA genome must be delivered to the cytosol across a cellular membrane. To achieve this the virion first attaches to the host cell via an integrin receptor at the cell surface (figure 1.4 step 1 – see section 1.6.1). Subsequently the virion is internalised, uncoated, and the viral RNA translocated to the cell cytoplasm by an as yet undefined mechanism (figure 1.4 step 2 – see sections 1.6.4.2 and 1.6.5.2).

1.4.2 Translation

An important feature of the FMDV RNA genome (shared with other picornaviruses) is that it is infectious, meaning that the viral RNA does not require any viral proteins to initiate the infection cycle. Once the viral RNA has been delivered to the cytosol of the host cell it is translated in order to produce the viral proteins required for replication (figure 1.4 step 3). Inside the cell the genome linked peptide VPg is removed by cellular factors and the viral RNA associates with ribosomes. The viral
Figure 1.4. Overview of the Life Cycle of a Picomavirus.

Virus binds to its receptor at the cell surface (1), and following particle uncoating the viral genomic RNA is delivered to the host cell cytoplasm (2). VPg is removed from the viral RNA, which is then translated (3). The polyprotein is cleaved nascently to produce individual viral proteins (4). RNA synthesis occurs on membrane vesicles (not drawn to scale). Viral positive (+) strand RNA is copied by the viral RNA-dependent RNA polymerase to form full length negative (-) strand RNAs (5), which are then copied to produce additional positive strand RNA (6). Early in infection newly synthesised positive strand RNA is translated to produce additional viral proteins (7), but later in infection the positive strands associate with structural protein to form new virus particles (8). Newly synthesised virus particles are released from the cell by lysis (9).

Figure adapted from (261)
RNA genome is positive sense and so is translated directly by the host cell's translation machinery. The polyprotein synthesised is processed co-translationally by virus-encoded proteases via a number of stable intermediates to give 15 mature polypeptides (see section 1.4.3).

All eukaryotic messenger RNA (mRNA) molecules have a cap structure at the 5' end which plays an essential role in the initiation of translation. The cap is recognised by the translation initiation factor eIF4F (95). eIF4F is a heterotrimeric complex of the translation initiation factors eIF4E, eIF4A, and eIF4G. eIF4E binds directly to the 5' cap structure, eIF4A is an RNA helicase, and eIF4G binds to both eIF4A and eIF4E and acts as a bridge between the two. Since the genomic RNA of FMDV lacks a 5' cap structure, and the translation initiation start site is located some 800 nucleotides downstream of the 5' end of the genome, translation must be initiated by a different mechanism to cellular mRNA. Translation of FMDV RNA is initiated at a structure known as the internal ribosome entry site (IRES). The IRES is a highly structured RNA element of approximately 450 nucleotides in length and is located immediately upstream of the translation start sites and downstream of the cre structure (see figure 1.3 b) (26, 164). The IRES facilitates translation by direct recruitment of the translational apparatus (including eIF4A, eIF4B, eIF4G, eIF2, and eIF3) to a site very close to the site of translation initiation.

Within 1–2 hours of the initiation of infection of the host cell by FMDV, the synthesis of host cell proteins declines to almost zero, due to the “shutoff” of cap-dependent protein synthesis. Shutoff of cap-dependent protein synthesis is due to the cleavage of the translation initiation factors eIF4G1 and eIF4GII by the virus encoded L protease (65, 100, 176, 201). However, IRES mediated translation of FMDV RNA is not affected by eIF4G cleavage, since the C-terminal fragment of eIF4G binds
directly to the IRES and is sufficient to mediate IRES dependent initiation of translation (177)

1.4.3 Polyprotein Processing

The full length polyprotein is never detected in infected cells or in vitro translation reactions since primary proteolytic processing of the nascent polypeptide begins cotranslationally (figure 1.4 Step 4). The processing of the FMDV polyprotein is depicted in figure 1.5. The FMDV polyprotein undergoes three primary cleavage events. The first is mediated by the L protease which cleaves the junction between its own C-terminus and the N-terminus of VP0 (1AB) (303). The second "cleavage" occurs at the junction between the C-terminus of the short 2A peptide and the N-terminus of the 2B region, and is mediated by the 2A sequence together with the first amino acid of 2B (280). It has been proposed that this event is not in fact a proteolytic cleavage of an existing peptide bond, but instead results from a modification of translation such that the bond is never formed but translation of the downstream sequence still continues (71). The properties of the 2A oligopeptide together with the first residue of 2B (a proline) can also mediate cleavage in artificial polyprotein systems (70, 279). The third cleavage event occurs at the 2C/3A boundary and is mediated by the virus encoded 3C protease. The four products of primary cleavage of the FMDV polyprotein are therefore as follows: The L protease, the capsid protein precursor P1-2A, and the 2BC and P3 replicative protein precursors.

The P1-2A, 2BC and P3 precursors undergo further "secondary" cleavages, which are also mediated by the 3C protease (12, 322). Although the 3C protease efficiently cleaves the sites mentioned above, processing of the capsid protein precursor (P1-2A)
Figure 1.5. Proteolytic processing map for Foot-and-mouth disease virus

The FMDV polyprotein is shown as an open box. The positions of the virus-encoded proteases and their cleavage sites are indicated. The 2A "cleavage" site is marked by an open arrow, 3C cleavages by thin closed arrows, and the L protease cleavage site by a wide closed arrow. Figure adapted from (23)
occurs more efficiently with 3CD, indicating that this precursor also possesses protease activity (278). Processing of the P3 and 2BC precursors is thought to occur both in cis and trans, with the cis reaction presumably predominating during the early stages of infection when the concentration of free 3C protease is at its lowest.

Processing of the P1-2A capsid precursor can only occur in trans since it is released from the ribosome before the 3C or 3CD proteases have been synthesised.

As shown in figure 1.5, full processing of the FMDV polyprotein results in the production of 15 mature polypeptides. A variety of cleavage intermediates are also observed, some of which have distinct biological roles. The 3CD precursor for example is relatively stable (278) and is required for the in vitro uridylylation of FMDV VPg in addition to the free 3D polymerase (221), as well as for maximally efficient cleavage of the P1-2A precursor (12). The 3AB precursor is very stable in vitro (278) and is thought to be the form in which the VPg peptide primer is delivered and anchored to the membranes on which viral RNA replication occurs (see section 1.4.4.3). The 3A protein itself may help to determine the host range of FMDV strains (238).

The 2BC precursor of FMDV (but not the mature 2B, 2C, or 3A proteins) has recently been shown to inhibit protein secretion in BHK cells (207). In poliovirus, the 2B, 2BC and 3A proteins can all perform this function (67), and the 3A protein has been shown to reduce the release of antiviral interferon by preventing its secretion (66). FMDV 2BC also causes remodelling of the ER into large vesicular structures close to the nucleus (207). This suggests that 2BC plays a role in the formation of replication vesicles required for RNA replication during virus infection. Consistent with this theory, co-expression of the poliovirus 2BC and 3A proteins has been shown to induce the formation of vesicles identical to those observed during poliovirus
infection (306). Within poliovirus infected cells both the 2B and 2C proteins are found within membrane associated viral replication complexes, suggesting they play a role in RNA replication (31, 33). The FMDV 2C protein is the target for guanidine hydrochloride, an antiviral compound which inhibits replication of FMDV RNA (263, 285, 286). These observations provide direct evidence that the FMDV 2C protein plays a role in RNA replication.

1.4.4 Viral RNA Replication

1.4.4.1 The Switch from Translation to Replication

After the FMDV genome has passed into the cytoplasm of the host cell, it is first translated to yield the proteins required for RNA replication, and then used as a template for negative strand RNA synthesis. It is thought that translation of the viral RNA must be terminated prior to negative-strand synthesis since translating ribosomes inhibit viral RNA replication by the 3D polymerase (14, 90). The mechanism of the switch from RNA translation to negative strand synthesis is unclear although a model for this process has been proposed. The poliovirus genome is thought to become circularised due to the interaction between the ternary complex of the 5’ cloverleaf, PCBP2, and 3CD at one end of the genome with PABP bound to the 3’ poly(A) tail at the other (15, 120, 180, 309). The formation of this structure has been proposed to inhibit the binding of translation initiation factors and ribosomes to the 5’ terminal IRES, meaning that the viral RNA would be naturally cleared of translating ribosomes over time, and hence would then be free to act as a template for negative strand RNA synthesis (15). Genome circularisation could therefore be the event which mediates the switch from translation to replication of viral RNA. A
similar mechanism is also possible in FMDV, since it has been proposed that the FMDV genome could become circularised via the interaction of PCBP bound to the poly(C) tract in the 5' UTR, with PABP bound to the 3' poly(A) tail (191).

1.4.4.2 Initiation of Positive and Negative Strand RNA Synthesis

Replication of the FMDV RNA genome follows translation and occurs via a two-step process common to all lytic viruses with a single stranded positive-sense genome.

The viral genomic RNA is transcribed to form a full-length negative-sense copy of the genome (figure 1.4 Step 5), which is used as a template for the production of new positive-sense genomes (figure 1.4 Step 6) (258).

Synthesis of both negative- and positive-strands is catalysed by the virus-encoded 3B primer and RNA dependent RNA polymerase, but several other virus non-structural proteins, and possibly host proteins, also participate in this process. RNA replication is asymmetric since many more positive strand than negative strand genome copies accumulate in infected cells. The viral peptide VPg (3B) acts as the primer for the synthesis of both negative and positive sense RNA. Unlike other picornaviruses, FMDV encodes three tandem similar but distinguishable copies of VPg all of which have been shown to be linked to FMDV genomic RNA (155).

In both poliovirus and FMDV infected cells a pool of uridylylated forms of VPg (VPgpU and VPgpUpU) can be detected. Formation of VPgpU and VPgpUpU is known as uridylylation, and is also carried out by the virus-encoded 3D polymerase. Uridylylation of VPg is dependent on a conserved stem loop known as the cis replication element (cre) (221). In vitro, uridylylation of FMDV VPg requires UTP, divalent cations, RNA containing the cre stem loop, the 3D polymerase and its 3CD precursor (221). All three copies of VPg encoded by the FMDV genome can be uridylylated in vitro, although the reaction is most efficient when the third of the
tandemly encoded copies is utilised. The efficiency of uridylylation is also significantly enhanced when the entire 5' UTR (including the cre) is included in the reaction rather than the cre alone (221). The requirements identified for cre-dependent in vitro uridylylation of FMDV VPg are similar to those previously identified for poliovirus (244, 245). Uridylylation of FMDV, poliovirus, and human rhinovirus VPg is dependent upon a conserved AAACA motif found in the non-base paired loop region of the cre stem loop (221, 244, 339). Mutation of each of the first three A residues of this motif drastically reduces VPg uridylylation in vitro as well as replication of an FMDV replicon in BHK cells, although mutation of the first A residue has the greatest effect (190, 221). These observations are consistent with studies in poliovirus which lead to the proposal of a “slide back” mechanism of uridylylation. In this model the first A residue of the cre AAACA motif is used as the template for the addition of both the first and second U residues to VPg (247). It should be noted that it is not clear whether the species uridylylated is VPg (3B) itself or a precursor which contains VPg, such as 3AB.

Based on studies carried out using poliovirus, negative strand synthesis has been proposed to occur following translocation of VPgpUpU formed on the cre structure to the 3' poly (A) tail of the infectious positive sense genome (245). VPgpUpU may become anchored to the poly (A) tail by Watson-crick base pairing, and the negative strand copy of the positive sense genome is then synthesised by the 3D polymerase using the positive strand as a template, and VPgpUpU as a primer (245). Priming of negative strand synthesis is thought to occur at an internal site since an authentic 3' end of the genome is not required for maximal efficiency negative strand synthesis (119).
More recent studies on poliovirus however indicate that negative strand synthesis is not dependent upon the cre structure in vitro, since mutations which disrupt cre-dependent uridylylation of VPG do not inhibit negative strand synthesis (99, 212, 218). This suggests that negative-strand synthesis by the 3D polymerase is not primed by VPGpUpU, but by the unmodified form of VPG (212, 217). By this model, the poly (A) tail would act as a template for uridylylation of VPG by the 3D polymerase to give VPG poly (U), which would subsequently be extended to give the full-length negative-strand genome copy. This is consistent with a previous study which showed that reactions containing 3D, VPG, UTP, Mg2+, and poly(A), result in the formation of VPG-poly(U) (246).

The above studies also showed that synthesis of positive-sense infectious poliovirus RNA from the negative sense template is dependent on the cre element, since mutations which prevent cre-dependent uridylylation of VPG also prevent positive strand synthesis (although not negative strand synthesis) in vitro (99, 212, 217). In addition, it has been shown that, unlike the initiation of negative strand synthesis, the initiation of positive strand synthesis occurs at the 3’ terminus of the template RNA, since an authentic 5’ end to the poliovirus genome is required for efficient positive strand synthesis (119). Following uridylylation, the elongation phase of both positive and negative strand synthesis is carried out by the virus encoded 3D polymerase.

1.4.4.3 The Site of RNA Synthesis

Infection of tissue culture cells by FMDV and other picornaviruses causes dramatic intracellular membrane rearrangements, resulting in the proliferation of vesicular structures within the host cell cytoplasm (208). This process has been most extensively studied in poliovirus infected cells, in which double-membraned vesicles
of between 200-400nm in diameter are seen to accumulate in tight clusters (60, 287). Virus RNA synthesis has been shown to be associated with these membranous structures (32, 44, 45), and is thought to take place on the outer cytoplasmic surface (31). Formation of vesicles with similar biochemical and morphological characteristics to those seen in poliovirus infected cells can be induced by co-expression of the poliovirus 2BC and 3A proteins in tissue culture cells (306).

Poliovirus-induced vesicles share a number of features with cellular structures called autophagosomes, including their double membranes, and the presence of cytoplasmic material within the vesicles. Autophagic vacuoles are double membraned structures which envelop cytoplasmic proteins and organelles, and become degradative upon maturation and fusion with lysosomes (171). The vesicles induced by poliovirus (and those of human rhinovirus-2 and -14 also) are thought to be derived from the ER by a process which subverts the cellular autophagosomal machinery, since the two structures share a number of characteristics (148, 306).

Vesicular structures induced by FMDV infection differ slightly from those induced by poliovirus infection. The majority of induced vesicles appear to be bounded by a single membrane rather than a double membrane, are relatively few in number, and are less tightly clustered than those induced by poliovirus (208). Secondly it has been observed that in cells infected with FMDV virtually all cytoplasmic organelles collapse into a region located on one side of the nucleus which also contains the vesicles on which RNA replication is thought to take place (208). This accumulation of organelles was not observed when the same cell line was infected with bovine enterovirus (208) and has not been observed in previous work carried out on poliovirus infected cells (e.g. (31)). The significance of this difference is as yet unclear. It should also be noted that FMDV and poliovirus differ in their sensitivity to
Brefeldin A (BfA). BfA inhibits membrane transport between the ER and the Golgi apparatus by preventing the formation of COPI-dependent secretory transport vesicles (274). Replication of poliovirus is extremely sensitive to the action of BfA (139), whereas FMDV replication is unaffected by this agent (210, 233), suggesting that the mechanism of formation and/or the source of the vesicles on which replication occurs may differ between the two viruses.

The precise role played by membranous structures in the replication of picornavirus RNA is still unknown. One possible role for the vesicles induced by picornaviruses is to act as a scaffold on which the virus-encoded proteins and template RNA required for genome replication can be assembled in the correct context. In poliovirus infection all non-structural proteins (including the 3CD precursor) except the viral proteases 2A and 3C have been detected on the surface of replication vesicles (77, 287, 306, 310). The 2C and 2B proteins of enteroviruses have been shown to bind membranes peripherally via amphipathic helices (243), whereas poliovirus 3A and its 3AB precursor are integral membrane proteins which interact with membranes via a hydrophobic region of 3A (314). The hydrophobic domain of 3A, in the form of the 3AB precursor, is thought to serve to deliver and anchor the VPg peptide primer of negative and positive strand synthesis to the membrane vesicles on which RNA replication occurs. The 3D polymerase and its 3CD precursor have been shown to be associated with replication vesicles in poliovirus infected cells and are required for RNA replication, yet have not been shown to interact with membranes. The membrane binding precursor 3AB binds to 3D and 3CD, and stimulates the polymerase activity of the former (257). 3AB may therefore serve to recruit and anchor 3D and 3CD to the virus induced vesicles on which viral RNA replication takes place.
1.4.5 Particle Assembly, Maturation, and Release

The first step in assembly (figure 1.4 step 8) is the formation of the 12S intermediate termed a “pentamer”. Pentamers are formed by the irreversible association of five protomers in a urea-sensitive fashion. The second stage in assembly sees the association of 12 pentamers in an acid-sensitive fashion to yield the final capsid structure. The stage at which RNA encapsidation occurs is unclear. Mutations which prevent the myristoylation of VP0 prevent the assembly of FMDV empty capsids, indicating that this lipid modification is required for particle assembly (1). The final stage of assembly is the maturation cleavage of VP0 (see below).

A number of intermediate particles have been identified in picornavirus infected cells, including a particle containing RNA but uncleaved VP0 (the provirion) (111), and a particle with uncleaved VP0 which lacks RNA (empty capsid) (105). The role of these particles, together with the signals necessary for encapsidation, remains unclear. In one model of assembly the genomic RNA is inserted into preformed empty capsids to yield the provirion. In the second model the provirion is formed by the assembly of 12 pentamers around a single copy of viral positive sense RNA. Evidence for the first model includes the ability to chase a radioactive label from FMDV structural proteins found in protomers, to pentamers, empty capsids, and ultimately virions (336). In addition the capsid proteins of both FMDV (1) and poliovirus can self-assemble into empty capsids in the absence of viral RNA. However, more recent work using the poliovirus cell-free replication system supports the second model, since only pentamers can interact with viral RNA to form virions in this system (327). This indicates that empty capsids may be a by-product of the assembly reaction. Picornaviruses package only newly synthesised, VPg linked,
positive strand RNA (229, 230), suggesting there is a link between active RNA replication and encapsidation, perhaps mediated by a cis-acting packaging signal. Any packaging signal may lie in the P1 region of the genome, since, in contrast to replicons based on poliovirus (259), FMDV replicons lacking virtually all of this region are inefficiently packaged when capsid proteins are provided in trans (199). Recently a cis-acting encapsidation element has been identified within a stem loop located at the 5’ end of the Aichi virus genome, which is the first report of such an element in a picornavirus (283).

Once the viral RNA has been packaged the final stage in virion assembly is the cleavage of VP0 to yield VP4 and VP2, an event termed the maturation cleavage. The degree of VP0 cleavage is higher in infectious virus particles than either naturally or artificially produced empty particles, indicating that the viral RNA plays a role in this process (56, 58, 275). VP0 cleavage is thought to be autocatalytic, and has been proposed to result from the activation of local water molecules by a conserved histidine residue (His 195 of poliovirus VP2 or His 145 of the FMDV protein), leading to a nucleophilic attack on the scissile bond and cleavage (16, 58, 125). Consistent with this model, mutation of histidine 195 of poliovirus results in the formation of unstable particles containing poliovirus RNA and uncleaved VP0 (125). The increased stability of mature particles over empty capsids results from increased ordering of the network of structural protein N-termini due to both the physical presence of RNA, and the increased degree of VP0 cleavage (58).

Recombinant FMDV particles in which the maturation cleavage is prevented from occurring by site-directed mutagenesis are non-infectious (160). The introduced mutations did not prevent normal RNA replication, protein expression, assembly, cell binding, or acid-mediated particle uncoating, but the acid breakdown products of the
particles were considerably more hydrophobic than wild type, presumably due to a failure to release myristoylated VP4 (160). These data appear to indicate that the maturation cleavage is required in order that the viral RNA is translocated into the cytoplasm, a process known as RNA escape.

The final step in the FMDV replication cycle is the release of the newly formed mature virus particles from the host cell (figure 1.4 Step 9). Infection of a cell monolayer by FMDV results in cell lysis and plaque formation, hence it is believed that progeny virions are primarily released upon lysis of the infected host cell (24). The mechanism by which lysis is triggered is unclear, although in the case of poliovirus the virus 3A protein has been implicated in this process (165). When poliovirus replicates in polarised epithelial cells resembling those that line the gastrointestinal tract (a natural site of infection), progeny virions are released at the apical surface of the cells by a non-destructive mechanism (320). It is likely that FMDV can also be released by non-lytic as well as lytic mechanisms.

1.5 Endocytosis Pathways

Mammalian cells take up extracellular material by a variety of different mechanisms that are collectively termed endocytosis. Endocytic mechanisms serve many important cellular functions including the uptake of extracellular nutrients, regulation of cell-surface receptor expression, and the maintenance of cell polarity. Endocytosis pathways are also exploited by viruses in order to gain entry to and infect cells. The endocytic pathways exploited by viruses, and the distinguishing features used to classify them, are depicted in figure 1.6. Each pathway is discussed individually below.
Figure 1.6. Different endocytosis pathways used by cells to internalise ligands.

The endocytosis pathways shown are classified according to four criteria: the dependence on clathrin, caveolae, dynamin and lipid rafts. The best-characterised pathways are (a) the clathrin-mediated pathway and (b) the caveolar pathway. The lipid-raft-mediated uptake pathways can be dynamin-dependent (c), or dynamin-independent (d). In addition, there are pathways that do not seem to depend on any of these factors (e). The intracellular routing for each of the pathways is also shown, but for all except the clathrin-mediated pathway they are poorly defined (indicated by thin arrows). Figure adapted from (245).
1.5.1 Clathrin-dependent Endocytosis

The most well known and intensively studied endocytosis mechanism is the clathrin-dependent endocytosis pathway. This pathway is characterised by the formation of shallow coated-pits at the plasma membrane, which become deeply invaginated and then pinch off from the plasma membrane to form coated vesicles within the cell cytoplasm. The coat found on the cytoplasmic surface of coated pits and vesicles is a honeycomb-like lattice composed of both hexagonal and pentagonal faces (295), and is formed by the oligomerisation of clathrin triskelions. The clathrin triskelions which make up the clathrin coat are three legged structures composed of three copies of the 190kDa clathrin heavy chain (CHC) and three copies of the smaller (~15kDa) clathrin light chain (156, 321).

Clathrin coated pit formation, invagination, and membrane fission require an array of accessory proteins in addition to clathrin. A wide variety of proteins have been identified as components of coated pits including the AP-2 adaptor complex, AP180, Epsin, Eps15, Dab2, Hip1, and Numb-1. In addition, proteins that are not components of the coated pit, but are involved in later stages of clathrin-dependent vesicle formation include dynamin, amphiphysin and endophilin. Dynamin is a large GTPase that self-assembles into a helical collar around the neck of invaginated coated pits. It is unclear however whether dynamin triggers vesicle fission directly, or regulates the activity of other factors that mediate the fission reaction.

Many of the above proteins can act as dominant negative inhibitors of clathrin-dependent endocytosis when full length or truncated forms are overexpressed in tissue culture cells. One of the most widely utilised specific dominant negative inhibitors of clathrin-dependent uptake is AP180. AP180 binds directly to the ear domain of AP-2 α subunit (113, 206, 316), and in doing so forms a complex which co-operatively
recruits clathrin (113). Over-expression of a full length version of AP180, or a C-terminal fragment containing only the clathrin-binding domain, potently inhibits clathrin-dependent endocytosis by mislocalizing clathrin and preventing its incorporation into nascent coated pits (82, 342).

Membrane proteins that are internalised in clathrin coated vesicles generally contain one or more signal sequences in their cytoplasmic domain that direct the protein to clathrin coated pits. Three such sorting signals have been indentified in single membrane spanning receptors thus far: 1) Tyrosine based signals of the form NPxY or YXXØ (Ø = a bulky hydrophobic residue, x = any residue), 2) Dileucine (LL) based motifs and 3) motifs involving serine phosphorylation and the ubiquitination machinery. Receptors bearing tyrosine or dileucine based endocytosis motifs within their cytoplasmic domains interact with the AP-2 adaptor complex, which itself binds directly to clathrin (via the β2 ear and hinge regions). It is unclear whether receptors carrying such motifs recruit AP2, resulting in clathrin coated pit formation, or rather the receptors are recruited to coated pits via interaction of their cytoplasmic domain with AP-2. The interactions mediated by serine based internalisation motifs are unclear.

Once a ligand or receptor has been delivered to a clathrin coated vesicle, the vesicle uncoats. The uncoated vesicle is then able to fuse with an early endosome (also known as sorting endosomes) in a Rab5 GTPase dependent fashion, and its contents are delivered to the endosomal lumen. Early endosomes are tubulo-vesicular structures bounded by a single membrane, whose lumen is mildly acidic (pH ~6.0). Many are located at the cell periphery close to the plasma membrane. The limiting membrane of early endosomes contains a peripheral membrane protein called early
endosomal antigen-1 (EEA-1), and this protein is used as a specific marker for this compartment (215).

Material delivered to early endosomes can either be delivered to late endosomes and lysosomes for degradation or recycled back to the cell surface. The relationship between early and late endosomes is unclear. An intermediate compartment with distinct properties (the endosomal carrier vesicle) has been defined (107), but it has proved difficult to distinguish whether early endosomes are formed de novo and mature into late endosomes (via ECV's) or whether material is transported between stable early and late endosomes by ECV's. Late endosomes are more acidic (pH ~5.5), more spherical, and tend to adopt a more juxtanuclear distribution than early endosomes. The cation independent mannose-6-phosphate receptor is concentrated within late endosomes and is a characteristic marker of this compartment (102). Late endosomes also contain a number of internal vesicles, thought to play a role in the degradation of a number of membrane proteins by enzymes delivered to this compartment following fusion with lysosomes (256).

Receptors and ligands that are not passed to late endosomes and lysosomes for degradation, can be recycled back to the cell surface for reuse. This process has been most intensively studied using transferrin and the transferrin receptor. After leaving early endosomes recycling receptors are found in tubular recycling endosomes, from which they are delivered back to the cell surface. These compartments are less acidic than early endosomes and are located close to the centrioles in some cell types (88, 337). Marker proteins for this compartment include recycling receptors such as the transferrin receptor, and the Rab11 GTPase (51). Kinetic studies have identified both a slow and a fast recycling pathway (112), leading to the suggestion that
recycling receptors may be able to traffic back to the cell surface directly from early endosomes as well as via recycling endosomes.

1.5.2 Lipid Raft-dependent Endocytosis

Lipid rafts are sphingolipid and cholesterol rich microdomains found at the cell surface and can be envisaged as islands of highly ordered saturated lipids that are laterally mobile in a more disordered and fluid bilayer of unsaturated lipids. They are defined biochemically by their insolubility in the non-ionic detergent Triton X-100 at 4°C and their sedimentation with low density fractions following centrifugation through sucrose gradients. A subset of endocytosis pathways operating in tissue culture cells (highlighted in green in figure 1.6) are dependent on the presence of intact lipid rafts at the cell surface, and are outlined briefly below.

1.5.2.1 Caveolae-dependent Endocytosis

Caveolae are a specialised form of lipid raft and typically appear as flask shaped invaginations of the plasma membrane of between 50-80nm in diameter by electron microscopy (249). Caveolae at the cell surface are characterised by the presence of the integral membrane protein caveolin-1 (or caveolin-3 in muscle cells) (273). Formation of caveolae appears to be dependent on caveolin since cell lines which do not express this protein do not possess any morphological caveolae at their cell surface.

The best characterised example of caveolae-dependent endocytosis of a ligand is the internalisation of Simian Virus 40 (SV40) by tissue culture cells (see figure 1.6
pathway b). Unlike clathrin-dependent endocytosis, this uptake pathway is not thought to be constitutive but instead is triggered. SV40 binds to the cell surface via MHC class I molecules and then moves laterally within the plasma membrane until trapped in caveolae (8, 251). The caveola and associated virus are then internalised, an event which is dependent on GM1 ganglioside (319), the actin cytoskeleton and the recruitment of dynamin (252). The internalised caveolar vesicles (cavicles) subsequently deliver internalised virus to a membrane-bound cytoplasmic organelle, termed the caveosome (251). This organelle is devoid of markers of classical endocytosis pathways, has a neutral pH, and possesses multiple flask-shaped caveolar domains enriched in caveolin-1. After a few hours, virus filled tubules devoid of caveolin-1 are released from the caveosome and traffic to the ER in a microtubule and COPI dependent manner (251, 268), and it is from this compartment that SV40 infection is initiated.

Other ligands known to be taken up by caveolae-dependent endocytosis include murine polyomavirus (a close relative of SV40) (182) and echovirus-1 (183, 254), both of which are thought to be delivered to caveosomes. Recent work utilising cholera toxin suggests that internalised caveolar vesicles can also deliver internalised ligands to early endosomes (248). Upon docking with early endosomes caveolar membrane domains do not disassemble but instead remain as distinct and stable membrane domains. The low pH within the early endosome allows cholera toxin to diffuse out of the caveolar domain to the surrounding membrane, and it is then delivered to the ER via the golgi apparatus.
1.5.2.2 Other Lipid-raft Dependent Endocytosis Pathways

A number of ligands which have been reported to utilise caveolae-dependent endocytosis pathways are also internalised normally by cells which do not express caveolin-1 in a lipid raft dependent fashion. This suggests that cells can internalise material via caveolae and clathrin independent but lipid raft dependent endocytosis pathways (see figure 1.6 pathways d and e). A good example is the virus SV40 which has been shown to be taken up by a caveolae-dependent uptake pathway (see above) yet will readily infect cells lacking caveolin-1 expression (62). The endocytosis pathway utilised by SV40 in cells lacking caveolae was rapid, dependent on lipid rafts and tyrosine kinase activity, but was independent of clathrin, and dynamin (62). The virus is internalised in small tight fitting vesicles and was delivered to pH neutral organelles with similar properties to caveosomes bar the absence of caveolin-1 and caveolin-2. A pathway with similar properties has been shown to mediate the internalisation of the cholera toxin B-subunit (CTB) in caveolin-1 knockout mouse embryonic fibroblasts, albeit that this molecule is delivered to the golgi rather than the ER (158). The lipid raft dependent but caveolar independent uptake of both CTB and SV40 were both also shown to occur in cells expressing caveolin-1, indicating that this pathway coexists with the caveolae dependent uptake pathway (62, 158).

In addition to the dynamin independent pathways described above, internalisation of other ligands has been shown to be mediated by caveolae-independent but lipid raft- and dynamin-dependent pathways. Examples of ligands taken up by this mechanism include interleukin-2 in cells lacking caveolae or following reduction of caveolin-1 expression by RNAi (166, 227), and autocrine motility factor (AMF) in transformed NIH-3T3 cells which express little caveolin-1 (167).
A recent review has suggested that caveolae-dependent endocytosis and the lipid raft dependent endocytosis mechanisms described above constitute a common endocytosis system in which caveolin-1 acts as a negative regulator of the internalisation of caveolae/raft domains at the cell surface (220). This hypothesis is consistent with the observation that decreased expression of caveolin-1 in ras- and abl-transformed NIH-3T3 cells is associated with an increased rate of delivery of AMF to the ER, and that the reintroduction of caveolin-1 results in a reduction in the rate of AMF internalisation (167). Similarly the rate of uptake of the DNA virus SV40 is much greater in caveolin-1 knockout mouse embryonic fibroblasts than the wild type cell line (62). The available evidence suggests that lipid rafts are constitutively internalised in the absence of caveolin-1, but are only internalised following the induction of cellular signalling events in cells which do express caveolin-1 (220).

### 1.5.3 Other Endocytosis Pathways

The final category of endocytosis pathway observed in cells (figure 1.6 pathway e) covers those internalisation mechanisms that are independent of clathrin, caveolin, and lipid rafts, about which relatively little is known. One such pathway is macropinocytosis, a triggered pathway used by cells to internalise large amounts of fluid and membrane (307). This internalisation pathway is strictly dependent upon actin polymerisation but is dynamin independent, and is exploited by vaccinia virus which is too large to enter via clathrin coated pits.

Other clathrin and lipid raft independent internalisation pathways are less well defined. Murine polyoma virus has been reported to utilise such a pathway in cells lacking caveolin-1 expression (94). Recent work has shown that around one third of influenza virus particles are also taken up by a clathrin and lipid raft independent
endocytosis mechanism, and the remainder by clathrin-dependent endocytosis (277, 294). These studies show that influenza virus uses this alternative pathway not only when clathrin-dependent endocytosis is blocked, but also in parallel under normal conditions (277).

1.6 Picornavirus Cell Entry

In order to initiate the infection cycle all that is required is the delivery of the viral RNA genome to the host cell cytoplasm across a host lipid membrane. First the virus particle must attach to the host cell via a specific cell surface receptor. The virus particle is then delivered to the membrane across which the viral RNA will pass into the cellular cytoplasm. This process is mediated either by the initial primary attachment receptor or by secondary cell surface receptors. Once the viral genome has reached the cytoplasm, it is translated and replicated, and progeny virus particles are assembled and released. The process by which the FMDV genome is delivered to the cytoplasm of the host cell is discussed in detail below.

1.6.1 FMDV Receptors

1.6.1.1 Background

The viral structural protein VP1 contains the major antigenic site on the surface of the FMDV capsid, which also encompasses the major receptor attachment site on the virion surface (47, 331). Early sequencing studies revealed that VP1 contains a conserved Arginine-Glycine-Aspartic Acid (RGD motif) within its GH loop (84, 93).
This motif is conserved across all natural isolates of FMDV (141), and is located at
the apex of the GH loop (2). Proteolytic cleavage of the GH loop of VP1 abolishes
cell attachment and synthetic RGD-containing peptides have been shown to inhibit
binding of FMDV to BHK cells (19, 84) indicating that the RGD motif may be
important for receptor binding. The role of the RGD in cell attachment was
confirmed by the creation of mutant viruses lacking this motif using an infectious
cloned, which were uninfected and were unable to bind susceptible cell lines (192).

A subset of the integrin family of cell-adhesion molecules are known to interact
with an RGD-motif within their ligand, which lead to the proposal that the receptor
for FMDV may be an integrin.

1.6.1.2 Integrin Attachment Receptors

Integrins are a family of cell surface, α-β heterodimeric glycoproteins composed of
at least 18 α and 8 β subunits which associate to form over 24 different α-β
combinations (135). Integrins contribute to a variety of processes, including adhesion
between cells, and between cells and the extracellular matrix, and induction of signal
transduction pathways that modulate various processes, including cell proliferation,
morphology, migration, and apoptosis. Of the possible heterodimer combinations,
ανβ1, ανβ3, ανβ2, ανβ5, ανβ6, ανβ8, α5β1 and α8β1 have been shown to interact
with their ligands in an RGD-dependent fashion (136, 228, 288, 299). Of the RGD-
dependent integrins, ανβ1 (145), ανβ3 (29, 222-224), ανβ6 (141, 204), and ανβ8
(142) have been shown to act as attachment receptors for FMDV. The integrins ανβ5
and α5β1 however do not act as receptors for FMDV infection, despite the fact that
virus binds to purified $\alpha 5\beta 1$ in an in vitro ELISA assay (141). The ability of $\alpha 8\beta 1$ to act as a receptor for FMDV has not been tested.

The first integrin reported to act as a receptor for FMDV was $\alpha v\beta 3$ (29). Berinstein et al. showed that FMDV shares a common receptor binding site with Coxsackievirus A9 (previously reported to utilise $\alpha v\beta 3$ as a receptor), when utilising cells permissive to both viruses (29). In addition, binding and infection of LLC-MK2 cells by FMDV was shown to be partially inhibited by a polyclonal serum directed against $\alpha v\beta 3$. The virus has also been shown to bind avidly to purified $\alpha v\beta 3$ in vitro, a process which is inhibited by RGD-, but not RGE-, containing peptides (146).

Subsequently a number of non-FMDV permissive cell lines such as K562 (224), CHO (224) and COS (222, 223) cells have been shown to become permissive upon transfection with the human $\alpha v$ and $\beta 3$ integrin subunits, indicating that one or both of these subunits are required for the infection process. In the latter system, transfection with bovine $\alpha v$ and $\beta 3$ lead to more efficient infection than transfection with the equivalent human subunits, a property that can be localised to the C-terminal one-third of the bovine $\beta 3$ subunit (223). Since the $\beta 3$ subunit is limited to pairing with the $\alpha v$ chain in this system it follows that $\alpha v\beta 3$ must play an active role in infection. This conclusion is supported by the observation that the infection of COS-1 cells transfected with the bovine $\alpha v$ and $\beta 3$ subunits is inhibited by a function blocking monoclonal antibody directed against the $\alpha v\beta 3$ heterodimer (222).

More recently, three other RGD-dependent integrins have been reported to act as receptors for FMDV: $\alpha v\beta 1$ (145), $\alpha v\beta 6$ (142, 145, 147, 204), and $\alpha v\beta 8$ (142). These experiments utilised two cell lines, a human colon carcinoma cell line (SW480 cells) and CHOB2 cells, both of which are normally non-permissive for FMDV infection. SW480 cells and wild type CHO cells normally express only the $\alpha v\beta 5$ and $\alpha 5\beta 1$
integrins as their RGD-binding integrins, but the CHOB2 variant cell line lacks endogenous α5 production and so expresses only αvβ5. SW480 cells become susceptible to infection by FMDV upon transfection with either the β6 or β8 integrin subunits and the consequent expression of the integrins αvβ6 or αvβ8 at the cell surface respectively (142, 147, 204). In addition, CHOB2 cells become susceptible to infection by FMDV upon transfection with either the β6 or αv integrin subunits and the consequent expression of the integrins αvβ6 or αvβ1 at the cell surface respectively (145). The αvβ1, αvβ6, and αvβ8 integrins were shown to serve as receptors for FMDV attachment to the host cell as this process was inhibited by RGD-containing peptides and also by function blocking antibodies specific for the transfected integrin (142, 145, 147). The same function blocking antibodies which blocked attachment of FMDV to the above integrins also blocked infection, confirming the role of αvβ1, αvβ6 and αvβ8 in infection (142, 145, 147). It should however be noted that the level of virus binding and infection mediated by αvβ1 is relatively low unless this integrin is activated using manganese ions or an activating antibody (145).

The relative efficiency with which FMDV utilises different integrin receptors may vary from serotype to serotype. For example, Type A viruses have been reported to use αvβ6 and αvβ3 with equal efficiency and αvβ1 to a lesser extent, whereas type O viruses have been reported to utilise both αvβ6 and αvβ1 more efficiently than αvβ3 (74). The basis for this difference is unclear since a type A virus carrying a type O GH loop showed virtually the same receptor utilisation pattern as the parent type A virus. However, experiments using soluble integrins showed that both type A and type O viruses are neutralised much more effectively by soluble αvβ6 than soluble
αvβ3 (75). This may suggest that the interaction between FMDV and αvβ6 is of much higher affinity than that between FMDV and αvβ3.

1.6.1.3 The Role of Integrins in Post-Attachment Events

As described above, four RGD-dependent integrins have been shown to act as attachment receptors for FMDV on cells. However, the role of integrin receptors in post-attachment events is not clear from these studies. In the case of αvβ6, mutant receptors containing certain deletions within the β6 cytoplasmic domain (the T1 and T5 deletion mutants – see section 7.1) are still able to bind FMDV but are no longer competent to mediate infection (204). This suggests that sequences within the cytoplasmic domain of the β6 subunit are required to mediate post-attachment steps of infection. More recently it has been shown that a chimeric αvβ6 receptor (αvβ6/8), in which the β6 cytoplasmic domain has been replaced by the equivalent region from the β8 integrin subunit, also binds FMDV but fails to mediate infection (142). Certain epitopes recognized by antibodies against the αvβ6 heterodimer are absent from the chimeric receptor, indicating that the β6 cytoplasmic domain is required for normal heterodimer assembly. Although the chimeric receptor binds FMDV, it is a less effective receptor for virus attachment and has altered specificity for RGD peptides compared to the wild type integrin. Interestingly a chimeric αvβ8 receptor, in which the β8 cytoplasmic domain is replaced by that from β6, is able to mediate both virus binding and infection similar to wild type αvβ8 (142). Following these observations it was shown that the same epitopes are lost from cells expressing αvβ6 with truncations in the β6 cytodomain (The T1 and T5 deletion mutants). There are three possible explanations for the above data.
(1) The β6 cytoplasmic domain is required to maintain the ectodomain of αvβ6 in a conformation that is necessary for both high-affinity binding of FMDV and subsequent infection. Such high affinity binding may be required to mediate cross-linking of the integrin receptor and/or internalisation of the integrin:virus complex by endocytosis.

(2) The cytoplasmic domain of the β6 integrin subunit is required for membrane penetration by FMDV, a role proposed previously for the cytodomain of the β5 chain in αvβ5-mediated infection by adenovirus (328). This activity is thought to be mediated by the three C-terminal residues of the β5 subunit. However these residues are different in the β6 subunit chain, and their deletion does not inhibit αvβ6-mediated infection by FMDV (204). Therefore, it would appear that, if the β6 cytodomain is needed for virus penetration into the cell, it works by a mechanism distinct from that used by αvβ5.

(3) The β6 cytoplasmic domain contains endocytosis motifs required for internalisation of the integrin:virus complex. Consistent with this notion both the T1 and T5 deletion mutants, and the αvβ6/8 chimeric receptor, lack a conserved NPXY motif known to mediate clathrin-dependent endocytosis. However, the integrin αvβ8 is able to mediate binding and infection of the same cell line (SW480 cells) by FMDV, despite the fact that the sequence of the β8 cytoplasmic domain is divergent from other β cytoplasmic domains and lacks the NPXY putative endocytosis motif. It is possible that internalisation of αvβ8 is mediated by a novel, as yet unidentified endocytosis motif.

As outlined above the cytoplasmic domain of the β6 integrin subunit is required for post-attachment events in αvβ6-mediated infection of tissue culture cells by FMDV,
although the precise role played by this domain is unclear. By contrast, removal of both the αv and β3 cytoplasmic domains (including the conserved NPXY motif) appears to have no effect on αvβ3-mediated infection by FMDV (222). This would suggest that either αvβ3 mediates only binding but not internalisation of FMDV, or that this integrin can be internalised in the absence of the αv and β3 cytoplasmic domains. Further work is required to clarify the role of integrin receptors of FMDV in post-attachment events.

1.6.1.4 Other Receptors

Heparan sulphates are randomly sulphated (and hence negatively charged) polysaccharides formed from an L-iduronic acid D-glucosamine disaccharide repeat. They form the carbohydrate component of proteins called heparan sulphate proteoglycans which are expressed on virtually all cell types as integral membrane proteins or as part of the extracellular matrix.

Heparan sulphate was originally identified as a potential co-receptor or enhancer of the entry of certain strains of type O FMDV into tissue culture cells (143). At this time it was thought that heparan sulphate acts as the first point of contact of the virus en route to the integrin receptor. It was subsequently shown however that field strains do not bind heparan sulphate with high affinity, and that this phenotype is acquired rapidly upon adaptation to tissue culture (224, 281). Such viruses do not require integrin receptors at all since their replication is not inhibited by mutation of the RGD integrin binding motif (13, 186, 224). In addition, strains that acquire the ability to bind heparan sulphate are attenuated in cattle, and infection of cattle with such strains yields revertant viruses which lack the ability to bind heparan sulphate (281).
The available data described above suggests that whilst high efficiency interaction of FMDV with heparan sulphate confers a clear advantage for growth in tissue culture cells, this phenotype is disadvantageous in the natural host animal. In subtype O-strains passage in tissue culture causes a single amino acid change (VP3-residue 56 from arginine to histidine), which causes a dramatic increase in the affinity of virus for heparan sulphate (281). It is not known whether a switch from integrin to heparan sulphate receptors late in infection, or a low affinity interaction of FMDV with heparan sulphate, play any role in infection.

FMDV has also been shown to be able to utilise artificial receptors in tissue culture. For example, FMDV is unable to infect CHO-B2 cells transfected with the Fc receptor, unless first coated with antibody (192). By contrast antibody coated poliovirus could not infect this cell line. The Fc receptor at the cell surface mediates the attachment of FMDV by binding the constant region of the antibodies coating the virion, and in doing so mediates infection of the host cell (192). Another artificial receptor that can be utilised by FMDV is a genetically engineered receptor consisting of ICAM-1 fused to a virus-binding single chain antibody (269). CHO cells are normally not susceptible to non-heparin binding strains of FMDV, but become susceptible upon transfection with this chimeric receptor (269). Both artificial receptors can mediate infection by RGD-deleted viruses. It has also been reported that a highly tissue culture adapted virus has been identified which does not use heparan sulphate or integrins as receptors but an unidentified third class of receptor (341). The plasticity of receptor use by FMDV suggests that unlike poliovirus and major group rhinoviruses, the FMDV receptor simply binds the virion rather than catalysing conformational changes required for uncoating.
1.6.2 Attachment to the host cell by other Picornaviruses

Picornaviruses have evolved to utilise a wide variety of cell surface proteins as receptors. The receptors utilised include members of the immunoglobulin superfamily such as the poliovirus receptor (PVR), complement-inactivating proteins such as decay acceleration factor (DAF), lipoprotein receptors, and integrins.

A number of picornaviruses have been shown to utilise RGD-dependent integrins as receptors, including: Human parechovirus 1 \((\text{parechovirus})\) (151), echovirus 9 \((\text{echovirus})\) (225, 264) and coxsackievirus A9 \((\text{coxsackievirus})\) (332), and of course foot-and-mouth disease virus \((\text{aphthovirus})\). In the case of FMDV, the RGD motif is located in the prominent GH loop of VP1, whereas in the other viruses it is located close to the VP1 C-terminus (117). Both FMDV and Coxsackievirus A9 have been shown to utilise the integrin \(\alpha v \beta 6\) as a receptor, and interestingly the sequence context of the RGD motif found in the two viruses is very similar to each other, and also to that of other ligands of \(\alpha v \beta 6\) such as latency associated protein-1 \((\text{LAP-1})\) (141, 147). This suggests that the residues surrounding the RGD motif may influence integrin specificity. Other viruses also shown to utilise RGD-dependent integrins as receptors include hantaanvirus, and certain adenoviruses.

The poliovirus receptor \((\text{CD 155})\) is a member of the immunoglobulin superfamily of receptors, and its ectodomain consists of three immunoglobulin-like domains (202), of which only domain 1 (the largest domain nearest the N-terminus of the protein) is required for virus binding (162, 213). Structures of all three poliovirus serotypes bound to soluble derivatives of the poliovirus receptor have been determined by cryoelectron microscopy (23, 116). Domain 1 of the poliovirus receptor penetrates the canyon at a glancing angle and makes contacts with both the south and north walls, contacts which involve all three surface exposed capsid proteins. This confirms
previous genetic data which indicated that the primary contact points between virus and receptor were located within the canyon.

The rhinoviruses can be divided into two groups based on receptor usage. The major receptor group rhinoviruses include human rhinovirus-3,-14, and -16 (HRV-3,-14,-16). These viruses utilise intercellular adhesion molecule-1 (ICAM-1) as their receptor (101). ICAM-1 is a member of the immunoglobulin supergene family which mediates the attachment of leukocytes to a number of cell types by binding to the integrin αLβ2 (76). The ectodomain of ICAM-1 contains five immunoglobulin-like domains, of which only domain 1 (the domain nearest the N-terminus of the protein) is thought to be directly involved in the binding of virus (197, 267) and the integrin αLβ2 (197). Cryostructures of ICAM-1 bound to HRV-16 (163, 236) and HRV-14 (163) have been determined and show that, like the poliovirus receptor, the major group rhinovirus receptor binds in the canyon surrounding the 5-fold symmetry axis, and makes more extensive contacts with the south rim of the canyon than the north rim. In contrast to the poliovirus receptor, ICAM-1 is oriented perpendicular to the surface of the virus particle.

The minor receptor group rhinoviruses include human rhinovirus-2 (HRV-2) and utilise members of the low density lipoprotein receptor family to attach to the host cell (126, 184). The LDL receptor family are a group of molecules which mediate the clathrin-dependent uptake and lysosomal targeting of a diverse range of ligands including lipoproteins, proteinases and hormones (11). All members of this family identified to date contain at least one clathrin-dependent endocytosis motif (FXNPXY) within their cytoplasmic domain. The very low density lipoprotein receptor (VLDL receptor) contains eight imperfect ligand binding repeats (each of 40 amino acids) in its ectodomain. A truncated version of the VLDLR, consisting of the
three ligand binding repeats closest to the N-terminus of the receptor, has been visualised in complex with HRV-2 by cryoelectron microscopy (123). This work revealed that this receptor does not bind in the canyon which encircles the 5-fold symmetry axis, as is the case for ICAM-1 binding to major group rhinoviruses, but instead the ligand binding repeats interact with the star shape dome on the icosahedral 5-fold axis. Further cryo-electron microscopy structures have revealed that virus binding is mediated by ligand binding repeats 2 and 3 (226), and a crystal structure of these domains bound to HRV-2 has been determined (324).

As described above, the receptors of poliovirus and the major group rhinoviruses bind to sites within the canyon surrounding the 5-fold symmetry axis. It was originally proposed that the reason receptors bound to a depression on the virion surface was to protect epitopes required for receptor binding from neutralisation by antibodies ("the canyon hypothesis") (271). However, this hypothesis is not consistent with the finding that the minor group rhinovirus receptor binds not within the canyon but to the highly exposed star shaped dome at the 5-fold axis itself. In addition the surface of FMDV possesses few significant surface depressions, and its integrin receptors bind to the exposed GH loop of VP1. The different receptor binding sites may reflect differences in the uncoating mechanisms of the respective viruses. Uncoating of major group rhinoviruses and polioviruses are at least partially receptor dependent, whereas uncoating of minor group rhinoviruses (and also FMDV) is dependent on exposure to acidic pH alone (see section 1.6.5). It has therefore been proposed that binding of the receptor within the canyon on the surface of poliovirus and major group rhinoviruses helps to destabilise the viral particle and facilitates the structural changes required for uncoating (127) (see section 1.6.3), changes which are triggered solely by exposure to acidic pH in minor group rhinoviruses.
Other receptors utilised by picornaviruses include vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin superfamily utilised by EMCV (134), and hepatitis A virus cellular receptor 1 (HAVcr1) (153), a mucin like molecule which carries an immunoglobulin-like domain at its N-terminus. The attachment of a number of enteroviruses to the host cell has been shown to be mediated by decay acceleration factor (DAF), the coxsackie adenovirus receptor (CAR), or a combination of the two. Examples include echovirus 11 which utilises DAF alone (304), and coxsackievirus B3 which utilises both DAF and CAR (although DAF cannot mediate infection in the absence of CAR), or CAR alone (292).

1.6.3 A-Particle Formation

Upon binding to its receptor at physiological temperatures, poliovirus undergoes an irreversible structural change resulting in the formation of a particle which sediments at 135S (rather than 160S for the native virion), termed the A-particle. A-particle formation results in a decreased affinity for the PVR, the externalisation of VP4 and the relocation of the N-terminus of VP1 from the inner to the outer surface of the capsid (86). The structure of the 135S A-particle of poliovirus has recently been solved to 10Å and this structure revealed that the externalised N-termini of VP1 lie around the 3-fold symmetry axis, rather than the 5-fold axis as previously thought (42). A-particle formation is thought to be a prerequisite for the release of the viral genomic RNA. It has been a matter for debate in recent years as to whether or not A-particles are an intermediate in poliovirus cell entry, but a number of lines of evidence suggest that this is the case (see section 1.6.5).
Decay acceleration factor (DAF) serves as an attachment receptor for a number of enteroviruses, including coxsackievirus A21, some isolates of coxsackie B3 (CB3) and several echoviruses. However, the above DAF-binding viruses all fail to infect normally non-permissive cells transfected with DAF, indicating that binding to DAF is insufficient to trigger postattachment events required for infection (27, 28, 291, 292). Coxsackievirus B3 is also able to bind to the Coxsackievirus and Adenovirus receptor (CAR) and, can attach to and infect non-permissive rodent cells transfected with this receptor (187). Interaction of CB3 with CAR, both in solution and at the cell surface, induces the formation of A-particles, whereas interaction with soluble or cell surface DAF does not (98, 205). This would explain why attachment to cell surface CAR, but not DAF, leads to infection of the host cell, since A-particle formation is thought to be required for genome release. It is likely that the same is true for the other viruses which bind but do not infect DAF-transfected cells. Echovirus 7 for example has been shown to form A-particles upon binding susceptible cells transfected with DAF, but not soluble DAF. This is consistent with A-particle formation being mediated by a second receptor (260). Interaction with DAF is not a dead end pathway for CB3 however, since non-permissive CHO cells transfected with CAR and DAF are more susceptible to infection than those transfected with DAF alone (205). This indicates that the virus may be concentrated at the cell surface by an initial attachment to DAF, before passing to a second receptor (CAR), which initiates cell entry and the conformational changes required for infection.

The human rhinoviruses can be divided into two groups based upon receptor usage. Major group rhinoviruses (e.g. HRV-14, HRV-3) utilise ICAM-1, whereas minor group rhinoviruses (e.g. HRV-2) utilise receptors of the low density lipoprotein receptor family. All rhinoviruses are thought to form A-particles prior to uncoating
and release of the viral RNA genome into the cytoplasm. For the minor group rhinoviruses, A-particle formation does not appear to be receptor-dependent, but instead is triggered on exposure to low pH (37). The conditions required for A-particle formation amongst the major group rhinoviruses varies from serotype to serotype (130). Human rhinovirus 14 for example will readily form A-particles at neutral pH when incubated with soluble ICAM-1, whereas complexes of human rhinovirus 16 and soluble ICAM-1 are relatively stable at pH 7 (130). It should be noted however that in all major group serotypes optimal A-particle formation and uncoating requires a mildly acidic pH in addition to receptor binding (231).

As described above, rhinoviruses and poliovirus form A-particles upon binding their cell surface receptor, upon exposure to low pH, or both. There is no evidence however for A-particle formation by FMDV, and indeed such a conformational change is unlikely to be required by FMDV, given that the virion dissociates into pentamers, VP4 and viral RNA at pH values just below neutrality. Unlike rhinoviruses and polioviruses, FMDV has been shown to be capable of using a range of receptors including an artificial receptor consisting of a single chain antibody fused to ICAM-1, and when pre-coated with antibody, the Fc receptor. It is therefore clear that uncoating of FMDV is not receptor dependent, but instead is triggered at a post-attachment stage.

1.6.4 Particle Uptake

All picornaviruses must deliver their genomic RNA across a cellular membrane to the cytosol of the host cell. The membrane across which the viral RNA passes may be either the plasma membrane or an internal organelle membrane, and differs between picornaviruses. Following attachment to the host cell, picornavirus virions must be
delivered to a compartment in the host cell in which the prevailing conditions are suitable to trigger A-particle formation, particle uncoating and delivery of the viral RNA to the cytoplasm. The endocytosis pathways exploited by viruses to achieve this are described in section 1.5

1.6.4.1 Uptake of Picornaviruses

Infection of the host cell by the minor group of human rhinoviruses (e.g. HRV-2) is mediated by the low density lipoprotein receptor (LDLR) and certain other members of the LDLR family (126, 184). This family of receptors are delivered to early and late endosomes by clathrin-dependent endocytosis, a process mediated by a conserved NPXY internalisation motif within the receptor cytoplasmic domain (49). The low pH within intracellular endosomes (pH <6) causes the release of the natural ligand from the receptor, the ligand being directed to lysosomes for degradation (276), and the receptor being recycled back to the cell surface. HRV-2 is also thought to be taken up by clathrin dependent endocytosis, since virus internalisation is inhibited by overexpression of dominant negative versions of AP180 and amphiphysin (296), both of which block internalisation of ligands by clathrin-dependent endocytosis. In addition, HRV-2 has been shown to colocalise with clathrin at the cell surface by immunofluorescence, consistent with uptake via clathrin-coated pits (296).

Formation of A-particles, and subsequent uncoating and RNA delivery, by minor group rhinoviruses is not dependent on receptor binding but is instead triggered by pH values below 5.6 (37). Consistent with this, infection by this virus is blocked by agents which dissipate the prevailing acidic pH within endosomes, such as bafilomycin A1 (21, 181, 231, 261), ammonium chloride (181), and the ionophore monensin (181). Human rhinovirus-2 is released from its receptor at mildly acidic pH
in early endosomes (37), before being delivered to late endosomes via endosomal
carrier vesicles where the more acidic pH triggers uncoating (21, 261).

Another picornavirus thought to enter cells via clathrin-dependent endocytosis is
human parechovirus-1 (151). Infection of susceptible cells by this virus is inhibited
by chlorpromazine, a drug which inhibits clathrin-dependent endocytosis. Also, viral
protein was shown to colocalize first with markers of early endosomes, and
subsequently with markers of late endosomes, two compartments associated with the
clathrin-dependent endocytosis pathway. However, in contrast to HRV-2, it is likely
that infection occurs from within early endosomes, since infection is not inhibited by
nocodazole, a drug which inhibits transport from early to late endosomes.

Infection of the host cell by major group rhinoviruses (e.g. HRV-3, -14, and -16) is
mediated by intracellular adhesion molecule 1 (ICAM-1) (101). HRV-14 has been
detected within endosomes inside cells (290), and infection by this virus has been
reported to be dependent on both dynamin (64) and a low pH step (231, 253). Since
dynamin is required for both clathrin and caveolae-dependent endocytosis, this
implies that uncoating of this virus (and subsequent RNA delivery) does not occur at
the cell surface, but instead requires delivery to an acidic endosomal compartment.
However, unlike the minor group rhinovirus receptors, ICAM-1 does not contain any
known endocytosis motifs within its cytoplasmic domain. In addition, neither the
cytoplasmic domain nor the transmembrane domain of ICAM-1 are required for
infection of Hela cells by HRV-14, since a genetically engineered receptor, consisting
of the ectodomain of ICAM-1 fused to a GPI anchor, is just as effective at mediating
infection as the wild type receptor (302). This would either imply that infection
occurs from the plasma membrane, or that internalisation of the receptor:virus
complex is triggered by receptor clustering or natural membrane turnover. Some
investigators report that HRV-14 infection can still occur in the presence of bafilomycin, indicating that low pH is not essential for infection by minor group rhinoviruses (20, 290) but instead merely reduces yield and delays host cell protein shutoff, consistent with the ability of this virus to uncoat upon binding soluble ICAM-1 at neutral pH (130). However, this could be explained by the observation that although uncoating of some minor group serotypes does occur at neutral pH, the maximum efficiency of uncoating requires both receptor binding and exposure to mildly acidic pH (231). The available data indicates that HRV-14 may infect cells by more than one route, namely direct penetration through the plasma membrane, and following uptake into acidic endosomes.

The entry pathway utilised by poliovirus is not well defined. Infectious intact virus particles have been detected within clathrin-coated vesicles within 10 minutes of adsorption to HeLa cells, and can be recovered from endosomal structures up to 30 minutes after adsorption (333). This data would suggest that infection of HeLa cells by poliovirus is dependent on the clathrin-dependent delivery to acidic endosomes. However, later studies showed that clathrin-dependent endocytosis (and also caveolae-dependent endocytosis) may not be essential for infection of this cell line by poliovirus, since expression of dominant-negative dynamin does not abolish infection (64). This would suggest that poliovirus can deliver its genomic RNA across the plasma membrane to the host cell cytoplasm, meaning that only the genomic RNA enters the host cell. It should be noted however that expression of dominant negative dynamin can lead to the upregulation of other (non-dynamin dependent) endocytosis pathways (61). In addition, both A-particle formation and infection of cells by poliovirus do not appear to require a low pH step since these processes are not inhibited by agents which raise endosomal pH (96, 103, 253). This would also
suggest that (in HeLa cells at least) delivery to acidic endosomes is not required for poliovirus infection, or at least that acidic pH within this compartment is not the trigger for uncoating.

More recent studies using transgenic mice expressing the poliovirus receptor, have shown that intact poliovirus can be incorporated into intracellular vesicles at neuromuscular junctions following intramuscular inoculation, a process dependent on the poliovirus receptor (234). Vesicles containing poliovirus and the poliovirus receptor have also been detected within neuronal tissue culture cells, and are transported in a retrograde direction along axons in a microtubule dependent manner, due to interactions between the cytoplasmic domain of the poliovirus receptor and dynein (234). Replication of poliovirus then occurs in the neural cell body. These observations suggest that infection of neuronal cells by poliovirus is dependent on delivery of intact virions to intracellular endosomes by clathrin-dependent endocytosis. There is also evidence for direct delivery of poliovirus RNA across the limiting membranes of neurones in vivo, since naked poliovirus replicon RNA has been shown to be delivered from neuromuscular junctions to the neural cell body (140). It seems likely that poliovirus can infect tissue culture and neuronal cells by multiple mechanisms, including direct delivery of the genomic RNA across the plasma membrane, or following clathrin-dependent delivery to endosomes.

The viruses described above either infect cells by direct penetration of the plasma membrane, or following clathrin-dependent delivery to intracellular endosomes. Infection by several other picornaviruses requires lipid raft dependent endocytosis pathways, the best studied of which is the caveolae-dependent pathway (see section 1.5.2.1). Echovirus-1 and its receptor, the integrin α2β1, have been localised to caveolae and infection is inhibited by methyl-β-cyclodextrin (MβCD) and a
dominant-negative version of caveolin, both of which inhibit caveolae formation and hence caveolae-dependent endocytosis (183). Echovirus-11 and its receptor DAF are found in lipid rafts, and infection and virus internalisation are inhibited by pretreatment of the cells with the raft disrupting reagent, nystatin, indicating that infection by this virus requires uptake via a lipid raft dependent endocytosis pathway (304). Similarly, infection by two other picornaviruses, coxsackievirus B4 and coxsackievirus A9 have been reported to require intact lipid rafts for infection (317, 318).

1.6.4.2 Uptake of FMDV

The pathways utilised by FMDV to enter tissue culture cells are not well defined, but the available evidence would suggest that FMDV is delivered to endosomes by receptor-mediated endocytosis. A number of factors would suggest this. One is the extreme acid-lability of the viral particle. The proteinaceous capsid dissociates into its constituent pentamers, releasing VP4 and viral RNA, at pH values below neutrality (43, 56). This suggests that viral uncoating would readily be triggered following uptake into mildly acidic early endosomes via receptor mediated endocytosis. A structural model which helps to explain how acid-induced disassembly of viral capsids occurs has been developed, and is based on the protonation of histidine residues found at inter-pentamer boundaries. (see section 1.6.5.2). Delivery to endosomes may be mediated by the virus' integrin receptor, since the cytoplasmic domain of the β6 integrin subunit is required for postattachment events in the αvβ6-mediated infection of cells by FMDV (142, 204).

Further evidence for virus entry through endosomes comes from the use of chemical compounds that raise endosomal pH. Both the ionophore monensin (which raises pH by exchanging protons for Na^+), and concanamycin A (a specific inhibitor of the
vacuolar ATPase), inhibit infection but not binding of FMDV to cells. These experiments imply that endosomal acidification is required for post attachment steps in infection of cells by FMDV. However, these experiments were carried out using a high multiplicity of infecting virus per cell which can cause infection to proceed via endocytic pathways that are not normally used by the virus (304). In addition, these studies did not clearly establish whether the inhibitory effect of these reagents on infection resulted directly from raising the endosomal pH or non-specific effects of these reagents.

1.6.5 Particle Uncoating and RNA delivery

1.6.5.1 Uncoating of Picornaviruses

Poliovirus undergoes a major structural rearrangement upon binding its receptor, to form the 135S A-particle, a transition that is thought to be an essential precursor to virus uncoating. This conformational change does not appear to be dependent upon exposure to low pH. A substantial portion of A-particles formed are eluted from the cell surface upon incubation at 37°C, however this particle is also the major form of the virus within infected cells at early times of infection and can be chased into the 80S empty capsid. The available evidence suggests that A-particles are an important intermediate in cell entry, and are converted to 80S empty capsids upon delivery of the viral RNA to the cytoplasm. However, the precise role of A-particles has remained an area of great debate.

Poliovirus A-particles differ from the native virion due to the externalisation of VP4, and the N-terminus of VP1 (86), and no longer bind to any form of the poliovirus receptor (175). In addition, unlike native virions, poliovirus A-particles
possess the ability to bind directly to lipid membranes, a property ascribed to the N-terminus of VP1, which is thought to form an amphipathic α-helix (86). A-particles have been shown to be able to infect tissue culture cells in a receptor-independent fashion (57, 133). Although the level of infectivity is low in comparison to that of native poliovirus virions, infectivity can be improved by 2-3 logs if A-particles are concentrated at the surface of cells expressing the Fc receptor by coating the altered virus particles with a monoclonal antibody (133). This indicates that A-particles display low infectivity because they do not interact with the poliovirus receptor and so do not become concentrated at the cell membrane, since infectivity is enhanced when they are artificially concentrated at the cell surface.

Addition of both native 160S poliovirus virions, and 135S A-particles to an artificial lipid bilayer results in the formation of cation specific, pH independent ion channels, albeit that the two types of particle form ion channels with differing properties (312). However, in artificial membranes containing the poliovirus receptor, addition of native virions, but not A-particles, resulted in the formation of ion channels, presumably since A-particles do not bind to the poliovirus receptor (313). The properties of the ion channels formed in lipid bilayers containing the poliovirus receptor differ from those formed in the absence of the poliovirus receptor, and are consistent with the binding to the membrane of a complex of the poliovirus and its receptor (313). The biggest difference is that the conductance of the channels is temperature dependent in the presence of the poliovirus receptor. Raising the temperature from 21°C to 31°C results in a large increase in the conductance of the ion channels, and the activation energy for this change is in the same order of magnitude as that calculated for the formation of 135S particles from 160S native virions (313). It has therefore been suggested that the conformational change
observed represents the transition from a complex in which only binding of virus occurs (a complex of 160S poliovirus, its receptor and the membrane) to one at which infection can start (a complex of 135S poliovirus, its receptor and the membrane) (313). Given that the formation of A-particles from native virions is receptor dependent, this theory is consistent with the observation that 135S particles are infectious in a receptor-independent fashion, whereas native poliovirus virions are not.

It is thought that the ion channels formed by poliovirus are formed by sequences from the N-terminus of VP1 and also VP4. The N-terminus of VP1 has been proposed to be involved in channel formation since these regions are thought to mediate the attachment of A-particles to membranes and can also be modelled as membrane interacting amphipathic helices (86). VP4 is thought to be directly involved in channel formation, and remains present in the plasma membrane following elution of A-particles from the surface of the cell (63). Mutants of threonine 28 of VP4 have been shown either to abolish ion channel formation or to result in the formation of ion channels with altered electrical characteristics, suggesting that VP4 is a key player in channel formation (63).

The ion channels formed by poliovirus upon membrane binding are thought to serve to bring about the delivery of the viral genomic RNA to the host cell cytoplasm. Viruses in which threonine-28 is replaced by glycine assemble correctly and form A-particles, but do not form ion channels in artificial membranes, and are also unable to transfer their genomic RNA to the host cell cytoplasm (63). It is therefore likely that the defect in cell entry previously identified for this mutant virus (214) lies at the stage of uncoating and/or RNA delivery. Mutations within the N-terminus of VP1
have also been shown to slow RNA delivery (157), providing further evidence for the role of poliovirus-induced channels in this process.

The precise role of the ion channels induced by poliovirus in RNA delivery is unclear but two models for how this could occur have been proposed (22, 266): 1) The viral RNA is extruded from the viral capsid to the host cell cytoplasm via the channel, or 2) The virion is taken up into endosomes and channel formation changes the ionic permeability of the endosomal membrane resulting in endosome lysis. Further work is required to elucidate which of these models is correct.

In contrast to poliovirus, the mechanism by which the minor receptor group rhinovirus HRV-2 releases its genome into the cytoplasm has begun to be revealed. As outlined in sections 1.6.3 and 1.6.4.1, HRV-2 is taken up by clathrin-dependent endocytosis and delivered to early endosomes. In early endosomes HRV-2 is released from its receptor and delivered to late endosomes, where the acidic pH (<5.6) triggers A-particle formation. When virus internalisation is carried out for 2 hours at 20°C (conditions where virus particles accumulate in late endosomes) the particles found in purified endosomes were either C-antigenic (A particles lacking VP4; 40%) or B particles (virions lacking VP4 and RNA; 60%) (261). In the presence of bafilomycin the particles isolated were exclusively native virions, indicating that uncoating was pH dependent. The above data suggests that HRV-2 undergoes low pH mediated conformational changes within late endosomes leading to particle uncoating and the delivery of the genomic viral RNA (but not its protein coat) to the cytosol across the endosomal membrane, via a virally induced membrane pore. This model is supported by work which has shown using in vitro (262) and in vivo (38) experimental systems, that uptake of HRV-2 into endosomes causes the release of co-internalised 10kDa dextran, but not co-internalised 70kDa dextran, from this compartment. This process
is dependent on the low pH within endosomes (38), and suggests that the virus forms a size restricted pore in the endosomal membrane which permits the release of molecules of sufficiently small size (including the viral RNA in extended form) from the endosomal lumen. The virus does not induce endosomal lysis since this would result in the release of both 10kDa and 70 kDa dextran, as observed with adenovirus (a virus known to induce endosomal lysis).

The nature and composition of the pore formed by HRV-2 is unclear. Uncoating of HRV-2 results in the externalisation of the N-terminus of VP1 at the 3-fold symmetry axis, the loss of VP4 from the capsid interior, and the formation of a channel of around 10Å by an iris like movement of the 5 copies of VP1 at the 5-fold symmetry axis (122). The channel at the 5-fold symmetry axis is large enough to accommodate an extended molecule of single stranded RNA and so the viral RNA is presumed to exit the particle by this route. It is not known whether the pore formed by HRV-2 within the endosomal membrane is contiguous with the viral capsid or is formed by VP4 alone following its release from the virion after A-particle formation. It is worth noting that the major group rhinovirus HRV-14 is thought to escape from endosomes by lysis of the endosomal membrane rather than by pore formation. This is because HRV-14 particles accumulate in endosomes under conditions which prevent uncoating, but not under conditions under which uncoating can occur (290). In addition infection with HRV-14, unlike infection with HRV-2, causes a reduction in the number of dextran labelled endosomes (290).

1.6.5.2 Uncoating of FMDV

Unlike other picornaviruses, there is no evidence for A-particle formation by FMDV. Instead, FMDV uncoats when exposed to pH values below neutrality, resulting in the dissociation of the proteinaceous capsid into its constituent pentamers.
and the release of VP4 and the viral RNA (43, 56). Given the extreme acid lability of the FMDV capsid, it is thought that uncoating is likely to be triggered by the mildly acidic pH found within early endosomes.

A structural model which helps to explain how acid-induced disassembly of viral capsids occurs has been developed (2, 56, 78). The 2-fold interfaces between pentamers contain a high proportion of histidine residues, which have a pKa of 6.8 in solution, which is the pH at which the FMDV particle disassembles. It was thus postulated that at pH values below neutrality, the imidazole side chains become protonated, and the resultant repulsion leads to uncoating (2). It is now believed that His-142 of VP3 is particularly important for this process, since it lies at the end of the positive pole of an α-helical dipole formed by residues 89-98 of VP2 (56). Each two-fold symmetry axis contains two such interfaces. When protonated, the histidine residue would repel the α-helix, resulting in disassembly. This model is supported by mutational data, which shows that if this residue (His-142) is mutated to aspartate (which is negatively charged), acid sensitivity of the capsid is markedly reduced, whereas mutation to arginine (positively charged) leads to a great reduction in capsid assembly (78).

Following uncoating of the FMDV virion, the viral RNA must be transferred to the cytoplasm of the host cell. The maturation cleavage of VP0 is reported to be essential for this process, since mutant virions in which VP0 cleavage does not occur bind cells normally, have normal acid sensitivity, but are non-infectious (160). These particles only differ from the wild type virus in that the acid breakdown products are considerably more hydrophobic than wild type, presumably due to the failure to release myristoylated VP4. It remains unclear however as to whether VP4 itself actually plays a role in RNA release into the cytoplasm, or whether its release allows
the capsid pentamers to facilitate this process. It is also unclear whether the viral RNA is transferred to the cytoplasm via a virus-induced pore in the endosomal membrane, as is the case for HRV-2 (38, 262), following virus induced lysis of the endosomal membrane, as reported for HRV-14 (290), or by another undefined mechanism.

1.7 Research Aims

The major objectives of the work presented in this thesis are to gain a better understanding of the entry pathways utilised by FMDV to enter and infect cells, and the role of the virus' integrin receptor in post-attachment events. Entry of FMDV was studied using immunofluorescence microscopy and inhibitors of specific endocytosis pathways (or steps within those pathways) were utilised.

The virus used to carry out these studies is FMDV strain O1Kcad2. Unlike some tissue culture adapted strains this virus does not bind heparin sulphate and is reliant on RGD-dependent integrins as its sole receptor family.

The cell lines used to study FMDV entry and infection in these studies are SW480 and Chinese hamster ovary-B2 (CHO-B2) cells transfected with the human β6 integrin subunit (SW480-β6 and CHO-β6 cells respectively). SW480 cells are a human colon carcinoma cell line which normally expresses αvβ5 and α5β1 as its only RGD-dependent integrins. The only RGD-dependent integrin expressed by CHO-B2 cells is αvβ5. Untransfected SW480 and CHO-B2 cells are non-permissive for infection by field strains of FMDV, but this is not as a result of a defect in intracellular virus replication since both cell lines are efficiently infected by the tissue culture adapted virus O1BFS, which uses heparan sulphate as its receptor. Both
CHO-B2 and SW480 cells become susceptible to infection by field strains of FMDV upon transfection with the human integrin β6 subunit and the consequent expression of the integrin αvβ6 at the cell surface. The integrin αvβ6 has been shown to act as the primary attachment receptor for field strains of FMDV on both SW480-β6 and CHO-β6 cells (145, 147).
Chapter Two: Materials and Methods

2.1 Cell Culture

The human colon carcinoma cell line SW480 transfected with full-length human β6 integrin subunit cDNA (SW480-β6) or a mutant form of the β6 subunit containing two mutations within the cytoplasmic domain (SW480-APLA) was grown in Dulbecco’s modified Eagle’s medium (DMEM-Sigma) supplemented with 10% foetal calf serum (FCS-Sigma), 20 mM glutamine, penicillin (100 SI units/ml), and streptomycin (100 μg/ml). The transfected cells were grown in the presence of Geneticin (1 mg/ml, Life Technologies) to maintain β6 expression. CHO cells transfected with the human β6 integrin subunit (CHO-β6) were cultivated using the same medium. Mock- and β6-transfected mouse embryonic fibroblasts (MEF’s) were grown in DMEM supplemented with 10% FCS, 20 mM glutamine, penicillin (100 SI units/ml), streptomycin (100 μg/ml), and Puromycin (4 μg/ml).

Baby hamster kidney (BHK) cells were grown in DMEM supplemented with 10% FCS, 20 mM glutamine, streptomycin (100 μg/ml), and penicillin (100 SI units/ml).

Primary bovine thyroid cells (BTY’s) were grown in Glasgow’s Modified Eagle’s medium (GMEM) supplemented with 10% adult bovine serum, streptomycin (100 μg/ml), and penicillin (100 SI units/ml).

2.1.1 Cell Passage

Confluent cell monolayers were washed twice with 0.25% trypsin-versene, and incubated at 37°C in the presence of trypsin-versene for 5-10 minutes. Cells in suspension were pelleted by centrifugation in a Sorvall Legend RT centrifuge for 3
minutes at 1000rpm. Cell pellets were resuspended in culture medium and seeded at an appropriate dilution in a fresh tissue culture flask.

2.2 Virus

Virus stocks were stored at -80°C. All experiments utilise the non-heparin binding FMDV strain O1Kcad2. Seed and working stocks were prepared using primary bovine thyroid cells (BTY's). Virus utilised for immunofluorescence and FACS studies was purified on sucrose gradients as described previously (56).

2.2.1 Preparation of viral working stocks

Monolayers of primary bovine thyroid cells (prepared in 175cm² tissue culture flasks) were washed with PBS, and infected with an aliquot of seed stock diluted in PBS at 37°C in a humidified 5% CO₂ incubator. After 15 minutes, ~20 ml of prewarmed viral growth medium (cell culture media containing 1% FCS) was added, and the flask incubated at 37°C, until full cytopathic effect was observed. The flask was then freeze-thawed to increase virus yield, and cell debris removed by centrifugation at 1000rpm for 3 minutes. The supernatant was aliquoted into screw capped 1.5ml eppendorf tubes and stored at -80°C.

2.2.2 Virus titration by plaque assay

Working stocks of virus prepared as above were titrated on BHK cells. In order to show that the virus stocks still utilised integrin receptors rather than heperan sulphate proteoglycans, they were also titrated on CHO cells. FMDV does not normally infect wild type CHO cells unless it has become adapted to use heperan sulphate as a
receptor. Duplicate BHK cell monolayers on 60mm tissue culture dishes were incubated with 10-fold dilutions of virus working stock in PBS for 15 minutes at 37°C in a humidified 5% CO₂ incubator. The cells were then overlaid with 4ml of Eagle's overlay (Eagle's medium supplemented with 0.6% indubiose, 5% tryptone phosphate broth, 1% foetal calf serum, 100 SI Units of penicillin/ml, and 100μg of streptomycin/ml) prewarmed to 42°C. After the overlay had set, the dishes were incubated for 48 hours at 37°C. Plaques were visualised by fixing and staining the cell monolayer with a solution of methylene blue and 4% paraformaldehyde in PBS, for at least 4 hours.

2.3 Antibodies, peptides, and other reagents.

The RGD peptide with its sequence derived from the GH loop of VP1 of type O FMDV (VPNLRGDLQVLAQKVAR) and the control RGB peptide were synthesized at the peptide synthesis facility at the Institute for Animal Health, Compton, United Kingdom.

The anti-FMDV monoclonal antibodies B2 (mouse IgG1) and D9 (mouse IgG2A), which recognize antigenic site 1 of type O FMDV (196) were purified using protein A (Pierce) according to the manufacturer's instructions. The anti-integrin antibodies used in these studies were 10D5 (mouse IgG2a; anti-αvβ6), E7P6 (mouse IgG1; anti-αvβ6) and P1F6 (anti-αvβ5) from Chemicon, 6.3G6 and 6.8G9 (both mouse IgG1; anti-αvβ6) from Biogen, and a rabbit monoclonal antibody, 4B5 (anti-β6) (131), obtained from Dean Sheppard (University of California).

In the course of these studies a number of antibodies directed against markers of cellular compartments were utilised. Early endosomes were detected using a monoclonal antibody (clone 14) which recognises early endosomal antigen-1 (mouse
IgG1; BD Transduction Laboratories). The transferrin receptor was detected using the monoclonal antibody H68.4 (mouse IgG1; Zymed Laboratories). Human LAMP-2 was detected using monoclonal antibody H4B4 whereas the hamster version was detected using the monoclonal antibody UH3 (both mouse IgG1; both obtained from the Developmental Studies Hybridoma Bank). Caveolin-1 was detected using a rabbit polyclonal antiserum (N-20) directed against the cytoplasmic domain of the human protein (Santa Cruz Biotech). All Alexa-fluor conjugated secondary antibodies were also obtained from Molecular Probes. Alexa fluor-488 and -568 conjugated transferrin, Alexa fluor-488 conjugated cholera toxin B (CTB), and acridine orange were also obtained from Molecular Probes.

Filipin (stock 5mg/ml in DMSO), nystatin (stock 100mg/ml in DMSO), cytochalasin D (stock 20mM in DMSO), nocodazole (stock 40mM in DMSO), wortmannin (stock 2mM in DMSO) and methyl-β-cyclodextrin (stock 100mM in DMEM) were all obtained from Sigma. Concanamycin A (stock 10mg/ml in DMSO) was obtained from Fluka.

### 2.4 Flow Cytometric Analysis

Virus binding and αvβ6 expression on SW480-β6 cells and MEF cell lines were determined by flow cytometry. Cells were harvested using a non-enzymatic cell dissociation solution (Sigma), and resuspended at $5 \times 10^6$ cells/ml in FACS buffer (20mM Tris, 1mM CaCl$_2$, 0.5mM MgCl$_2$, 1% Normal Goat Serum, 2% Bovine Serum Albumin, in 150 mM NaCl). 30μl of cells were placed in to a number of round bottomed wells of a 96-well plate. Cells were incubated sequentially with FMDV O1kcd2 (10μg/ml) where appropriate, primary antibodies (10μg/ml), and R-phycoerythrin conjugated anti-mouse isotype specific secondary antibodies (Southern
Biotechnology Associates) for 30 minutes on ice. Between each step cells were washed once with FACS buffer. Virus was detected with MAb B2 (10μg/ml), and αvβ6 with MAb 10D5 (10μg/ml). Finally, the cells were washed twice with FACS buffer and resuspended in PBS (pH 7.5)-2mM CaCl$_2$-1mM MgCl$_2$, containing 1% paraformaldehyde. The samples were then analysed by flow cytometry using a FACSCalibur (Becton Dickinson) instrument, with 6000 cells being counted per sample. Background fluorescence was measured by omitting the primary antibody or, in the case of virus binding, the virus itself.

### 2.5 Virus Infectivity Assays

FMDV infection was quantified using either an infectious centre assay, an elispot assay, or using immunofluorescence microscopy. Each assay is outlined below.

#### 2.5.1 Infectious Centre Assay

Mouse Embryonic Fibroblasts transfected to express αvβ6 were harvested using trypsin versene, collected by centrifugation (1000rpm 3mins), resuspended in cell culture medium, and incubated for 1 hour at 37°C with continuous rotation. One million cells were infected with FMDV type O1Kcad2 (m.o.i = 0.25) in a 200μl volume for 1 hour at 37°C with continuous rotation. Virus which had not been internalised was inactivated by the addition of 1.3 ml of 1M citric acid buffer (pH5.2) for 2 mins. The cells were neutralised by washing with PBS (pH 7.5) containing 2mM CaCl$_2$ and 1mM MgCl$_2$, and resuspended in 300μl of this buffer supplemented with 0.5% FCS. 10-fold dilutions of infected cells were made using the same buffer. 100μl of each dilution was mixed with 1ml of molten Eagle’s overlay and layered
over subconfluent BHK cells in 60mm dishes. Following solidification of the medium a further 3ml of overlay was laid over the top. Following incubation for 48 hours at 37°C and 5% CO₂, infectious centres were visualised as plaques by staining with a solution of methylene blue and 4% paraformaldehyde in PBS, for at least 4 hours. To verify the inactivation of cell surface virus by the acid wash step, control samples were incubated with virus in parallel at 4°C (to prevent virus internalisation). The number of infectious centres obtained for this control was <10 per million cells.

2.5.2 Elispot Infection Assay

2.5.2.1 Standard Assay

Cells were prepared in 96-well tissue culture plates and grown overnight at 37°C until approximately 70-80% confluent. The cells were washed once with DMEM and incubated with FMDV strain O1Kcad2 (m.o.i. ~0.3 pfu/cell) for either 0.5 or 1h at 37°C (see figure legends for details). The monolayers were washed once with DMEM and incubated with DMEM at 37°C for 4-4.5 hours before being fixed with 4% paraformaldehyde in PBS for 1 hour. The cells were washed five times with PBS after this and each subsequent step, and then permeabilised with 0.1% Triton X-100 in PBS for 15mins. After an incubation for 0.5 hours in blocking buffer (10mM Tris-HCl pH 7.5; 140mM NaCl; 1mM CaCl₂; 0.5mM MgCl₂; 10% normal goat serum; 1% fish gelatin), the cells were incubated with MAb 2C2 (Mouse IgG2a) diluted in block buffer (1/1000) for 1h at room temperature (RT). The cells were then incubated sequentially with a biotinylated, goat anti-mouse IgG secondary antibody (1/500-Southern Biotechnologies) and streptavidin-conjugated alkaline phosphatase (1/1000-Caltag Laboratories) prepared in block buffer, for 1h each at RT. 200μl of the alkaline phosphatase substrate (BIO-RAD) was added to each well and colour development
monitored using a light microscope. The cells were then washed four times with distilled water and allowed to air dry. The infected cells appear dark-blue and were counted using an ELispot plate reader (Zeiss). Non-specific labelling was determined by either omitting Mab 2C2 or performing the assay on mock-infected cells. The extent of non-specific labelling was similar for either condition. Where appropriate (see chapter 3), cells were treated with chemical inhibitors of endocytosis before, during, and after incubation with virus (see below).

2.5.2.2 Hypertonic Sucrose Treatment

The effect of hypertonic sucrose on infection of cells by FMDV was assayed as follows. Cells in triplicate wells were washed once with DMEM, before being treated with 0.1-0.4M sucrose (or mock treated with DMEM alone) for 15 or 30 minutes at 37°C. Following sucrose pre-treatment, cells were incubated with FMDV O1Kcad2 in DMEM (m.o.i ~ 0.3) for 30 or 60 minutes at 37°C in the presence or absence of sucrose (see chapter 3). Cells were then incubated with DMEM in the absence of sucrose for 4.5 or 4 hours, and fixed and infection quantified using the elispot assay as described in section 2.5.2.1. In the replication controls (marked RC in the relevant figures) cells were incubated with sucrose after the removal of the virus inoculum.

2.5.2.3 Nocodazole, Wortmannin, Filipin and Nystatin Treatment

The effect of nocodazole, wortmannin, filipin, and nystatin on infection of cells by FMDV was assayed as follows. Cells in triplicate wells were washed once with DMEM, before being incubated with nocodazole, wortmannin, filipin, or nystatin for 30 minutes at 37°C (see chapter 3). Since the stock solutions of each of these drugs were made up using DMSO, mock treated samples were treated with an equivalent
dilution of DMSO. Following drug treatment, cells were incubated with FMDV O1Kcad2 in DMEM (m.o.i ~ 0.3) for 60 minutes at 37°C in the presence of drug. Cells were then washed once with DMEM, and incubated with DMEM in the presence of drug for a further 4 hours at 37°C, before being fixed and infection quantified using the elispot assay as described in section 2.5.2.1. In the replication controls cells were not pre-treated with drug. Instead the drug was added to the cells after the removal of the virus inoculum.

2.5.2.4 Methyl-β-Cyclodextrin Treatment

The effect of methyl-β-cyclodextrin on infection of cells by FMDV was assayed as follows. Cells in triplicate wells were washed once with DMEM, and incubated with methyl-β-cyclodextrin in DMEM for 30 minutes. Mock treated samples were treated with DMEM alone. The cells were then incubated with FMDV O1Kcad2 (m.o.i ~ 0.3) for 60 minutes at 37°C in the presence or absence (mock-treated samples) of drug. Cells were then washed once with DMEM, incubated with DMEM in the absence of drug for a further 4 hours at 37°C, before being fixed and processed as described in section 2.5.2.1.

2.5.2.5 Concanamycin A Treatment

The effect of Concanamycin A on infection of cells by FMDV was assayed as follows. Cells in triplicate wells were washed once with DMEM, before being incubated with Concanamycin A for 30 minutes at 37°C. Since the stock solution of concanamycin A was prepared using DMSO, mock treated samples were treated with equivalent dilutions of DMSO. Following pre-treatment with concanamycin A, cells were incubated with FMDV O1Kcad2 in DMEM (m.o.i ~ 0.3) for 60 minutes at 37°C.
in the presence of drug. Cells were then washed once with DMEM, incubated with DMEM in the absence of drug for a further 4 hours at 37°C, before being fixed and processed as described in section 2.5.2.1. In the replication controls, drug was added after the removal of the virus inoculum.

2.5.3 Immunofluorescence Infection Assay

2.5.3.1 Standard Assay

Cells grown on 13mm glass coverslips were washed twice with DMEM and then infected at an m.o.i of ~0.5 with FMDV O1Kcad2 for five hours at 37°C. Cells were then fixed using 4% paraformaldehyde in PBS for 1 hour. Infected cells were identified with a rabbit polyclonal serum to FMDV and an Alexa-568 conjugated goat anti-rabbit IgG secondary antibody, by immunofluorescence microscopy (see section 2.6).

2.5.3.2 Dominant Negative AP180

SW480-B6 cells were prepared on glass coverslips and transiently transfected using lipofectamine 2000 (Invitrogen) with a plasmid containing the coding sequence for the C-terminal fragment (residues 530-915) of AP180 (AP180C) fused to a c-myc tag. The protocol for transfection is as described in section 2.7.11. At 7.5 hours post-transfection, the cells were infected at an m.o.i ~0.5 with FMDV O1Kcad2 for 5 hours at 37°C (chapter 3). The cells were fixed and processed for the confocal microscope as described in section 2.6.1. Cells transfected with AP180C were detected using the anti-c-myc tag antibody (9E10) and Alexa-488 conjugated goat anti-mouse IgG secondary antibody. Infected cells were identified with a rabbit polyclonal serum to
FMDV and an Alexa-568 conjugated goat anti-rabbit IgG. The proportion of non-transfected and infected cells that were also infected was then calculated.

2.6 Immunofluorescence Microscopy

2.6.1 Detection of intracellular antigen

Cells were grown on 13mm glass coverslips (BDH) to 40-60% confluence. The cells were washed twice in PBS, and the cells fixed for 40 minutes using 4% paraformaldehyde in PBS. Following two washes with Tris-buffered saline (TBS), cells were permeabilised for 15 minutes using 0.1% Triton X-100 or 0.5% saponin in block buffer (TBS, 1mM CaCl$_2$, 0.5mM MgCl$_2$, 0.1% Sodium Azide, 1% Fish gelatin (Sigma), and 10% normal Goat Serum (Sigma)). Following permeabilisation, cells were washed using TBS and non-specific binding sites were blocked for a further 30 minutes using block buffer. The cells were incubated with primary antibodies diluted as appropriate in block buffer for 60 minutes, with unbound antibody being removed at the end of this period by three five minute TBS washes. When saponin was used to permeabilise the cells, 0.1% saponin was included in this and all of the subsequent steps. Alexa fluor 488nm (green labelling) or 568nm (red labelling) conjugated secondary antibodies (Molecular Probes) were diluted 1:200 in block buffer, and incubated with the cells for a further 45 minutes, before being washed with TBS as before. Finally the coverslips were washed with distilled water and mounted in Vectastain mounting medium containing DAPI to label the nucleus, and sealed in place using nail varnish. For double labelling experiments the specificity of the secondary conjugated-antibodies was confirmed by showing a lack
of cross-reactivity of the anti-mouse-IgG1 secondary antibody for mouse IgG2a and vice versa.

2.6.2 Detection of surface antigen

To detect antigen present at the cell surface, the above procedure was modified. Cells, prepared on coverslips as above, were cooled on ice, and non-specific binding sites blocked by incubation with ice cold block buffer for 30 minutes on ice. Where appropriate ligands (e.g. virus, cholera toxin b subunit) were bound to cells on ice for the times indicated (see results chapters). The cells were then incubated sequentially with primary and secondary antibodies diluted in cold block buffer on ice for 60 and 45 minutes respectively. The cells were then fixed with cold 4% paraformaldehyde for 40 minutes on ice, and washed, mounted and sealed as above.

2.6.2.1 Lipid raft labelling using Cholera Toxin B

Cholera Toxin B binds GM1 ganglioside which is enriched in lipid rafts. Cells grown on coverslips were incubated with Alexa-488 conjugated cholera toxin B (0.2μg/ml) in cold DMEM for 45 minutes at 4°C. Excess unbound CTB was removed by three washes of five minutes each with cold DMEM. Surface bound CTB was cross-linked by incubation for 60 minutes at 4°C with a rabbit anti-cholera toxin antiserum (Sigma-diluted 1 in 125 in DMEM). Unbound antibody was removed by washing with cold DMEM as before. Coverslips were washed and mounted as described in section 2.6.1.
2.6.3 Ligand Internalisation

2.6.3.1 Virus binding and internalisation

Cells were incubated with FMDV (5μg/ml) at 4°C in DMEM for 45 mins. Excess unbound virus was removed by three washes of five minutes each with cold DMEM. For co-localization experiments with surface antigens, the cells were processed as described in section 2.6.2 above. Where virus internalisation was required, cells were washed twice with prewarmed DMEM and incubated at 37°C for the times indicated on the figures. Internalisation was halted by fixation with cold 4% paraformaldehyde for 40 minutes on ice. Coverslips were then processed as described in section 2.6.1. Virus was detected using Mab D9 (5μg/ml) and a goat anti-mouse IgG2a Alexa-conjugated antibody. In the experiment shown in fig 3.3, the pre-binding step at 4°C was omitted and the virus was added directly to the cells at 37°C.

2.6.3.2 Transferrin binding and internalisation

Cells were washed four times with serum-free DMEM, and incubated for 45 minutes at 37°C. This incubation in the absence of serum serves to deplete the cells of iron thus enhancing subsequent transferrin uptake. Alexa-488 or -568 conjugated transferrin (Molecular Probes-10μg/ml) in cold DMEM was bound to the cells for 45 min at 4°C. Excess unbound transferrin was removed by three washes of five minutes each with cold DMEM. In order to initiate internalisation of bound transferrin, cells were washed twice with prewarmed DMEM and incubated at 37°C for the times indicated on the figures. After this time internalisation was halted by fixation with a cold 4% paraformaldehyde/0.2% glutaraldehyde solution for 40 minutes on ice. Coverslips were then processed as described in section 2.6.1. Where transferrin uptake was used as a control for the effect of inhibitors of endocytosis on
the clathrin-dependent pathway, the pre-binding step was omitted and Alexa-conjugated transferrin was added directly to the cells at 37°C.

2.6.3.3 Cholera Toxin B binding and internalisation

Cells were incubated with alexa-488 conjugated cholera toxin B subunit (Molecular Probes – 0.2µg/ml) at 4°C in DMEM for 45mins. Excess unbound CTB was removed by three washes of five minutes each with cold DMEM. For co-localization experiments with surface antigens, the cells were processed as described in section 2.6.2 above. Where ligand internalisation was required, cells were washed twice with prewarmed DMEM and incubated at 37°C for the times indicated on the figures. Internalisation was halted by fixation with cold 4% paraformaldehyde for 40 minutes on ice. Coverslips were then processed as described in section 2.6.1. Where CTB uptake was used as a control for the effect of inhibitors of endocytosis on lipid raft dependent endocytosis pathways, the pre-binding step was omitted and Alexa-488 conjugated CTB was added directly to the cells at 37°C.

2.6.4 Acridine orange labelling of acidic vesicles

Cells on coverslips were treated with concanamycin-A (100nM) in DMEM for 0.5 hours at 37°C. Mock-cells were treated with an equivalent dilution of DMSO. The cells were then incubated with acridine orange (0.5 µg/ml in DMEM) in the presence or absence (Mock) of the drug for a further 15 min. The cells were washed in the cold with PBS and mounted and sealed as described in section 2.6.1 except that PBS was used in place of Vectashield. The cells were visualized immediately on the confocal
microscope ($\lambda_{ex} = 488$) collecting fluorescent emission in the green (490-550) and red (590-640) regions of the spectrum.

2.6.5 Image collection

All immunofluorescence images were collected using the 63x objective lens of a Leica SP2 confocal microscope. Each image represents a single confocal section through the middle of the cell(s) in question. In order to remove crosstalk between detection channels, data were collected sequentially. Images were processed using Adobe Photoshop software.

2.7 DNA Techniques

2.7.1 Restriction Endonuclease Digests

Digests were carried out in a total volume of 20µl according to the instructions supplied with each enzyme. All enzymes were supplied by Promega, except SunI which was from New England Biolabs.

2.7.2 Polymerase Chain Reaction

PCR was performed in a volume of 50µl. The reaction was constituted as follows:

- Template DNA
- 200 pmol Primer 1 and Primer 2 (MWG or Sigma)
- dNTP mix (Promega - Final concentration = 800 µM)
- 10x enzyme buffer
sterile filtered deionised water to 50μl
5 units Taq or Pfu polymerase (Roche and Promega respectively)

The template DNA utilised was either plasmid DNA, or in the case of overlap PCR, two overlapping PCR products. The reaction was overlaid with sterile mineral oil and incubated in a thermal cycler according to the following protocol:

Step 1  94°C  2 min
Step 2  94°C  1 min
Step 3  50°C or 60°C  1 min
Step 4  72°C  1 min
Step 5  cycle to step 4  30 Times
Step 6  72°C  10 min

PCR products were purified by agarose gel electrophoresis before use in ligation reactions or further rounds of PCR. Pfu polymerase was used when the PCR products were to be used as templates for further PCR, and Taq polymerase was used when the PCR product needed to be cloned into a vector. PCR products amplified using Taq polymerase were ligated without further modification into pGEMT Easy (Promega).

2.7.3 Agarose Electrophoresis of DNA

DNA was subjected to electrophoresis using gels of between 0.8 and 1.5% agarose dissolved in TAE buffer (0.04M Tris-Acetate, 0.001M EDTA). Ethidium bromide (Promega) was added to the gel mix before it set at a concentration of 0.5μg/ml. Gels were normally run at a constant voltage of 100V.
2.7.4 Purification of DNA Fragments

The desired ethidium bromide stained DNA bands were excised from agarose gels using a scalpel, having been visualised on a UV lightbox (Biorad). The DNA was purified using the QIAEXII kit from QIAGEN, as per the manufacturers instructions. The agarose was first solubilised in buffer QX1, and the DNA bound to QXII beads. The beads were then washed using an ethanol containing buffer, and the bound DNA eluted from the beads in 20μl tris buffer (10mM Tris-Cl pH 8.5).

2.7.5 Shrimp Alkaline Phosphatase Treatment

Vector DNA was dephosphorylated using shrimp alkaline phosphatase (Roche). This treatment improves the efficiency of ligation by preventing vector resealing. The reaction was constituted as follows:

7μl Linearised and gel extracted vector
0.9μl 10x reaction buffer
1μl Shrimp Alkaline Phosphatase

This reaction was incubated for 30 minutes at 37°C. The phosphatase enzyme was then heat inactivated by treatment at 65°C for 15 minutes.

2.7.6 Ligations

Ligation Reactions were performed using T4 DNA Ligase (Promega) and the supplied 10x buffer according to the manufacturers instructions. All ligations were performed
using a vector:insert ratio of 1:3 in a total volume of 10µl. Ligation mixes were incubated overnight at 4°C, and were transformed into *E.coli* DH5α or JM109 cells.

2.7.7 Preparation of Competent Cells

Competent *E.coli* DH5α and JM109 cells were prepared in bulk and frozen at -70°C as 100µl aliquots. The method used to prepare the cells is outlined below:

1. Single colonies of either *E.coli* strain were grown overnight at 37°C in 5ml of LB broth supplemented with 20mM MgSO₄ (LB-Mg).
2. 1ml of the overnight culture was added to 10ml of LB-Mg
3. 10ml cultures were grown until an OD₆₀₀ of 0.2 to 0.8 was reached.
4. The above 10ml cultures were transferred to a 1 litre flask, containing 50ml of LB-Mg, and the cultures incubated at 37°C until an OD₆₀₀ of 0.5 to 0.9 was reached.
5. At this point 200ml of LB-MG were added, and the culture was incubated at 37°C until an OD₆₀₀ of 0.6 was reached.
6. The cells were cooled on ice, before being centrifuged at 4200 rpm for 15 minutes at 4°C.
7. The pellet was resuspended in 50ml Fresh TFB1 (30mM Potassium Acetate, 50mM MnCl₂, 100mM KCl, 10mM CaCl₂, and 15% Glycerol) and incubated on ice for 5 minutes.
8. The cells were recentrifuged at 4200 rpm for 15 minutes at 4°C.
9. The pellet was resuspended in 10ml ice cold TFB2 (10mM MOPS, 75mM CaCl₂, 10mM KCl, and 15% Glycerol).
10. Cells were divided into 100μl aliquots, snap frozen on dry-ice and stored at -70°C until required.

2.7.8 Transformation of Competent Cells

Frozen competent cells were thawed for 5 minutes on ice. An aliquot of DNA (e.g. 5μl of a ligation reaction) was added to 50μl of competent cells. The mixture was incubated on ice for 30 minutes. The samples were heat shocked for 50 seconds at 42°C, and incubated for 2 minutes on ice. 1ml of LB was added and the culture incubated for 60 minutes at 37°C. The cultures were then centrifuged at 1000 rpm for 1 minute, and resuspended in 150μl of LB. The transformed bacteria were then plated onto LB/agar plates containing a suitable antibiotic (e.g. Ampicillin (100μg/ml)). Where the vector used was suitable for blue-white screening the LB/agar plates were also supplemented with 0.1mM IPTG, X-Gal (40μg/ml) and Ampicillin (100μg/ml). The plates were incubated overnight at 37°C.

2.7.9 Isolation of Plasmid DNA

2.7.9.1 Small scale plasmid purification (mini-preps)

5ml of LB broth was inoculated with a single bacterial colony with a sterile toothpick and incubated overnight at 37°C with shaking. 1.5ml of the culture was transferred to a fresh eppendorf tube and centrifuged at 14000rpm for 1 minute. The supernatant was removed and the DNA extracted using the Qiagen miniprep system as per the manufacturers instructions. DNA was routinely analysed on a 1% agarose gel.
2.7.9.2 *Large scale plasmid purification (Maxi-preps)*

5 ml of LB broth was inoculated with a single bacterial colony and grown up as if for a miniprep as described above. 200 µl of this culture was diluted into 100 ml of LB, and grown up overnight. The DNA was extracted using the Qiagen maxiprep system as per the manufacturers instructions, and analysed on a 1% agarose gel.

2.7.10 *Spectrophotometric Determination of DNA Concentration*

For quantitation of the concentration of DNA in a given sample, optical density readings were taken at 260 and 280 nm using an Ultrospec 2100 pro UV/visible spectrophotometer (Amersham Biosciences). Typically maxi-prep DNA was diluted 1/100 in distilled water before these readings were taken using 1 ml capacity quartz cuvettes with a 1 cm path length. Distilled water was used as a reference.

an OD$_{260}$ of 1 = A double stranded DNA concentration of 50 µg/ml (50ng/µl)

The OD$_{260}$/OD$_{280}$ ratio gives an estimate of the purity of the DNA sample. A pure DNA preparation has an OD$_{260}$/OD$_{280}$ ration of approximately 1.8.

2.7.11 *Transient Transfection of Mammalian Cells with plasmid DNA*

The cells to be transfected (SW480-B6 cells) were grown overnight on glass coverslips in the absence of Geneticin and Penicillin/Streptomycin. 1.5 µg of the plasmid DNA to be transfected and 8 µl of the transfection reagent lipofectamine 2000 (Invitrogen) were made up to 400 µl with OPTI-MEM (Gibco). This solution was
mixed by vortexing for 1 second and incubated for 45 minutes at room temperature.
After 45 minutes the transfection mixture was made up to a volume of 2 ml using
OPTI-MEM. The coverslips were washed twice with OPTI-MEM, and the
transfection mixture was divided equally between 2 coverslips (0.75 μg of DNA per
coverslip). The coverslips were then incubated with the transfection medium for 4
hours at 37°C. After this time the coverslips were washed twice with cell culture
medium lacking Geneticin, and incubated at 37°C for sufficient time for the protein
encoded by the plasmid DNA to be expressed to an appropriate level (see results
chapters). After this time period (typically 8 hours), the cells were fixed with 4%
paraformaldehyde for 40 minutes and processed for immunofluorescence microscopy.
Where appropriate cells were infected with FMDV for 5 hours prior to
paraformaldehyde fixation (chapter 3).

2.7.12 DNA Clean Up

The DNA to be treated was made up to 250 μl with Tris buffer (10 mM Tris-Cl pH 8.5).
An equal volume (250 μl) of Phenyl:Chloroform:Isoamyl alcohol (Life Technologies)
was added to the DNA, and mixed by shaking. The organic and aqueous phases were
separated by centrifugation for 2 minutes at 11000 rpm. The majority of the upper
aqueous phase (~220 μl) was removed and placed in a fresh tube, an equal volume of
chloroform was added, and the contents mixed by shaking. The phases were
separated by centrifugation as above, and the upper aqueous layer removed and
placed in a fresh tube.

In order to precipitate the DNA, 2.5 volumes of 100% ethanol and 0.1 volumes of
3 M sodium acetate (Sigma; pH 5.2) were added, and the mixture incubated at -20°C
for 3 hours. The precipitated DNA was pelleted by centrifugation at 13000 rpm for 10 minutes. The supernatant was removed and the pellet washed using 70% ethanol, and the mixture recentrifuged as above. The pellet was allowed to air dry and resuspended in RNase free water.

2.7.13 DNA Sequencing

Sequencing was performed using the CEQ2000 capillary sequencing system (Beckman-Coulter). Sequencing reactions were performed using the CEQ2000 dye terminator cycle sequencing quick start kit, as outlined in the manufacturers’ instructions. This system utilises four colour dideoxy-terminator cycle sequencing, using an unlabelled sequencing primer. The sequencing reactions were ethanol precipitated, resuspended in 40μl of sample loading solution, and loaded on to the 96-well sequencing plate. Samples were then run overnight. Sequence data was transferred to the UNIX GCG software package for manipulation.

2.8 RNA Techniques

2.8.1 In Vitro Transcription

Full length RNA genome copies were transcribed from both wild-type and recombinant versions of the infectious copy plasmid pT7S3 using the megascript kit (AMBION). Template DNA for this reaction was first phenol:chloroform extracted, and ethanol precipitated, before being linearised using the restriction endonuclease HpaI. Following proteinase K treatment, the linearised templates were run on a 1% agarose gel, and gel extracted. Each reaction was constructed as follows:
2µl ATP Solution
2µl CTP Solution
2µl GTP Solution
2µl GTP Solution
2µl 10x Reaction Buffer
1µg Template DNA
2µl T7 Polymerase Enzyme Mix
Make up to 20µl Nuclease free water

The reactions were constructed at room temperature, and then incubated for 4 hours at 37°C. 1µl DNaseI was then added, and the reaction mixture incubated for a further 15 minutes at 37°C.

2.8.2 RNA Purification

In vitro transcribed RNA was purified using the Megaclear kit (AMBION) as per the manufacturer's instructions. This kit uses a column based system to separate the RNA from the components of the in vitro transcription reaction.

2.8.3 RNA Transfection by Electroporation

BHK cells were grown to 90% confluency in a 175cm² tissue culture flask. The cells were removed using trypsin-verse, and pelleted by centrifugation (1000rpm for 3 minutes at 4°C) and resuspended in ice cold electroporation buffer (21mM Hepes pH 7, 137mM NaCl, 5mM KCl, 0.7mM Na₂HPO₄, and 6mM Glucose, in RNase free water). The cells were then counted using a haemocytometer and resuspended at 2
million cells per ml in ice cold electroporation buffer. 0.8ml of this cell suspension was transferred to a pre-chilled electroporation cuvette (BioRad) with a 0.4cm gap length and stored on ice. Viral RNA or RNA transcripts derived from the infectious copy plasmid (chapter 8) were added to the cells in the cuvette, mixed gently, and immediately electroporated at 750V with a capacitance of 25μFD. Each sample was pulsed twice, and a time constant of between 0.6 and 0.7 was typically recorded. All electroporations were carried out using a BioRad gene pulser. Following electroporation, the cuvette was allowed to stand at room temperature for 10 minutes, and the cells were then added to 6ml cell culture medium in a 25cm² tissue culture flask and incubated at 37°C and 5% CO₂.

2.8.4 Total RNA Extraction

CHO cells and SW480-β6 cells were grown to 100% confluency in 35mm tissue culture dishes. Total RNA was extracted using the trizol reagent (Life Technologies) according to the manufacturers protocol. The final RNA pellet was dissolved in 20ul of RNase free water in an RNase free tube (AMBION) and stored at -80°C.

2.8.5 Reverse transcription of total cellular RNA

5μl of total RNA isolated from CHO or SW480-β6 cells, 2μl of random hexanucleotide primers (Promega-50μg/μl) and 3μl of RNase-free water were added to an RNase-free tube and incubated at 70°C for 5 minutes in order to melt secondary structure and allow primer annealing. The tube was then cooled by incubation at room temperature for 10 minutes. To each reaction the following were added:
4μl first strand synthesis buffer
2μl Acetylated BSA (Promega-Final Conc 0.1mg/ml)
2μl DTT (Promega-Final Conc 10mM)
1μl dNTP Mix (Promega)
1μl Moloney Murine Leukemia Virus reverse transcriptase (Promega)

This reaction mixture was incubated for 5 minutes at room temperature, and then the first strand synthesis reaction was allowed to proceed for 60 minutes at 37°C. Finally the reverse transcriptase enzyme was denatured by incubation at 70°C for 15 minutes.

The cDNA produced from CHO and SW480-β6 cells was used as a template for the amplification of Caveolin-1, and as a control, β-Actin. Amplification was carried out using Taq polymerase in conjunction with the primers shown below.

Caveolin1 Amplification:
Cav1 for = 5' - atg tct ggg ggc aaa tac gta ga-3'
Cav1 rev = 5' - gta aat gcc cca gat gag tgc ca-3'

Actin Amplification
Actin for = 5' - gag aag ctg tgc tac gtc gc-3'
Actin rev = 5' - cca gac agc actgt tgg gc-3'

The PCR method used was as described in section 2.7.2.
Chapter Three: The Effect of Pharmacological Inhibitors of Endocytosis on FMDV Uptake and Infection

3.1 Introduction

The route by which foot-and-mouth disease virus (FMDV) enters and infects tissue culture cells remains poorly defined. Material, such as virus particles, can be taken up into cells via a variety of different endocytic uptake pathways, such as clathrin-dependent endocytosis and caveolae-dependent endocytosis (see section 1.5). There are a number of pharmacological inhibitors and cell treatments capable of inhibiting specific endocytic uptake pathways, and indeed specific steps in those pathways. The aim of the work presented in this chapter is to look at the effects of the inhibition of specific pathways by these treatments on αvβ6-mediated infection of SW480-β6 cells. This has enabled us to gain a better insight into the endocytic uptake pathways required for αvβ6-mediated infection of cells by FMDV.

A number of viruses have been shown to utilise the clathrin-dependent endocytosis pathway to enter and infect cells. Examples include: influenza virus (194), hantaan virus (150), semliki forest virus (72), human rhinovirus 2 (296), human parechovirus 1 (151), and vesicular stomatitis virus (195). There are a number of methods by which clathrin-mediated endocytosis can be inhibited. One such method is the treatment of cells with hypertonic media (121). Following incubation in hypertonic medium the geodesic lattices of clathrin present under each coated pit are replaced by accumulations of small empty clathrin “microcages” and cell surface receptors, such as the LDL receptors, lose their normal clustered distribution at the cell surface (121). Upon return of cells to isotonic medium normal clathrin lattices begin to reappear.
within 2 minutes and recover to normal levels within 10-15 minutes. This method has been found to inhibit clathrin-dependent endocytosis in virtually all cell types studied.

A second method used to inhibit this pathway is to express dominant negative versions of proteins involved in clathrin-coated pit formation and internalisation in the target cells. The cellular protein, AP180 was first purified and characterised from coated vesicles taken from bovine brain tissue (3). It was found to bind clathrin triskelia \textit{in vitro} and to promote the formation of arrays of clathrin similar to the surface coat of coated vesicles (3). Subsequently it was also found to bind directly to the $\alpha_{c}$-appendage domain of the adaptor protein AP-2 (113, 206, 316) to form a complex that co-operatively recruits clathrin (113). AP180 is thought to be tethered to the plasma membrane via a phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$) binding site located in the N-terminal region of the molecule (82). Overexpression of either full length or a C-terminal fragment of AP180 inhibits clathrin-dependent endocytosis by mislocalizing clathrin and preventing its incorporation into nascent coated pits (82, 342). Hence clathrin-mediated endocytosis can be inhibited by transient transfection of cells with the C-terminal portion (residues 530-915) of AP180 (AP180-C).

Both of the above techniques (treatment of cells with hypertonic sucrose and transient transfection with AP180-C) are utilised in experiments described in section 3.2.1 to look at the role of clathrin-dependent endocytosis in the entry and infection of SW480-β6 cells by FMDV.

Recently a number of viruses have been reported to utilise lipid raft dependent uptake mechanisms to enter and infect cells. Reported examples include echoviruses 1 (254) and 11 (304), coxsackievirus B4 (317) and SV40 (9, 251, 252). Lipid rafts are envisaged as highly ordered "islands" of saturated lipids and cholesterol. The
non-raft portions of the membrane consist primarily of a bilayer of unsaturated lipids, which is disordered and fluid, allowing lipid rafts to move laterally in the plane of the bilayer (242). The straight acyl chains of the saturated lipids present in lipid rafts allow close packing with cholesterol, creating ordered microdomains. The acyl chains of the unsaturated phospholipids are crooked and as a result cannot pack together so tightly, resulting in a more disorganised and fluid nature. Cholesterol is therefore essential to maintaining the integrity of lipid rafts.

A number of agents can be used to deplete the cell membrane of cholesterol, including Nystatin, Filipin, and Methyl-β-cyclodextrin (MβCD). The sensitivity of raft-dependent endocytic pathways to nonacute cholesterol depletion by such agents distinguishes them from the clathrin dependent and constitutive pinocytic uptake pathways. Methyl-β-cyclodextrin is a water soluble heptasaccharide, consisting of (α -1,4)-linked α-D-glucopyranose units. The molecule has a hydrophilic outer surface and a lipophilic central cavity, in which cholesterol molecules bind. Methyl-β-cyclodextrin is utilised in experiments described below, in order to establish the role (if any) of lipid raft dependent uptake pathways in αvβ6-dependent infection of SW480 cells.

Foot-and-mouth disease virions are thought to be locked into the metastable state (For a review see (127)) by the cleavage of VP0 to give VP2 and VP4 (the so called maturation cleavage). Virtually all known viruses are released from the metastable state by interaction with the virus (co)receptor, exposure to low pH, or a combination of both. This is thought to expose hydrophobic residues which can interact with biological membranes, facilitating the transfer of the viral genome to the appropriate compartment of the cell (the cytoplasm in the case of FMDV). Other Picornaviruses, such as Poliovirus, undergo a structural change upon interaction with their receptor, to
form the so-called "Altered-" or A-particle (see section 1.6.3). FMDV has never been shown to form A-particles, or indeed to undergo any structural changes upon interaction with its integrin receptors. Interaction with its receptor is therefore unlikely to be the trigger for release of FMDV virions from the metastable state.

FMDV capsids are however extremely acid labile dissociating into their constituent pentamers and viral RNA at pH values just below neutrality. A structural model has been developed for the acid-induced disruption of the FMDV capsid, involving the protonation of histidine residues (pKa ~ 6.5) at inter-pentamer interfaces (56, 78)(see section 1.6.5.2). This sensitivity to acid lead to speculation that infection of cells by FMDV requires a low pH step, in order to release the virus from the metastable state, and that entry proceeds through endosomes. The low pH within endosomes is maintained by the vacuolar H⁺-ATPase, by means of ATP-dependent proton translocation (a process termed "Active Endosomal Acidification"). Concanamycin A blocks the action of this enzyme, and in doing so dissipates the acidic pH within endosomal compartments.

Concanamycin A, and the related compound Bafilomycin A1, have been used to study the role of low pH in infection by a number of viruses. Examples include enveloped viruses such as semliki forest virus (138), influenza virus (108-110), and non-enveloped viruses such as human rhinoviruses-2 (21) and -14 (253) (HRV-2 and HRV-14), poliovirus (253), and encephalomyocarditis virus (253). If the entry of FMDV does require a low pH step this compound would be expected to inhibit infection of susceptible cells by this virus. Concanamycin A is used as a tool to investigate whether cell entry by FMDV requires a low pH step in the experiments described below.
In non-polarized cells, internalized receptors and their ligands generally take one of two paths following uptake into early endosomes. Firstly membrane receptors and/or their ligands can be delivered to late endosomes and ultimately lysosomes, resulting in their degradation. Alternatively, the membrane rich tubular portions of the early endosome deliver material to the pericentriolar recycling endosome compartments, and thence back to the cell surface. In certain cell types, a pathway capable of delivering some receptors/ligands (e.g. the transferrin receptor/transferrin) directly back to the surface from early endosomes, without passage through pericentriolar recycling endosomes, has also been identified. The above pathways are affected differently by pharmacological reagents such as nocodazole and wortmannin, allowing the role (if any) of these pathways in entry of FMDV into susceptible cells to be investigated.

Transport of material from early to late endosomes has been reported to be dependent on microtubules in unpolarized BHK cells (107) and polarized MDCK cells (36) since transport between these two compartments is inhibited by the microtubule depolymerising drug Nocodazole. Recycling of receptors or their ligands back to the cell surface however is thought to be microtubule independent, since these pathways are not inhibited by treatment with Nocodazole(198, 308).

Depolymerisation of microtubules also interferes with caveolae-dependent endocytosis since treatment with Nocodazole causes the accumulation of Caveolin-1 and invaginated caveolae at the surface of CHO cells(216), indicating that internalisation of these structures is dependent on the microtubule network. However this remains controversial since delivery of SV40 to caveosomes via caveolae in CV-1 cells was not affected by the action of Nocodazole(251)
The phosphatidylinositol-3'-kinase (PI3-kinase) inhibitor wortmannin has been widely used to look at the role of these enzymes in vesicular trafficking. The initial stages of fluid phase uptake have been reported to be sensitive to wortmannin (284). In contrast, the available data suggests that wortmannin has only minimal effects on clathrin-dependent delivery of ligands, such as transferrin, to early endosomes (172, 188, 284, 298). However, the transport of proteins such as internalized platelet derived growth factor receptors and Semliki Forest Virus beyond early endosomes to late endosomes and ultimately lysosomes has been reported to be wortmannin sensitive (152, 188). Wortmannin also inhibits the recycling of transferrin from early endosomes back to the cell surface (188, 293, 298), and is thought to achieve this by reducing both the rate of influx and efflux from pericentriolar recycling endosomes.

It is worth noting however that some studies report that wortmannin has no impact on transferrin recycling (284), so this effect may be cell type dependent. To summarise the available data, wortmannin has no effect on clathrin-dependent delivery to early endosomes, but does interfere with transport beyond early endosomes, to either late endosomes/lysosomes or to recycling endosomes. Nocodazole and Wortmannin are useful tools to study the role of transport beyond early endosomes in infection, whether that be via late endosomes or recycling endosomes.

It is known that the cytoskeleton, including the microtubule network and actin filaments, plays a role in endocytic uptake pathways at distinct stages. The role of the microtubule network is discussed briefly above. The actin cytoskeleton has been shown to be required for caveolae-dependent uptake of ligands, including SV40 (252) and clustered alkaline phosphatase (241). Lipid raft dependent uptake in caveolin-1 deficient cells has very similar properties to that seen in caveolin-1 expressing cells (79, 114), and so is also dependent on the actin cytoskeleton. Macropinocytic uptake
pathways are also dependent on rearrangements of the actin cytoskeleton in a number of cell types. The precise role of actin in clathrin-dependent endocytosis is less clear since the sensitivity of clathrin-dependent endocytosis to microfilament disrupting drugs seems to vary according to cell type and whether the cells are grown in suspension culture or on a solid substrate (87). This indicates that actin plays a variable and non-obligatory role in clathrin-dependent endocytosis in mammalian cells. The fungal metabolite Cytochalasin D has been shown to disrupt the actin cytoskeleton by binding to the barbed growing end of microfilaments at which net polymerisation occurs, thereby preventing the addition of actin monomers (39, 55). This agent is thus a useful tool for examining the role of the actin cytoskeleton in entry and infection of cells by FMDV.

Described above are a number of pharmacological reagents and cell treatments which inhibit different endocytic pathways at distinct stages. The work described below is an investigation of the effects these reagents and treatments have on αvβ6 mediated entry and infection of SW480-β6 cells. In the majority of cases infection is quantified using the ELIspot assay (see methods section 2.5.2). In this assay SW480-β6 cells in a 96-well plate are incubated with FMDV (O1Kcad2; m.o.i ~0.3) for up to 1 hour at 37°C. The cells are then washed and incubated in culture medium for a further 4 hours, fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton-X-100. The infected cells are incubated sequentially with Mab 2C2 (which recognises the viral protein 3A, a marker for virus replication), a biotinylated goat anti-mouse IgG secondary antibody and streptavidin-conjugated alkaline phosphatase. Following incubation with enzyme substrate the infected cells appear dark-blue in colour and can be counted using an ELIspot plate reader. Examples of a mock infected cell monolayer (panel A) and an infected cell monolayer (panel B) subjected
to this assay are shown in figure 3.1. Infection is assayed at a low m.o.i (-0.3), as it has been hypothesised that vast excesses of virus may cause virions to be taken up by pathways which would not be utilised under normal physiological conditions (304).
Figure 3.1. Quantification of infection using the ELISpot assay. SW480-avp6 cells in a 96-well plate were incubated with FMDV (O1Kcap2; m.o.i ~0.3) for 1h at 37°C. The cells were washed and incubated in culture medium for a further 4h, fixed with 4% paraformaldehyde and permeabilised with 0.1% Triton-X-100. The infected cells were incubated sequentially using Mab 2C2 (which recognises the viral protein 3A, a marker for virus replication), a biotinylated goat anti-mouse IgG secondary antibody and streptavidin-conjugated alkaline phosphatase. In the presence of enzyme substrate the infected cells appear dark-blue and were counted using an ELISpot plate reader. Panel (A) shows the background labelling of a mock infected cell monolayer. Omission of the primary antibody from the assay (rather than virus) produced a similar level of background labelling. Panel (B) shows an infected cell monolayer.
3.2 Effect of Pharmacological Inhibitors of Endocytosis on FMDV Entry and Infection

3.2.1 Inhibitors of Clathrin-Dependent Endocytosis

3.2.1.1 Hypertonic Sucrose Treatment

Treatment of cells with hypertonic concentrations of sucrose has been shown to inhibit clathrin-dependent endocytosis (121, 129). We therefore decided to investigate the effect of this treatment on infection of SW480-β6 cells by FMDV strain O1Kcad2. Unlike tissue culture adapted strains O1Kcad2 utilises the integrin αvβ6 as its receptor on this cell line and not heparan sulphate proteoglycans (147).

Figure 3.2 shows the effect of sucrose treatment on infection. Infection was assessed using the elispot infection assay (see above). Cells were pretreated with a range of concentrations of sucrose (0.1-0.4M) for 30 minutes, prior to incubation with virus in the presence of sucrose for 1 hour. Virus and sucrose were then washed away and the assay completed. Pre-treatment with 0.1M and 0.2M sucrose had no effect on infection. In contrast, pre-treatment with hypertonic concentrations of sucrose (0.3M and 0.4M) virtually abolished infection, leading to greater than 95% inhibition. In order to determine the effect of these treatments on intracellular virus replication, sucrose was added for 1.5 hours following removal of the virus inoculum, rather than prior to the addition of virus. At this time it would be expected that virus had already entered the cell and intracellular virus replication initiated. On figure 3.2 this data is marked RC followed by the relevant concentration of sucrose. These data show the same pattern as for pre-treatment, i.e. infection is abolished by 0.3 and 0.4M, but not by 0.1 and 0.2M sucrose (Fig 3.2). From this experiment it is not clear at which stage...
infection is being inhibited. The possibilities are: a) uptake of virus is blocked so any impact of sucrose on replication is irrelevant, b) sucrose treatment is blocking infection at the stage of intracellular virus replication. It should be noted however that the replication controls are not exactly analogous to the data shown when cells are pretreated with sucrose. In the samples pretreated with sucrose, no sucrose is present during the “replication stage” of the assay, that is after the virus inoculum has been removed, and virus has entered the cells and is undergoing cytoplasmic replication. For the replication controls sucrose is present for 1.5 hours of the 4 hour “replication stage” of the assay. Since cells rapidly recover from treatment with hypertonic sucrose (within 30 minutes), it is by no means clear that replication is being inhibited at all in the pre-treated samples in the manner implied by the replication controls.

In order to establish the stage at which hypertonic sucrose treatment inhibits infection of SW480-ß6 cells by FMDV, the effect of this treatment on uptake of Alexa-fluor labelled Transferrin (a ligand taken up exclusively by clathrin mediated endocytosis) and virus was investigated by confocal microscopy. Cells were either mock treated or pretreated with 0.4M sucrose for 30 minutes, prior to incubation with ligand (either transferrin or purified FMDV) for 20 minutes at 37°C in the presence of sucrose where appropriate. Cells were then fixed, and where appropriate virus was labelled using the monoclonal antibody D9 and an Alexa-568 labelled goat anti-mouse IgG2a secondary antibody. Figure 3.3 shows the results of these experiments. Panels A and B show the uptake of Transferrin in both mock treated (panel A) and sucrose treated cells (panel B). It is clearly evident that hypertonic sucrose has blocked the uptake of Transferrin, confirming that this treatment does indeed block clathrin-dependent endocytosis. Transferrin is not seen at the cell surface in panel B
since the extensive washing steps performed in this assay result in the removal of transferrin remaining at the cell surface. Panels C and D show the uptake of virus in mock treated (panel C) and sucrose treated cells (Panel D). In mock treated cells virus was seen in vesicular structures in the cell cytoplasm, whereas in sucrose treated cells the vast majority of virus remained at the cell surface. This shows that sucrose treatment blocks the uptake of virus as well as transferrin, suggesting that virus is taken up by clathrin-dependent endocytosis. This also clarifies the data shown in figure 3.2 panel A. The block in infection shown here must be at the stage of virus entry, since pre-treatment with 0.4M sucrose prevents virus from entering the cell, making any impact on cytoplasmic replication less important.

The above evidence strongly suggests that pre-treatment of cells with hypertonic sucrose treatment inhibits infection by FMDV by preventing clathrin-dependent uptake of virus particles. We were interested to see however if the use of milder conditions for sucrose treatment could separate the effects on entry and cytoplasmic replication of FMDV. Figure 3.4 shows the effect on infection of two concentrations of sucrose (0.3M and 0.4M), using two different treatment protocols for each. The treatment protocols were as follows 1) 15 minutes pre-treatment with sucrose followed by a 30 minute incubation with virus in the absence of sucrose and 2) 15 minutes pre-treatment with sucrose followed by a 30 minute incubation with virus in the presence of sucrose. In both assays, virus and sucrose were then washed away and the assay completed. In order to look at the effect of these treatments on intracellular replication, sucrose (either 0.3 or 0.4M) was added for a period of 15 minutes (the control for treatment 1) or 45 minutes (the control for treatment 2) following the removal of the virus inoculum. On Fig 3.4 this data is marked RC followed by the concentration of sucrose used, with the assay conditions indicated.
below. Fig 3.4 shows that treatment of the cells with 0.4M sucrose for 15 minutes (treatment 1) inhibited infection by ~80% when sucrose was added prior to infection but only by ~30% when added after infection had been initiated (RC treatment 1). A further two experiments were then carried with 0.4M sucrose using treatment 1. The results of these experiments are shown in figure 3.5 panel A, and as an average of the three experiments in figure 3.5 panel B. These experiments confirm that 0.4M sucrose (using treatment1) inhibits infection between 68-80% (mean = 74%), whereas the equivalent replication control showed an inhibition of between 5 and 29% (mean = 16%). These data show that it is possible to separate the impact of sucrose on entry and replication, using milder conditions than those used in figure 3.2, albeit that the inhibition of infection is less dramatic.

Pre-treatment with hypertonic sucrose inhibits infection of SW480-β6 cells by FMDV, which indicates that entry of FMDV is clathrin-dependent. The fact that the same treatment regime inhibits uptake of transferrin (a marker for clathrin-dependent endocytosis) and virus by these cells, strongly supports this conclusion. When sucrose is added after the addition of virus rather than before (the replication controls), inhibition of infection is also seen. This indicates that sucrose treatment can inhibit intracellular replication of FMDV as well as entry of the virus. However, when milder treatment conditions are used inhibition of infection is much greater when sucrose is added prior to the virus inoculum rather than after it, suggesting that under these conditions the major inhibitory effect of sucrose is on the entry stage of infection. Taken together this evidence suggests that hypertonic sucrose treatment inhibits clathrin-dependent endocytosis, and in so doing prevents uptake of virus from the cell surface and hence infection of the target cells.
Figure 3.2. Hypertonic Sucrose Inhibits infection by FMDV
SW480-ovp6 cells were treated with sucrose for 0.5h prior to incubation (m.o.i ~0.3) with FMDV for 1h at 37°C in the presence of sucrose. Mock cells were treated with DMEM. Data points marked RC ("Replication Control") represent samples that were not pre-treated with sucrose but instead were treated with the indicated concentration of sucrose for 1.5h following removal of the virus inoculum. Following removal of virus, samples were incubated for a further 4h at 37°C, and then fixed with 4% paraformaldehyde. Infection was quantified as described in figure 3.1. The number of infected cells in the sucrose-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. The mean and ±SD of three observations is shown.
Figure 3.3. Sucrose inhibits uptake of both FMDV and transferrin. SW480-OV16 cells were treated with 0.4M sucrose (panels B and D) or Mock Treated (panels A and C) for 0.5h. The cells were then incubated with Alexa-488 conjugated transferrin (green - Panels A and B) or FMDV (red - Panels C and D) for 20min at 37°C in the presence (Panels B and D) or absence (Panels A and C) of sucrose. DAPI stained nuclei are shown in blue. Bars = 5μm

Sucrose treatment inhibited uptake of both transferrin (compare panel B with A) and FMDV (compare panel D with C).
Figure 3.4. Hypertonic Sucrose Inhibits infection by FMDV

SW480-cvJ8 cells were treated with sucrose for 15 minutes prior to incubation (m.o.i ~0.3) with FMDV for 0.5h at 37 °C in the absence (Treatment 1) or presence (Treatment 2) of sucrose as indicated. Mock cells were treated with DMEM. Data points marked RC ("Replication Control") represent samples treated with the indicated concentration of sucrose for 15 (Treatment 1) or 45 minutes (Treatment 2) following removal of the virus inoculum.

Following removal of virus, samples were incubated for a further 4.5h at 37°C, and then fixed with 4% paraformaldehyde. Infection was quantified as described in figure 3.1. The number of infected cells in the sucrose-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. The mean and ±SD of three observations is shown.
Figure 3.5. Hypertonic Sucrose Inhibits infection by FMDV
SW480-cov6 cells were treated with sucrose for 15 minutes prior to incubation (m.o.i ~0.3) with FMDV for 0.5h in the
absence of sucrose. Mock cells were treated with DMEM. Data points marked RC ("Replication Control") represent samples
treated with the indicated concentration of sucrose for 15 minutes following removal of the virus inoculum. Following
removal of virus, samples were incubated for a further 4.5h at 37°C, and then fixed with 4% paraformaldehyde. The
number of infected cells in the sucrose-treated samples was expressed as a percentage of the number of infected cells in
mock-treated samples.

Panel A. Shown are the results of three independent experiments. Experiment 1 is represented by unfilled bars,
experiment 2 by grey bars and experiment 3 by black bars. The mean and ±SD of three observations is shown.
Panel B. Shown is the mean and ±SD of the data shown in panel A.
3.2.1.2 Dominant Negative AP180C

In order to confirm the role of clathrin-dependent endocytosis in infection of SW480-β6 cells by FMDV, a more specific method of inhibiting this pathway was employed, namely transient transfection of dominant negative AP180C. Overexpression of either full length or a C-terminal fragment of AP180 inhibits clathrin-dependent endocytosis by mislocalizing clathrin and preventing its incorporation into nascent coated pits (82, 342).

SW480-β6 cells, prepared on coverslips, were transiently transfected with a construct containing the C-terminal portion (residues 530-915) of AP180 (AP180-C). At 12.5 hours post-transfection, the transfected cells were infected with FMDV strain O1Kcad2. Infection was allowed to proceed for 5 hours, before the cells were fixed and processed for immunofluorescence microscopy. The AP180C expressed in the cells was c-myc tagged and so was detected under the confocal microscope using the anti-c-myc antibody 9E10. Infected cells were identified using a rabbit polyclonal serum that recognises FMDV capsid proteins. Input virus was not detected since cells infected in the presence of guanidine hydrochloride (a reagent that inhibits FMDV replication) showed little or no fluorescent labelling (not shown). Figure 3.6 shows the results of these experiments. Panels A and B show representative regions where cells had been transfected with AP180C (shown as green) and subsequently infected with FMDV (shown as red), as viewed down the confocal microscope. None of the transfected cells shown were infected, and none of the infected cells are also transfected to express AP180C. On each coverslip the proportion of non-transfected cells which were infected, and the proportion of transfected cells infected was calculated by sampling a number of fields of view down the confocal microscope.
These data are shown in panel C. Approximately 19% of non-transfected cells were infected with FMDV (n=901), whereas only 1.6% of transfected cells were also infected. This means that expression of dominant negative AP180 C, which specifically inhibits clathrin-dependent endocytosis, inhibited infection by approximately 91%. This data, taken together with the sucrose data outlined in section 3.2.1.1, shows that clathrin-dependent endocytosis is required for entry and infection of SW480-β6 cells by FMDV.
Figure 3.6. Expression of AP180C inhibits infection by FMDV.

Panels A and B. SW480-uv36 cells were transfected to express c-myc tagged AP180C and subsequently infected with FMDV for 5 hours. Infected and/or transfected cells were detected by indirect immunofluorescence using the confocal microscope. Transfected cells (green) were detected using an anti-c-myc monoclonal antibody (9E7) and infected cells (red) with a rabbit polyclonal serum to FMDV. Nuclei were stained with DAPI and are shown as blue. As shown in these images, in general, transfected cells were not found to be infected, and infected cells were not found to be transfected. Bars = 10µm

Panel C. Pictures of random fields of view were taken using the 63x objective lens. In the fields of view sampled, the total number of transfected cells and the number of transfected and infected cells were counted, and used to work out the percentage of transfected cells that were also infected. Similarly, the total number of untransfected cells and the number of untransfected and infected cells were counted, and used to work out the proportion of untransfected cells that were infected. This data is presented in the table shown in panel C.

Expression of AP180C inhibited infection as the proportion of transfected cells that were infected was greatly reduced compared to the equivalent figure for untransfected cells.

<table>
<thead>
<tr>
<th></th>
<th>Untransfected Cells</th>
<th>AP180C Transfected Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Infected Cells</td>
<td>171</td>
<td>4</td>
</tr>
<tr>
<td>Total Number of Cells Counted</td>
<td>901</td>
<td>244</td>
</tr>
<tr>
<td>% of cells infected</td>
<td>19.0</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Red = Virus  Green = AP180C
3.2.2 Effect of Inhibition of Lipid Raft-dependent Endocytosis on FMDV Infection

3.2.2.1 Cholesterol Depletion by Methyl-β-Cyclodextrin.

As described in section 3.1, plasma membrane lipid rafts are rich in cholesterol and are disrupted by agents that deplete the membrane of cholesterol or sequester cholesterol within the membrane. Consequently, lipid raft-dependent endocytic pathways (including the caveolae dependent uptake pathway) are also sensitive to such agents. We therefore decided to use methyl-β-cyclodextrin, filipin, and nystatin, all of which are agents that bind plasma membrane cholesterol and inhibit lipid-raft dependent endocytosis, to investigate the role (if any) of lipid raft dependent uptake pathways in infection of SW480-β6 cells.

Figure 3.7 shows the effect of methyl-β-cyclodextrin on infection of SW480-β6 cells by FMDV. Infection was measured using the elispot infection assay (see figure 3.1). Cells were pretreated with methyl-β-cyclodextrin for 30 minutes prior to incubation with virus (m.o.i=0.3) for 30 minutes in the presence of drug. Both the virus and drug were then washed away, and the assay completed. The drug was not present throughout the assay because it proved toxic to the cells if present for this length of time. The concentrations of drug utilised (5 and 7.5mM) did not inhibit infection by FMDV, indicating that infection was not reliant on lipid raft dependent endocytic pathways.

Further experiments were carried out to confirm that methyl-β-cyclodextrin treatment disrupts lipid rafts and lipid raft dependent endocytosis at the concentrations utilised in figure 3.7. Lipid rafts were visualised at the cell surface using the cholera toxin B subunit (CTB). CTB binds the ganglioside GM1 which is located
predominantly within lipid rafts. Fluorescently conjugated CTB can therefore be used as a marker for lipid rafts at the cell surface. Raft resident molecules (including GM1) adopt a diffuse distribution at the cell surface rather than a punctate pattern when visualised by immunofluorescence methods (255). This is thought to be because of extensive coverage of the plasma membrane surface by lipid rafts and the fact that the diameter of lipid rafts is below the resolution limit of the light microscope, resulting in an apparently even distribution of raft localized proteins. Therefore, to aid raft identification the rafts were visualised as puncta by antibody cross-linking alexa-conjugated CTB at the cell surface. Janes et. al. have utilised this method of lipid raft visualisation previously and showed that CTB labelled membrane patches display characteristics consistent with biochemically isolated lipid rafts(149).

SW480-β6 cells were either treated with methyl-β-cyclodextrin or mock treated, and subsequently CTB was allowed to bind the cells for 45 minutes at 4°C and then crosslinked with a rabbit anti cholera toxin polyclonal serum. The resulting data is shown in figure 3.8. Panel A shows that lipid rafts visualised by crosslinked CTB have a punctate distribution at the surface of mock treated SW480-β6 cells. In contrast, panel B shows that a diffuse labelling pattern is observed at the surface of SW480-β6 cells pre-treated with 7.5 mM methyl-β-cyclodextrin. This indicates that treatment with methyl-β-cyclodextrin results in the disruption of lipid rafts, since the punctate structures corresponding to lipid rafts on the surface of mock treated cells are not observed in drug treated cells.

The impact of methyl-β-cyclodextrin treatment on lipid-raft dependent endocytosis pathways was investigated by examining the effect of the drug on the uptake of alexa-fluor labelled CTB by SW480-β6 cells using confocal microscopy. Since the cell surface receptor for CTB, GM1 ganglioside, is highly enriched in lipid rafts, CTB
predominantly enters cells by lipid raft dependent endocytosis in a number of different cell types (115, 211, 289, 311, 315). Methyl-β-cyclodextrin has also been reported to inhibit clathrin-dependent endocytosis under certain conditions (270, 305). In order to investigate this possibility, the effect of methyl-β-cyclodextrin on the uptake of Alexa-fluor labelled transferrin (a marker for clathrin-dependent endocytosis) was also examined. Figure 3.9 shows the results of these experiments.

Panels A and C show that Alexa-labelled CTB (green) and Alexa-labelled transferrin (red) are both taken up by mock-treated SW480-p6 cells following a 20 minute incubation at 37°C. Panel B shows that pre-treatment with 7.5mM methyl-β-cyclodextrin inhibits uptake of CTB, since the majority of ligand remained at the cell surface, indicating that lipid-raft dependent endocytosis had been inhibited. Panel D shows that methyl-β-cyclodextrin does not inhibit clathrin-dependent endocytosis since pre-treatment with this drug did not prevent the uptake of transferrin. In summary, treatment with methyl-β-cyclodextrin inhibits uptake of CTB (a marker of lipid raft-dependent endocytosis), but not transferrin (a marker of clathrin-dependent endocytosis). Since the same treatment did not inhibit infection of SW480-p6 cells by FMDV (see figure 3.6), this indicates that FMDV does not enter cells by lipid-raft dependent endocytosis.

In order to reinforce the data shown above, we also investigated the effect of nystatin and filipin on FMDV infection of SW480-p6 cells. Like methyl-β-cyclodextrin these agents bind cholesterol and inhibit lipid raft-dependent endocytosis, but they act via a different mechanism. Methyl-β-cyclodextrin selectively extracts cholesterol from cells but does not become incorporated into the plasma membrane (137, 235, 338). This results in depletion of cholesterol from the lipid bilayer. Nystatin and Filipin however become incorporated into lipid
membranes where they chelate cholesterol (91, 203, 237). This means that the cholesterol is still present in the membrane but is sequestered by the drug molecules such that it cannot form part of the structure of lipid rafts, resulting in the inhibition of lipid raft-dependent uptake mechanisms. Unlike methyl-β-cyclodextrin, these agents have not been reported to inhibit the clathrin-dependent endocytosis pathway (270).

Figure 3.10 shows two independent experiments investigating the effect of nystatin on infection of SW480-β6 cells by FMDV. The cells were pretreated with a range of nystatin concentrations (5.25-100μg/ml), and infection was assessed using the elispot infection assay. Samples were pretreated with Nystatin for 1 hour and the drug remained present throughout the assay. Nystatin treatment did not inhibit infection in comparison to mock treated samples at any of the concentrations tested. In comparison, infection by SV40 is inhibited by ~60% at 25μg/ml (9) and infection by Echovirus 11 is inhibited by ~60% at 23.2μg/ml (304). Both of these viruses utilise lipid raft dependent endocytic uptake mechanisms.

Figure 3.11 shows the effect of filipin on infection of SW480-β6 cells. The cells were pretreated with a range of concentrations of filipin (1.25-10μg/ml), and infection assessed by the elispot assay using the same experimental design as for Nystatin. Filipin treatment did not inhibit infection in comparison to mock treated samples at any of the concentrations tested. Together the experiments with nystatin and filipin support the methyl-β-cyclodextrin data and show that virus entry and infection of SW480-β6 cells by FMDV is not dependent on lipid raft dependent endocytic pathways.
Figure 3.7. MβCD does not inhibit FMDV infection.

SW480-cyβ6 cells were treated with MβCD in DMEM for 0.5h and infected with FMDV (m.o.i - 0.3) for 0.5h in the presence of the drug and infection quantified as described in figure 3.1. The mock cells were treated with DMEM. Following removal of virus, samples were incubated for a further 4.5h (Panel B) at 37°C, and then fixed with 4% paraformaldehyde. Infection was quantified as described in figure 3.1. The number of infected cells in the MβCD-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. Two independent experiments are shown, and each data point represents the mean and ±SD of three observations.
Figure 3.8. MβCD disrupts plasma membrane lipid rafts.
SW480-36 cells were pretreated with 7.5mM MβCD (Panel B) or mock treated with DMEM (Panel A) for 0.5h. Alexa-488 conjugated CTB (green) was allowed to bind to the cell surface for 45 mins on ice, and crosslinked using an anti cholera toxin polyclonal antibody for 1h on ice. The cells were fixed using paraformaldehyde and viewed under the confocal microscope. Nuclei are stained with DAPI and are shown as blue.
Figure 3.9. MjICD blocks uptake of cholera toxin B (CTB) but not Transferrin.
SW480-uviβ cells were treated with 7.5mM MjICD (panels B and D) or Mock Treated (panels A and C) for 0.5h. The cells were then incubated with Alexa-488 conjugated CTB (green – Panels A and B) or Alexa-568 transferrin (red – Panels C and D) for 20min at 37°C in the presence (Panels B and D) or absence (Panels A and C) of MjICD. DAPI stained nuclei are shown in blue. Bars = 10μm
MjICD treatment inhibited uptake of CTB (compare panel B with A) but not Transferrin (compare panel D with C).
Figure 3.10. Nystatin does not inhibit FMDV infection.

SW480-c3B6 cells were treated with Nystatin for 1h and infected with FMDV (m.o.i = 0.3) for 1h in the presence of the drug. The mock cells were treated with DMSO. Following removal of virus, samples were incubated for a further 4h at 37°C in the presence of drug, and then fixed with 4% paraformaldehyde. Data points marked RC represent samples treated with 100µg/ml Nystatin for 4h following removal of the virus inoculum. Infection was quantified as described in figure 3.1. The number of infected cells in the Nystatin-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. Two independent experiments are shown, and each data point represents the mean and ±SD of three observations.
Figure 3.11. Filipin does not inhibit FMDV infection.

SW480-cvβ6 cells were treated with Filipin for 1 h and infected with FMDV (m.o.i ~0.3) for 1 h in the presence of the drug. The mock cells were treated with DMSO. Following removal of virus, samples were incubated for a further 4 h at 37°C in the presence of drug, and then fixed with 4% paraformaldehyde. Data points marked RC represent samples treated with 10μg/ml Filipin for 4 h following removal of the virus inoculum. Infection was quantified as described in figure 3.1. The number of infected cells in the Filipin-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. The mean and ±SD of three observations is shown.
3.2.3 Inhibition of Endosomal Acidification

The experiments described above strongly suggest that FMDV utilises the clathrin-dependent endocytosis pathway but not lipid-raft dependent endocytosis pathways to enter and infect cells. Having been taken up by clathrin-dependent endocytosis, ligands such as transferrin are delivered to early endosomes (sometimes termed "sorting" endosomes pH~6.0). We next sought to establish whether FMDV requires the low pH within endosomes in order to successfully infect SW480-β6 cells. As described in the introduction (section 3.1), because of the extreme acid lability of the FMDV capsid it has long been thought that FMDV may require such a low pH step during entry in order to infect cells successfully. This theory has been in part tested previously using the ionophore monensin (18) and the macrolide antibiotic Concanamycin A (204). Concanamycin A is a potent and highly specific inhibitor of the vacuolar H+-ATPase, and hence raises the pH within endosomal compartments, including early endosomes. However, the previous experiments (18, 204) were carried out at a high m.o.i, which can cause infection to proceed via endocytic pathways not usually utilised by the virus (304). In addition, these studies did not establish whether the inhibitory effects of these reagents could be attributed to a rise in the pH within these compartments, or to non-specific effects of the reagents. We therefore decided to investigate the effect of concanamycin A on FMDV infection more rigorously.

To investigate whether FMDV infection requires the low pH within endosomes, we investigated the effect of Concanamycin A on infection of SW480-β6 cells by FMDV strain O1Kcad2. Cells were pre-treated with Concanamycin A for 0.5h prior to the 1h incubation with virus in the presence of drug (see methods). Infection was
determined using the ELIspot infection assay at low m.o.i (~0.3). Figure 3.12 shows the results of three independent experiments. FMDV infection was inhibited by Concanamycin A in a dose-dependent manner in all experiments. At 1μm and 100nM Concanamycin A drastically reduced infection in all three experiments. At these concentrations, infection was inhibited by between 83 and 97% relative to mock treated cells. The effect of 10nM concanamycin A was variable between experiments ranging from a 5-75% inhibition. This suggests that a concentration of 10nM may be close to the Kᵢ for the drug under the conditions used in the assay, which would mean that slight variations in the conditions due to experimental error could make a large difference to the level of inhibition observed. When 1μM Concanamycin A (the highest concentration of drug used) was added for a period of 1.5h following the removal of the virus inoculum (RC in figure 3.12), no inhibition of infection was observed. This indicates that the drug is inhibiting an early stage in infection of cells by (i.e. cell entry) rather than intracellular virus replication. The data in figure 3.12 show that at a low multiplicity of infection, successful infection SW480-β6 cells by FMDV requires the low pH within an endosomal compartment.

In order to verify that Concanamycin A raised the pH within the acidic endosomal compartments of SW480-β6 cells, experiments were performed using the vital fluorescent dye Acridine Orange. Acridine orange is a membrane permeant weak base whose spectral properties vary according to its local concentration. At relatively low concentrations, the dye emits fluorescence in the green region of the spectrum upon excitation at 488nm. The dye becomes trapped within the acidic endosomal compartments of live cells due to protonation, leading to an increase in the local concentration of dye within these compartments. At these elevated concentrations fluorescence emission is observed in the red region of the spectrum as well as the
green region. Hence acridine orange has frequently been used to stain acidic endosomes within live cells (6, 7, 340), since these compartments show up as red/orange coloured vesicular structures in fluorescence images.

SW480-β6 cells were either treated with 100nM Concanamycin A or with an equivalent concentration of DMSO (mock treatment), and stained with Acridine Orange. Figure 3.13 panel A shows SW480-β6 cells which have undergone mock-treatment. The acidic endosomal compartments show up as the orange/red vesicular structures seen in this image. Panel B shows cells which have undergone treatment with 100nM Concanamycin A prior to Acridine Orange staining. This concentration of concanamycin A was used since it is the lowest concentration which inhibited virus infection by >95% (see figure 3.12). As is clearly evident, there are no orange/red labelled acidic vesicles to be seen following treatment with Concanamycin A, indicating that at this concentration this reagent has raised endosomal pH.

One possible consequence of raising the pH within endosomes is to inhibit the recycling of internalised cell surface receptors back to the plasma membrane, since a low pH step is often required to trigger separation of a receptor and its cargo. If this were the case, the inhibitory effect of Concanamycin A on FMDV infection could result from depletion of the viral receptor, αvβ6, from the cell surface rather than as a direct consequence of the inhibition of endosomal acidification. In order to eliminate this possibility, we used FACS to investigate the level of αvβ6 expression and virus binding at the cell surface following treatment of cells with 100nM Concanamycin A or an equivalent concentration of DMSO (mock treatment). Figure 3.14 shows the results of two such experiments. Panel A shows that treatment with Concanamycin A does not result in a reduction in the level of αvβ6 expressed at the cell surface. Panel B depicts representative histograms taken from experiment 2. The level of surface
αvβ6 expression on concanamycin A treated cells is represented by the black histogram, the equivalent data for mock treated cells by the white histogram, and the level of background fluorescence (measured by omission of the primary antibody) by the grey histogram. In agreement with this, panel C shows that the level of virus binding in both experiments at the cell surface is not altered by treatment with Concanamycin A. Panel D depicts representative histograms from experiment 2. The level of virus binding at surface of concanamycin A treated cells is represented by the black histogram, the equivalent data for mock treated cells by the white histogram, and the level of background fluorescence (measured in the absence of virus) by the grey histogram. Since αvβ6 expression and virus binding at the cell surface are not altered by Concanamycin A treatment, the notion that the inhibition of FMDV infection by this agent is due to depletion of its receptor from the cell surface can be discounted.

The experiments described in this section show that Concanamycin A (i) raises endosomal pH, (ii) potently inhibits FMDV infection of SW480-β6 cells (iii) does not inhibit intracellular virus replication, and (iv) does not reduce virus binding or αvβ6 expression at the cell surface. Taken together, these data show that FMDV infection requires exposure to the low pH within endosomes for successful infection. The block to infection following treatment with Concanamycin A lies after binding of virus to the cell surface, but prior to translation and replication of the viral RNA. Given the acid lability of the virus capsid, it is likely that the acid pH within endosomes is required for uncoating and/or transfer of the viral genome into the cytosol. However, it is also possible that Concanamycin A blocks infection by altering intracellular trafficking of virus particles following virus binding. This issue is addressed in section 4.3.
Figure 3.12. Concanamycin A inhibits FMDV infection.
SW480-oxf6 cells were treated with Concanamycin A for 0.5 h and infected with FMDV (m.o.i ~0.3) for 1 h in the presence of the drug. The mock cells were treated with DMSO. Following removal of virus, samples were incubated for a further 4 h at 37°C, and then fixed with 4% paraformaldehyde. Data points marked RC represent samples treated with 1μg/ml Concanamycin A for 4 h following removal of the virus inoculum. Infection was quantified as described in figure 3.1. The number of infected cells in the drug-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. Three independent experiments are shown, and each data point represents the mean and ±SD of three observations.
Figure 3.13. Concanamycin-A raises the intra-endosomal pH. SW480-cry6 cells, prepared on coverslips were treated with either concanamycin-A (100nM) or DMSO (mock-treated) for 0.5h at 37°C. The cells were then incubated with acridine orange and visualized on the confocal microscope. Fluorescence emission was collected for green and red regions of the spectrum. The figure shows an overlay of the green and red fluorescence. Panel A) shows the intracellular acidic vesicles (orange labelling) in the mock-treated cells. This staining was completely abolished in the concanamycin-A treated cells (panel B) showing that intra-endosomal pH had been raised. Bars = 10µm.
Figure 3.14. Concanamycin A treatment does not reduce αvβ6 expression or virus binding at the surface of SW480-β6 cells.

SW480-β6 cells were pre-treated with DMSO (Mock) or 100 nM Concanamycin A for 0.5h and then prepared for flow cytometry. Surface Integrin expression was detected using Mab 10D5 and cell-bound virus with Mab B2, followed by R-phycoerythrin conjugated secondary antibodies. Background fluorescence was determined by omitting the primary antibody (αvβ6 expression) or virus (virus binding).

Panels A and C. Shown are the level of αvβ6 expression (Panel A) and virus binding (Panel C) on the surface of mock (DMSO) treated and Concanamycin A treated cells. Data are expressed as a percentage of the MFI (mean fluorescence intensity) values obtained for mock treated cells. Shown are the mean ±SD of three observations for two independent experiments: Experiment 1 (open bars) and Experiment 2 (shaded bars).

Panels B and D. Shown are representative histograms for surface expression of αvβ6 (Panel B) and virus binding (Panel D) on SW480-β6 cells; Virus binding or integrin expression on the Mock Treated Cells is shown as an open histograms. Virus binding or integrin expression Concanamycin A treated cells is shown as a black histogram. Background Fluorescence is shown as a grey histograms.
3.2.4 Inhibition of PI 3-kinases

PI 3-kinases are a group of enzymes that phosphorylate the membrane lipid phosphatidylinositol and its phosphorylated derivatives at the 3-position of the inositol head group. Wortmannin is a specific inhibitor of human class I, II β and γ, and III PI 3-kinases at nanomolar concentrations (50-200nM). However, Class II C2α PI 3-kinases are less sensitive to Wortmannin, since complete inhibition is only achieved at a concentration of 10mM (IC50 = 420 nM) compared to a value of 100nM for the type Iα enzyme (68). At such high concentrations wortmannin is no longer a specific inhibitor since the PI 4-kinases, PI-4Kα and PI-4Kβ are inhibited by Wortmannin concentrations of 150 nM and above(68).

Figure 3.15 shows the effects of Wortmannin on infection of SW480-β6 cells by FMDV O1Kcad2. The drug concentrations used were specific for PI 3-kinases (25-100nM) and do not inhibit PI 4-kinases. Infection by FMDV was not inhibited at any of these concentrations suggesting that the activities of PI 3-kinases (except Class C2α which is less sensitive to wortmannin) are not essential for entry to and infection of SW480-β6 cells. As described in section 3.1, clathrin dependent delivery to early endosomes is not wortmannin sensitive, whereas traffic from early endosomes to either late- or recycling-endosomes is wortmannin sensitive for most cell types. The inability of wortmannin to inhibit infection implies that traffic of virus beyond early endosomes to late- or recycling-endosomes is not required for successful infection of SW480-β6 cells.
Figure 3.15. Wortmannin does not inhibit FMDV infection.

SW480-ocv6 cells were treated with Wortmannin for 0.5h and infected with FMDV (m.o.i -0.3) for 1h in the presence of the drug. The mock cells were treated with DMSO. Following removal of virus, samples were incubated for a further 4h at 37°C in the presence of drug, and then fixed with 4% paraformaldehyde. Data points marked RC represent samples treated with 200 nM Wortmannin for 4h following removal of the virus inoculum. Infection was quantified as described in figure 3.1. The number of infected cells in the Wortmannin-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. The mean and ±SD of three observations is shown.
3.2.5 Disruption of the Cytoskeleton

3.2.5.1 Microtubule Disruption by Nocodazole

As described in section 3.1, nocodazole causes the complete disassembly of the microtubule network. This does not inhibit clathrin-dependent delivery of receptors and ligands to early endosomes, or their recycling back to the cell surface, but does inhibit traffic from early endosomes to late endosomes.

To investigate whether FMDV infection requires an intact microtubule network, we investigated the effect of nocodazole on infection of SW480-β6 cells by FMDV strain O1Kcad2. The cells were pre-treated for 0.5h with Nocodazole, or an equivalent concentration of DMSO (mock treatment), prior to the addition of virus. Infection was measured using the elispot infection assay. The drug, or DMSO, was present throughout the remainder of the assay up until paraformaldehyde fixation. The results of two identical experiments are shown in figure 3.16. It is clear that neither 20μM nor 40μM nocodazole has any inhibitory effect on infection of SW480-β6 cells by FMDV. In order to verify that the treatments used in these experiments had disrupted the microtubule network, cells on coverslips were treated with 40mM Nocodazole or DMSO (mock treatment) for 30 minutes, before being fixed with paraformaldehyde. Microtubules were then fluorescently labelled using a monoclonal antibody directed against β-tubulin, and an Alexa-488 conjugated goat anti mouse IgG1 antibody. The results of these experiments are shown in figure 3.17. A group of mock treated cells is shown in panel A, and a group of nocodazole treated cells in panel B, β-tubulin is shown in green. The mock treated cells contain thin filamentous structures which represent intact microtubules. The nocodazole treated cells show only a diffuse green fluorescence throughout the cytoplasm which represents depolymerised tubulin.
subunits. This shows that treatment of SW480-β6 cells with 40μM nocodazole does
cause disassembly of the microtubule network.

At a concentration of 40μM nocodazole causes disassembly of the microtubule
network as observed by immunofluorescence, but does not inhibit infection as
measured using the elispot infection assay. Since nocodazole was present at all stages
of the infection assay, this indicates that an intact microtubule network is not essential
for any step of the infection cycle, be that entry of virus particles, translation and
replication of the viral RNA, or particle assembly.
Figure 3.16. Nocodazole does not inhibit FMDV infection.

SW480-xw66 cells were treated with Nocodazole for 0.5h and infected with FMDV (m.o.i ~0.3) for 1h in the presence of the drug. The mock cells were treated with DMSO. Following removal of virus, samples were incubated for a further 4h at 37°C in the presence of drug, and then fixed with 4% paraformaldehyde. Infection was quantified as described in figure 3.1. The number of infected cells in the Nocodazole-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. Two independent experiments are shown, and each data point represents the mean and ±SD of three observations.
Figure 3.17. Nocodazole disrupts the microtubule network

SW480-cy16 cells were treated with 40\,\mu M Nocodazole for 0.5h (Panel B) or mock treated with an equivalent concentration of DMSO (Panel A). The cells were then fixed with 4\% paraformaldehyde. The microtubule network (green) was labelled by immunofluorescence microscopy using a monoclonal antibody raised against α-tubulin (clone DM1a; Sigma) and an Alexa-488 conjugated goat anti-mouse secondary antibody. Nuclei were stained with DAPI and are shown as blue. Bars = 10\,\mu m. In nocodazole treated cells (Panel B) tubulin labelling is diffuse, indicating that the filamentous microtubules visible in mock treated cells (Panel A) have been disrupted by the drug treatment.
3.2.5.2 Actin Disruption by Cytochalasin D

Actin has been shown to play a role in the uptake of ligands by a number of endocytic pathways including lipid raft and caveolae dependent endocytosis (241, 249), and macropinocytosis. However, the actin cytoskeleton is not thought to play an obligatory role in uptake of ligands via clathrin-dependent endocytosis in all cell types (87). The fungal metabolite Cytochalasin D has been shown to disrupt the actin cytoskeleton by binding the growing barbed end of microfilaments at which net polymerisation occurs, thus preventing the addition of actin monomers (39, 55). This agent is therefore a useful tool to study the role of the actin cytoskeleton in cellular processes.

Given the involvement of the actin cytoskeleton in certain endocytic uptake pathways, we sought to investigate the effect of the disruption of microfilaments by Cytochalasin D on infection of SW480-β6 cells by FMDV. Cells were either mock treated or pre-treated with Cytochalasin D or for 1h prior to the addition of virus at an m.o.i~0.3. The drug remained present throughout and infection was measured using the elispot infection assay. Figure 3.18 shows the results of two independent experiments investigating the effect of Cytochalasin D on infection of SW480-β6 cells by FMDV. Taken together, these two experiments show that infection is enhanced by Cytochalasin D concentrations ranging from 2.5 to 40μM. The extent of this enhancement is variable, ranging from 1.5 to 3-fold. Infection of cells treated with drug after the addition of virus was also enhanced, although less so compared with when the drug was added prior to infection. In experiment 2, virus particles left at the cell surface following removal of the virus inoculum were neutralised using an acid cell wash step. This treatment appeared to make no difference to the results.
obtained (compare experiments 1 and 2). This observation suggests that the enhancement in infection observed in the replication controls cannot simply be ascribed to enhancement of the uptake of virus remaining at the cell surface following removal of the virus inoculum. This indicates that Cytochalasin D may enhance both the entry and replication stages of infection of SW480-β6 cells by FMDV.

The enhancement of infection by Cytochalasin D is not consistent with uptake of FMDV via lipid rafts, caveolae, or macropinocytosis, since uptake of ligands via these pathways is thought to be dependent on the actin cytoskeleton. Clathrin-dependent endocytosis however is not inhibited by Cytochalasin D in all cell types, meaning that this data does not preclude against uptake of virus by this pathway.
Figure 3.18. Cytochalasin D enhances FMDV infection.

SW480-oxp6 cells were treated with Cytochalasin D for 1h and infected with FMDV (m.o.i ~0.3) for 1h in the presence of the drug. The mock cells were treated with DMSO. Samples in experiment 2 (but not experiment 1) were treated for 2 minutes with acid cell wash to destroy any virus particles left at the cell surface. Following removal of virus, samples were incubated for a further 4h at 37°C in the presence of drug, and then fixed with 4% paraformaldehyde. Data points marked RC represent samples treated with the indicated concentration of Cytochalasin D for 4h following removal of the virus inoculum. Infection was quantified as described in figure 3.1. The number of infected cells in the Cytochalasin D-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. Two independent experiments are shown, and each data point represents the mean and ±SD of three observations.
3.3 Summary and Discussion

In this chapter experiments were carried out using inhibitors of specific endocytosis pathways in order to determine the cellular uptake route of FMDV. The experiments described in section 3.2.1 strongly suggest that infection of SW480-β6 cells by FMDV strain 01Kcad2 requires clathrin-dependent endocytosis. Infection was blocked by pre-treatment of SW480-β6 cells with hypertonic concentrations of sucrose, a treatment that is known to block clathrin-dependent endocytosis in a number of cell lines (121). Using SW480-β6 cells this treatment was shown to block the uptake of fluorescently labelled transferrin as observed by confocal microscopy, confirming that clathrin-dependent endocytosis had been inhibited. The observed block to infection was confirmed to be at the level of virus entry since pre-treatment with hypertonic sucrose also blocked virus uptake. The role of clathrin-dependent endocytosis in infection was confirmed by showing that overexpression of the C-terminal fragment of AP180 (AP180C), which acts as a specific dominant negative inhibitor of clathrin-dependent endocytosis (82, 342), inhibited FMDV infection.

Following uptake by clathrin-mediated endocytosis, receptors and their ligands are delivered to the acidic early endosome. The experiments outlined in section 3.2.3 investigate whether the early stages of infection of SW480-β6 cells by FMDV requires the prevailing low pH within these compartments. Treatment with concanamycin A, an agent that raises endosomal pH by blocking the activity of the vacuolar H+-ATPase, inhibited infection by up to 95%, indicating that infection requires a low pH step. The inhibitory effect of the drug was exerted at an early stage of infection since addition of drug immediately following removal of the virus inoculum had virtually no effect on infection, indicating that intracellular virus
replication was not altered. The ability of concanamycin A to raise the pH within acidic endosomal compartments was confirmed using Acridine Orange, a dye which accumulates in low pH compartments and alters its spectral properties as a result.

Raising endosomal pH can inhibit receptor recycling, resulting in the depletion of these molecules at the cell surface. It is therefore possible that the inhibitory effect of concanamycin A could have resulted from reduced virus binding to the cell as a consequence of depletion of the αvβ6 integrin receptor from the cell surface. This possibility was ruled out however since treatment with concanamycin A did not reduce either virus binding or the level of αvβ6 expression at the cell surface, as measured by flow cytometry.

Bafilomycin A1 (a compound related to concanamycin A that also inhibits the vacuolar H+-ATPase) has been shown to interfere with vesicular transport and to prevent the transport of HRV-2 from early to late endosomes (21). It is therefore possible that the inhibition of FMDV infection by concanamycin A is a result of the inhibition of intracellular trafficking events rather than the elevation of endosomal pH. This issue was investigated by immunofluorescence microscopy in section 4.3.1. These experiments showed that delivery of FMDV to compartments positive for markers of early and recycling endosomes was not inhibited in concanamycin A treated cells. It is therefore unlikely that the inhibition of FMDV infection by concanamycin A is due to the inhibition of virus trafficking. To summarise, concanamycin A raises endosomal pH and inhibits infection, but does not alter expression of the integrin αvβ6, virus binding, delivery of virus to early and recycling endosomes or intracellular replication of the virus. The low pH within endosomes is therefore required for a step prior to the initiation of intracellular virus replication but post attachment of the virus to the cell surface.
Clathrin-dependent endocytosis is one of a number of distinct endocytic pathways that are exploited by viruses in order to gain entry to cells (see section 1.5.1). A number of viruses have been shown to be taken up by endocytic pathways that depend on cholesterol rich lipid rafts (see section 1.5.2). Lipid-raft dependent endocytosis pathways are sensitive to the action of drugs which bind cholesterol. The experiments shown in section 3.2.2 strongly suggest that FMDV does not require lipid raft dependent endocytosis pathways in order to infect SW480-β6 cells. Infection was not inhibited by three reagents (Methyl-β-Cyclodextrin, Filipin, Nystatin) which inhibit lipid raft dependent endocytic uptake pathways. Methyl-β-cyclodextrin treatment was shown to block uptake of Cholera Toxin B (a marker for lipid raft dependent uptake) but not transferrin (a marker for clathrin-dependent uptake), confirming the specificity of the treatment for raft-dependent endocytosis. 

Nocodazole and wortmannin have been used to investigate trafficking of ligands from early endosomes to late- and recycling-endosomes. Nocodazole depolymerises microtubules and blocks transport from early to late endosomes in BHK(107) and MDCK (36) cells. Nocodazole treatment of SW480-β6 cells resulted in the depolymerisation of microtubules, as judged by immunofluorescence microscopy, but did not block infection by FMDV. These observations indicate that FMDV does not require a functional microtubule network and implies that transport from early to late endosomes is not needed for FMDV infection of SW480-β6 cells. The PI-3 kinase inhibitor wortmannin does not block clathrin dependent delivery of ligands such as transferrin to early endosomes(172, 188, 293, 298), but does interfere with transport beyond early endosomes to late- and recycling-endosomal compartments (152, 188, 293, 298). Like nocodazole treatment, wortmannin did not inhibit infection of
SW480-β6 cells by FMDV, indicating that transport beyond early endosomes to late or recycling endosomes is not essential for infection.

As described above, experiments using nocodazole indicate that the microtubule network is not required for infection of SW480-β6 cells by FMDV at any stage. The role of the actin cytoskeleton was also investigated using the drug Cytochalasin D which disrupts the actin cytoskeleton by binding the barbed growing end of microfilaments (39, 55). The experiments outlined in section 3.2.5.2 show that disruption of the actin cytoskeleton using cytochalasin D enhances infection. The actin cytoskeleton has been shown to play a role in caveola-dependent endocytic uptake (241, 252), and the equivalent pathway in cells which do not express caveolin-1 (114). Macropinocytosis is also dependent on the actin cytoskeleton. The enhancement of infection by Cytochalasin D is therefore inconsistent with uptake of virus by these pathways. Clathrin-dependent endocytosis however is not inhibited by Cytochalasin D in all cell types (87), meaning that this data does not preclude uptake of virus by this pathway.

There are a number of possible explanations for the enhancement in infection seen following treatment with Cytochalasin D. The plasma membrane of cells is linked directly to an underlying actin-rich cytoskeleton (the cell cortex), so the actin cytoskeleton may act to regulate endocytic events. The virus receptor αvβ6 has been shown to localize to focal contacts in SW480-β6 cells when plated on Fibronectin (54, 132) and associates with the actin cytoskeleton upon ligand binding. Association of the cytoplasmic domain of αvβ6 with the actin cytoskeleton may constrain the lateral mobility of the integrin in the cell membrane, thereby regulating its entry into endocytic vesicles. Disruption of the actin cytoskeleton by Cytochalasin D would remove this constraint, allowing αvβ6 and its ligand (the virus) to more readily
migrate to sites of clathrin-coated pit formation, thereby possibly enhancing virus uptake and hence infection. Alternatively, actin may act as a molecular "fence", acting as a barrier to the formation of endocytic vesicles. Examination of the plasma membrane by quick-freeze deep-etch techniques reveals that few of the actin filaments lying in the plane of the membrane are observed in the immediate vicinity of clathrin-coated pits. Disruption of cortical actin filaments by Cytochalasin D may clear areas of the plasma membrane of this "molecular fence" allowing endocytic vesicles to form more readily at the cell surface. This would increase the rate of uptake of FMDV and hence enhance infection as a result. However, Cytochalasin D also appeared to have a small stimulatory effect on the cytoplasmic replication of FMDV. Infection of BHK cells by FMDV has been shown to cause accumulation of cytoplasmic organelles and viral proteins in a region to one side of the cell nucleus. It is possible that disruption of the actin cytoskeleton by Cytochalasin D aids the rearrangements of the cell interior observed in FMDV infected cells, thus enhancing infection.

In summary, the data presented in this chapter show that infection of SW480-β6 cells by FMDV requires clathrin-dependent delivery to early endosomes, and the low pH within this compartment, but not lipid rafts or lipid raft dependent endocytosis pathways. The data presented suggest that transport beyond early endosomes to late or recycling endosomes is not essential for infection.
Chapter Four: Investigation of the Entry Pathways Utilised by FMDV by Immunofluorescence

4.1 Introduction

In the previous chapter, the effect of inhibitors of endocytosis on infection of SW480-β6 cells by FMDV (O1Kcad2) was investigated. These studies have shown that αvβ6-mediated infection of SW80-β6 cells is dependent on clathrin-dependent endocytosis and active endosomal acidification, but not on intact lipid rafts and suggest that FMDV most likely infects its host cell following virus uptake into early- or recycling-endosomes. These experiments were carried out using a low multiplicity of infecting virus per cell to limit the possibility of virus uptake (and hence infection) proceeding via endocytic pathways that are not normally used by FMDV.

To verify that on entry, FMDV is taken up into endosomes, the intracellular trafficking of virus in SW480-β6 cells at early times post-entry was investigated using indirect immunofluorescence confocal microscopy (Chapter 4). The virus used for these studies was FMDV O1Kcad2. Virus was purified by sucrose gradient sedimentation. To visualise the virus during entry the amount of virus used in the experiments described in this chapter was necessarily high. Virus, and/or fluorescently labelled ligands were allowed to bind to the cell surface at 4°C, (a temperature which does not allow endocytosis to occur), excess unbound virus (or ligand) was removed by washing, and virus internalisation initiated by a shift to 37°C for a defined period of time. The cells were then fixed with paraformaldehyde and permeabilised. Virus, and/or markers of particular cellular compartments were labelled using specific monoclonal or polyclonal primary antibodies, followed by Alexa Fluor conjugated secondary antibodies (see section 2.6 for details). The
monoclonal antibody D9 was used to label virus. This antibody recognises a linear epitope within the GH loop of VP1 and so would be expected to recognise dissociated pentamers as well as intact virus particles (196).

4.2 Uptake of Virus by SW480-β6 cells

4.2.1 Time Course of Virus Uptake

Figure 4.1 shows a time course of virus uptake by SW480-β6 cells. The times indicated on the figure are the time elapsed following the initiation of internalisation by the shift to 37°C, following a virus pre-binding step at 4°C. The virus adopts a punctate pattern at the cell surface, presumably reflecting the distribution of its receptor ανβ6. At 37°C virus is rapidly taken up into the cell. After 5 minutes (Panel A) virus has already begun to enter the cell and was observed in relatively small puncta deep of the cell membrane. By 10 and 15 minutes (Panels B and C) the majority of virus has entered the cell and is located in peripheral vesicle-like structures. After 30 minutes (Panel D), viral proteins have accumulated in larger structures in the perinuclear region. In the experiments described below, virus was allowed to enter the cell for defined periods of time as above, but the cells were dual labelled for virus and markers of particular cellular compartments.
Figure 4.1. FMDV entry into SW480-p6 cells. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) at 4°C for 45 mins, washed to remove excess unbound virus and shifted to 37°C. After incubation at 37°C for (A) 5, (B) 10, (C) 15, and (D) 30min, internalisation was halted by fixation on ice using 4% paraformaldehyde. Virus that had entered the cells was detected using the confocal microscope. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse IgG2A-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Shown is labelling of FMDV (red). DAPI stained nuclei are shown as blue.
4.2.2 Early Endosomal Antigen-1

Early Endosomal Antigen-1 (EEA-1) is a marker for early endosomes (215). Figures 4.2-4.5 show colocalisation of FMDV (labelled green) and EEA-1 (labelled red). On the figure, colocalisation of virus with EEA-1 does not always result in bright yellow labelling since the green and red signals can be of variable intensity. At 5 minutes (figure 4.2), intracellular virus can be seen to colocalise with peripheral EEA-1 positive early endosomes, although most virus remains on the cell surface. At 10 minutes (figure 4.3) a greater amount of virus has entered the cells and the extent of virus colocalisation with EEA-1 is greater than seen at 5 minutes. At 15 minutes (figure 4.4) virus and EEA-1 were also colocalised with one another indicating that virus is present in early endosomes up to 15 minutes post internalisation. By 30 minutes (figure 4.5), the labelling for virus and EEA-1 appeared close by one another, but extensive colocalisation was not observed. This indicates that the virus has either been delivered to a different compartment at this time, or has excluded marker proteins. These data are consistent with the rapid delivery of virus to early endosomes. The detection of virus in EEA-1 positive early endosomes is consistent with the inhibition of infection by treatments that inhibit clathrin-dependent endocytosis and endosomal acidification (see sections 3.2.1 and 3.2.2 respectively).
Figure 4.2. Dual labelling of SW480-β6 cells for early endosomes (EEA-1) and FMDV at 5 minutes after initiation of virus internalisation. SW480-β6 cells, prepared on coverslips were incubated with FMDV strain O1Kcald2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.3. Dual labelling of SW480-P6 cells for early endosomes (EEA-1) and FMDV at 10 minutes after initiation of virus internalisation. SW480-P6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C, internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.4. Dual labelling of SW480-p6 cells for early endosomes (EEA-1) and FMDV at 15 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain 01Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5µg/ml) and D9 (mouse IgG2A-5µg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.5. Dual labelling of SW480-p6 cells for early endosomes (EEA-1) and FMDV at 30 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
4.2.3 Lysosomal Associated Membrane Protein-2

Following transit through early endosomes one possible destination for viral protein is late endosomes and lysosomes. A marker protein used for lysosomes (and to a lesser extent late endosomes) is Lysosomal Associated Membrane Protein-2 (LAMP-2) (48). A time course of virus uptake was undertaken as above, and cells labelled for virus (green) and LAMP-2 (red). The results of these experiments are shown in figures 4.6-4.9. Little or no colocalisation could be detected at 5 (figure 4.6), 10 (figure 4.7), 15 (figure 4.8), or 30 (figure 4.9) minutes following the initiation of virus internalisation. This indicates that within the first 30 minutes of infection viral protein is not trafficked to lysosomes, and that the intracellular virus which was not colocalised with EEA-1 at 30 minutes (see Figure 4.5) is not present in lysosomes. These data are consistent with the lack of inhibition of infection of SW480-β6 cells by nocodazole and wortmannin (see sections 3.2.4 and 3.2.5.1), both of which are reported to inhibit transport from early- to late-endosomes and hence delivery to lysosomes. However, although virus was not detected in lysosomes, it is possible that the epitope recognised by the anti-virus monoclonal antibody is rapidly destroyed upon entry to lysosomes, hence resulting in no colocalisation being observed. However the antibody epitope recognised by mab D9 is linear (159, 335) making this less likely, and neither the intensity of virus labelling or the number of virus positive vesicles appeared to decline with time, indicating that large amounts of the epitope recognised by D9 are still present inside the cells at 30 minutes.
Figure 4.6. Dual labelling of SW480-p6 cells for late endosomes/lysosomes (LAMP-2) and FMDV at 5 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies H4B4 (mouse IgG1-0.25µg/ml) and D9 (mouse IgG2A-5µg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.7. Dual labelling of SW480-p6 cells for late endosomes/lysosomes (LAMP-2) and FMDV at 10 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies H4B4 (mouse IgG1- 0.25μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.8. Dual labelling of SW480-p6 cells for late endosomes/lysosomes (LAMP-2) and FMDV at 15 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain 01Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies H4B4 (mouse IgG1 0.25μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.9. Dual labelling of SW480-p6 cells for late endosomes/lysosomes (LAMP-2) and FMDV at 30 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5 pg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies H4B4 (mouse IgG1-0.25 pg/ml) and D9 (mouse IgG2A-5 pg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
4.2.4 Transferrin Receptor

Upon leaving early endosomes, another possible fate of internalised ligands and receptors is to pass to recycling endosomes which are usually located in the perinuclear region of the cell. Transferrin and the transferrin receptor are both delivered to early endosomes via clathrin-dependent endocytosis. Subsequently, both receptor and ligand are delivered back to the cell surface via recycling endosomes. The Transferrin receptor is therefore used as a marker for early- and recycling-endosomes. A timecourse of virus uptake was undertaken, with cells labelled for virus (green) and Transferrin Receptor (red). The results of these experiments are shown in figures 4.10-4.13. Virus was seen to partially colocalises with the transferrin receptor at 5 and 10 minutes post internalisation (figures 4.10 and 4.11 respectively). More extensive colocalisation was observed at 15 minutes (figure 4.12). This is consistent with the traffic of virus from the cell surface to early- and then recycling-endosomes. By 30 minutes however (figure 4.13), the level of colocalisation of virus with the transferrin receptor is reduced compared to that seen at 15 minutes. This indicates that virus passes from early- and recycling-endosomes to an unidentified compartment by 30 minutes, or has caused the exclusion of marker proteins from the recycling endosomes.
Figure 4.10. Dual labelling of SW480-P6 cells for the transferrin receptor and FMDV at 5 minutes after initiation of virus internalisation. SW480-P6 cells, prepared on coverslips were incubated with FMDV strain 01Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.11. Dual labelling of SW480-p6 cells for the transferrin receptor and FMDV at 10 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain O:Kc2 (5 pg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5 pg/ml) and D9 (mouse IgG2A-5 pg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.12. Dual labelling of SW480-p6 cells for the transferrin receptor and FMDV at 15 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5ug/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5ug/ml) and D9 (mouse IgG2A-5ug/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.13. Dual labelling of SW480-P6 cells for the transferrin receptor and FMDV at 30 minutes after initiation of virus internalisation. SW480-P6 cells, prepared on coverslips were incubated with FMDV strain 01 Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (red) and panels B and E show virus (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
4.2.5 Alexa Fluor Conjugated Transferrin

As described above, transferrin and the transferrin receptor are delivered to early endosomes by clathrin-dependent endocytosis, before being delivered back to the cell surface via recycling endosomes. Therefore transferrin is also commonly used as a marker for clathrin dependent endocytosis. Virus and Alexa-fluor conjugated transferrin were allowed to bind to the surface of SW480-β6 cells at 4°C, before being co-internalised by shifting the temperature to 37°C. At 5 minutes (figure 4.14), virus and transferrin can be seen to colocalise in vesicular structures close to the cell surface. At 10 minutes (figure 4.15), virus and transferrin appeared to have accumulated in the same compartment and colocalise extensively. At 15 minutes (figure 4.16), virus and transferrin are still colocalised but in vesicles in the perinuclear region. No data was obtained at 30 minutes because a transferrin signal was not observed at this time point, presumably because the transferrin had recycled out of the cells by this time. Virus and transferrin colocalise at all time points at which transferrin can be observed, indicating that the two ligands are trafficked by the same route, i.e. clathrin-dependent delivery to early- and recycling-endosomes. Colocalisation appeared lower at 5 minutes than other time points, but this could be due to the two receptors (transferrin receptor and αvβ6) recycling at different rates.

In order to observe the earliest stages of transferrin and virus endocytosis, uptake was slowed by allowing internalisation to occur at 19°C rather than 37°C. As shown in figure 4.17, at this early stage, virus and transferrin can be seen to colocalise just below the plasma membrane, indicating that virus and transferrin are delivered to early endosomes by the same, rather than different, routes.
Figure 4.14. Co-internalised transferrin and FMDV at 5 minutes after initiation of internalisation in SW480-P6 cells. SW480-P6 cells, prepared on coverslips were incubated with Alexa Fluor-488 conjugated transferrin (10µg/ml) and FMDV strain O1Kcad2 (5µg/ml) for 45 minutes at 4°C. Internalisation of virus and transferrin was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse IgG2A-5µg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show fluorescently labelled transferrin (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.15. Co-internalised transferrin and FMDV at 10 minutes after initiation of internalisation in SW480-J16 cells. SW480-J16 cells, prepared on coverslips were incubated with Alexa Fluor-488 conjugated transferrin (10μg/ml) and FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Internalisation of virus and transferrin was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse IgG2A-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show fluorescently labelled transferrin (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.16. Co-internalised transferrin and FMDV at 15 minutes after initiation of internalisation in SW480-36 cells. SW480-36 cells, prepared on coverslips were incubated with Alexa Fluor-488 conjugated transferrin (10 μg/ml) and FMDV strain O1Kcad2 (5 μg/ml) for 45 minutes at 4°C. Internalisation of virus and transferrin was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse IgG2A-5 μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show fluorescently labelled transferrin (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.17. Co-internalised transferrin and FMDV in SW480-P6 cells after 30 minutes incubation at 19 °C. SW480-P6 cells, prepared on coverslips were incubated with Alexa Fluor-488 conjugated transferrin (10 μg/ml) and FMDV strain O1Kcad2 (5 μg/ml) for 45 minutes at 4°C. Internalisation of virus and transferrin was initiated by shifting the temperature to 19°C. Following 30 minutes incubation at 19°C internalisation was halted at an early stage by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse IgG2A-5 μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show fluorescently labelled transferrin (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
4.2.6 Alexa Fluor Conjugated Cholera Toxin B

Cholera Toxin B (CTB) has been reported to be taken up into cells by lipid raft dependent endocytosis, and in particular the caveolae-dependent endocytic pathway (118, 211, 289, 311, 315). CTB is therefore often used as a marker for lipid raft dependent endocytosis. Therefore, the intracellular transport of FMDV and fluorescently labelled CTB were compared. Virus and Alexa-fluor conjugated CTB, were allowed to bind to the surface of SW480-β6 cells at 4°C, before being co-internalised by shifting the temperature to 37°C. At 5 minutes (figure 4.18), a relatively small amount of virus and CTB has been internalised, but there is a degree of colocalisation between the two. By 10 and 15 minutes (figures 4.19 and 4.20), slightly greater colocalisation between virus and the CTB was observed. At 30 minutes (figure 4.21), CTB has accumulated in perinuclear regions but was also-present in vesicular-like structures. At this time point, colocalisation of CTB with virus was observed in the small vesicular-like CTB positive structures, rather than the larger perinuclear accumulations. These observations were unexpected given that reagents (methyl-β-cyclodextrin, nystatin and filipin) that inhibit lipid-raft dependent endocytosis failed to inhibit infection of SW480-β6 cells by FMDV (see figures 3.6, 3.8, and 3.9 respectively). However, CTB has been shown recently to be taken up by a number of different endocytic pathways, and is no longer considered a specific marker for lipid raft- or caveolae-dependent endocytosis. For example, it has been shown that in certain cell types, CTB can be taken up by clathrin-dependent endocytosis (311), or by a dynamin- and caveolae-independent pathways (311). It has also been shown that cholera toxin that has been internalised via caveolae can be delivered to early endosomes in Hela cells (248). Therefore, it is possible that the
observed colocalisation of FMDV with CTB inside the cell results from CTB (or at least a fraction of it) being delivered to early and/or recycling endosomes, rather than a result of virus being taken up by lipid raft-dependent endocytic mechanisms (See section 4.2.7).
Figure 4.18. Co-internalised cholera toxin B subunit (CTB) and FMDV at 5 minutes after initiation of internalisation in SW480-P6 cells. SW480-P6 cells, prepared on coverslips were incubated with Alexa Fluor-488 conjugated CTB (0.2µg/ml) and FMDV strain O′Kcad2 (6µg/ml) for 45 minutes at 4°C. Internalisation of virus and CTB was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse IgG2A-5µg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show fluorescently labelled CTB (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.19. Co-internalised cholera toxin B subunit (CTB) and FMDV at 10 minutes after initiation of internalisation in SW480-P6 cells. SW480-P6 cells, prepared on coverslips were incubated with Alexa Fluor-488 conjugated CTB (0.2μg/ml) and FMDV strain O1Koad2 (5μg/ml) for 45 minutes at 4°C. Internalisation of virus and CTB was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse IgG2A-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti-mouse secondary antibody. Panels A and D show fluorescently labelled CTB (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.20. Co-internalised cholera toxin B subunit (CTB) and FMDV at 15 minutes after initiation of internalisation in SW480-P6 cells. SW480-P6 cells, prepared on coverslips were incubated with Alexa Fluor-488 conjugated CTB (0.2μg/ml) and FMDV strain 01Kcad2 (5μg/ml) for 45 minutes at 4°C. Internalisation of virus and CTB was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse lgG2A-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show fluorescently labelled CTB (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.21. Co-internalised cholera toxin B subunit (CTB) and FMDV at 30 minutes after initiation of internalisation in SW480-P6 cells. SW480-P6 cells, prepared on coverslips were incubated with Alexa Fluor-488 conjugated CTB (0.2μg/ml) and FMDV strain O1Koad2 (5μg/ml) for 45 minutes at 4°C. Internalisation of virus and CTB was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Following permeabilisation with 0.1% Triton, virus was detected using the monoclonal antibody D9 (mouse IgG2A-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show fluorescently labelled CTB (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
4.2.7 Endocytosis of Cholera Toxin B in SW480-β6 Cells

To determine whether CTB is delivered to early- and recycling-endosomes following uptake by SW480-β6 cells, the colocalisation of CTB with EEA-1 was investigated.

The results of these experiments are shown in figures 4.22–4.25. At 5 minutes (figure 4.22), a proportion of the CTB which had been internalised was colocalised with EEA-1. At 10 minutes (figure 4.23), CTB can again be found in vesicular structures which also label for EEA-1. At 15 minutes (figure 4.24), the level of colocalisation between CTB and EEA-1 appears to be slightly reduced compared with the earlier time points and CTB had begun to accumulate in the perinuclear region. By 30 minutes (figure 4.25), the majority of CTB was found in the perinuclear region and was not colocalised with EEA-1. These data suggest that a proportion of internalised CTB is delivered from the cell surface to early endosomes. The CTB found in the perinuclear region presumably represents CTB present in the golgi apparatus since CTB has been shown to be delivered to this compartment at later times (17).

Next, colocalisation of CTB with a marker of early- and recycling-endosomes, the Transferrin Receptor was investigated. These data are shown in figures 4.26-4.29. Internalised CTB can be seen to colocalise with the transferrin receptor at 5, 10 and 15 minutes (see figures 4.26, 4.27, and 4.28 respectively) following initiation of internalisation. There is also some colocalisation at 30 minutes (figure 4.29), but this is mostly restricted to more punctate structures, rather than the structures where CTB accumulates in the perinuclear region. Consistent with these observations, co internalised fluorescent Transferrin and CTB show colocalisation after 5 minutes and 15 minutes (see figures 4.30 and 4.31 respectively). This is again consistent with a proportion of the internalised CTB being delivered to early- and recycling endosomes.
Finally, colocalisations of CTB with a marker of lysosomes (Lamp-2) was investigated. These data are shown in figures 4.32-4.35. Little colocalisation was observed at 5, 10, 15, or 30 minutes. This indicates that CTB is not delivered to lysosomes within this time frame.

CTB (or at least a proportion of it) appears to be delivered from the cell surface to early/recycling endosomes. Colocalisation of CTB and FMDV particles is therefore consistent with the model of clathrin-dependent delivery of virus to acidic early and recycling endosomes (Chapter 3). It is likely that the majority of CTB that is delivered to early endosomes is delivered via a non-clathrin dependent pathway since methyl-β-cyclodextrin blocks uptake of CTB but not transferrin by SW480-β6 cells (see figure 3.7)
Figure 4.22. Labelling of SW480-p6 cells for EEA-1 at 5 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-488 conjugated CTB (0.2 μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 was detected using the monoclonal antibody 14 (mouse IgG1 5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show EEA-1 labelling (red) and panels B and E show Alexa-488 conjugated CTB (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.23. Labelling of SW480-p6 cells for EEA-1 at 10 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-488 conjugated CTB (0.2μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 was detected using the monoclonal antibody 14 (mouse IgG1-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show EEA-1 labelling (red) and panels B and E show Alexa-488 conjugated CTB (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.24. Labelling of SW480-p6 cells for EEA-1 at 15 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-488 conjugated CTB (0.2μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 was detected using the monoclonal antibody 14 (mouse IgG1-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show EEA-1 labelling (red) and panels B and E show Alexa-488 conjugated CTB (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.25. Labelling of SW480-p6 cells for EEA-1 at 30 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-488 conjugated CTB (0.2µg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 was detected using the monoclonal antibody 14 (mouse IgG1-5µg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show EEA-1 labelling (red) and panels B and E show Alexa-488 conjugated CTB (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.26. Labelling of SW480-p6 cells for the transferrin receptor at 5 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-488 conjugated CTB (0.2µg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor was detected using the monoclonal antibody H68.4 (mouse IgG1-5µg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show transferrin receptor labelling (red) and panels B and E show Alexa-488 conjugated CTB (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.27. Labelling of SW480-p6 cells for the transferrin receptor at 10 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-488 conjugated CTB (0.2μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor was detected using the monoclonal antibody H68.4 (mouse IgG1 5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show transferrin receptor labelling (red) and panels B and E show Alexa-488 conjugated CTB (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.28. Labelling of SW480-P6 cells for the transferrin receptor at 15 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-P6 cells, prepared on coverslips were incubated with Alexa-488 conjugated CTB (0.2ng/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor was detected using the monoclonal antibody H68.4 (mouse IgG1, 5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show transferrin receptor labelling (red) and panels B and E show Alexa-488 conjugated CTB (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.29. Labelling of SW480-p6 cells for the transferrin receptor at 30 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-488 conjugated CTB (0.2 μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor was detected using the monoclonal antibody H68.4 (mouse IgG1-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show transferrin receptor labelling (red) and panels B and E show Alexa-488 conjugated CTB (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.30. Co-internalised transferrin and Cholera Toxin B subunit (CTB) at 5 minutes after initiation of internalisation in SW480-P6 cells. SW480-P6 cells, prepared on coverslips were incubated with Alexa Fluor-568 conjugated transferrin (10μg/ml) and Alexa-488 conjugated CTB (0.2μg/ml) for 45 minutes at 4°C. Internalisation of CTB and transferrin was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Panels A and D show fluorescently labelled transferrin (red) and panels B and E show fluorescently labelled CTB (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.31. Co-internalised transferrin and Cholera Toxin B subunit (CTB) at 15 minutes after initiation of internalisation in SW480-P6 cells. SW480-P6 cells, prepared on coverslips were incubated with Alexa Fluor-568 conjugated transferrin (10μg/ml) and Alexa-488 conjugated CTB (0.2μg/ml) for 45 minutes at 4°C. Internalisation of CTB and transferrin was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde/0.2% glutaraldehyde. Panels A and D show fluorescently labelled transferrin (red) and panels B and E show fluorescently labelled CTB (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.32. Labelling of SW480-p6 cells for late endosomes/lysosomes (LAMP-2) at 5 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa fluor 488 conjugated CTB (0.2μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 was detected using the monoclonal antibody H4B4 (mouse IgG1-0.5μg/ml) followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show LAMP-2 labelling (red) and panels B and E show CTB (green). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.33. Labelling of SW480-p6 cells for late endosomes/lysosomes (LAMP-2) at 10 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-fluor 488 conjugated CTB (0.2μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 was detected using the monoclonal antibody H4B4 (mouse IgG1:0.5μg/ml) followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show LAMP-2 labelling (red) and panels B and E show CTB (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.34. Labelling of SW480-p6 cells for late endosomes/lysosomes (LAMP-2) and 15 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-p6 cells, prepared on coverslips were incubated with Alexa-fluor 488 conjugated CTB (0.2 μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 was detected using the monoclonal antibody H4B4 (mouse IgG1-0.5 μg/ml) followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show LAMP-2 labelling (red) and panels B and E show CTB (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.35. Labelling of SW480-P6 cells for late endosomes/lysosomes (LAMP-2) at 30 minutes after initiation of Alexa-488 conjugated Cholera Toxin B subunit (CTB) internalisation. SW480-P6 cells, prepared on coverslips were incubated with Alexa-fluor 488 conjugated CTB (0.2 μg/ml) for 45 minutes at 4°C. CTB internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 was detected using the monoclonal antibody H4B4 (mouse IgG1-0.5μg/ml) followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Panels A and D show LAMP-2 labelling (red) and panels B and E show CTB (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
4.2.8 Caveolin-1

Caveolin-1 is a marker for caveolae-dependent endocytosis (section 1.5.2.1). Caveolae are defined morphologically by electron microscopy as smooth surfaced invaginations of the plasma membrane of around 55-65 nm in diameter, and caveolin-1 forms a major protein constituent of caveolae (273). Caveolin-1 is also a major component of internalised caveolar vesicles (or cavicles) (216), and a neutral pH endocytic compartment called the caveosome, (251) to which the virus SV40 is delivered upon caveolae-dependent endocytic uptake. Therefore colocalisation of FMDV with caveolin-1 was investigated. However four different antibodies directed against human caveolin-1 failed to produce any caveolin-1 labelling under the confocal microscope.

The above observations prompted investigations of caveolin-1 expression in SW480-β6 cells by PCR. PCR primers complementary to conserved regions of human caveolin-1 were synthesized. Total RNA was extracted from both SW480-β6 and CHO cells; a cell line known to express caveolin-1 and possess caveolae. This RNA was then reverse transcribed and conventional PCR carried out using primers complementary to caveolin-1, and β-actin (a positive control). The resulting gel is shown in figure 4.36. A band of ~275bp corresponding to β-actin is present in lanes 2 and 4, indicating that β-actin is expressed in both SW480-β6 and CHO cells as expected. This confirms that the RNA extraction, reverse transcription and PCR processes have been successful. However a band of ~350 bp representing caveolin-1 is present in lane 3 (CHO cells) but not lane 1 (SW480-β6 cells) confirming that SW480-β6 cells express little or no caveolin-1 and therefore would be expected to lack caveolae. However, it is thought that the endocytic pathways associated with
caveolin-1 in cells that express this molecule have functional equivalents in cells lacking caveolin-1 expression and surface caveolae (see reviews (220, 242)). For example in cells lacking caveolin-1, SV40 is taken up to a neutral pH endocytic compartment in a lipid-raft dependent fashion, just as observed in cells expressing caveolin-1 (62). The only apparent difference is that this compartment lacks caveolin-1 on its surface. It is unlikely therefore that a lack of caveolin-1 expression has a major impact on the route utilised by FMDV to enter SW480-β6 cells.
Figure 4.36. SW480-P6 cells do not express caveolin-1. An RT-PCR was carried out for the presence of actin or caveolin-1 mRNA in CHO and SW480-P6 cells. A PCR product (~275bp) was detected for actin in both cell lines (lanes 2 and 4). In contrast a PCR product (~350bp) for caveolin-1 was detected for CHO (lane 3) but not for SW480-P6 cells (lane 1). M = size markers.
4.3 Effect of Pharmacological Inhibitors of Endocytosis on the uptake of Virus by SW480-β6 cells.

4.3.1 Concanamycin A

The experiments shown in chapter 3 established that concanamycin A inhibits infection of SW480-β6 cells by FMDV. It has been reported that agents that raise the pH within endosomes can also inhibit intracellular trafficking events. Therefore, the effect on FMDV trafficking and virus delivery to early- and recycling-endosomes was investigated in concanamycin A treated cells.

SW480-β6 cells were either pre-treated for 30 minutes with 100 nM concanamycin A or mock treated with an equivalent concentration of DMSO. Virus was then bound to the cell surface at 4°C, and allowed to enter cells for 15 minutes at 37°C. The cells were then fixed with paraformaldehyde with the drug being present throughout the period prior to fixation. Figures 4.37 to 4.42 show the results of these experiments. Virus appeared to colocalise with EEA-1 at 15 minutes to the same extent in both mock-treated (figure 4.37) and concanamycin A treated (figure 4.38) cells. Similarly, extensive colocalisation between virus and the transferrin receptor at 15 minutes was observed in both mock treated (figure 4.39) and concanamycin A treated (figure 4.40) cells. FMDV did not colocalise with LAMP-2 after 15 minutes in mock treated (figure 4.41) or concanamycin A (figure 4.42) treated cells. These data show that virus is still delivered to compartments positive for markers of early endosomes (EEA-1 and Transferrin Receptor) and recycling endosomes (Transferrin Receptor).
when treated with concanamycin A. These data (in conjunction with that shown in section 3.2.3) shows that concanamycin A raises endosomal pH and inhibits infection by FMDV, but does not inhibit surface integrin expression, virus binding to the cells, delivery of FMDV to early- and recycling-endosomes, or intracellular virus replication. Hence it appears that raising endosomal pH using Concanamycin A blocks FMDV infection of SW480-B6 cells by preventing virus uncoating and/or delivery of viral RNA across the endosomal membrane.
Figure 4.37. Dual labelling of DMSO treated SW480-p6 cells for early endosomes (EEA-1) and FMDV at 15 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were pre-treated with 0.001% DMSO for 30 minutes at 37 °C, and then incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C in the presence of DMSO, internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1, 5μg/ml) and D9 (mouse IgG2A, 5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.38. Dual labelling of Concanamycin A treated SW480-p6 cells for early endosomes (EEA-1) and FMDV at 15 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were pre-treated with 100nM Concanamycin A for 30 minutes at 37 °C, and then incubated with FMDV strain O1Kcad2 (5pg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C in the presence of Concanamycin A, internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse lgG1-5pg/ml) and D9 (mouse lgG2A-5pg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.39. Dual labelling of DMSO treated SW480-P6 cells for the transferrin receptor and FMDV at 15 minutes after initiation of virus internalisation. SW480-P6 cells, prepared on coverslips were pre-treated with 0.001% DMSO for 30 minutes at 37 °C, and then incubated with FMDV strain O1Kcad2 (5ug/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C in the presence of DMSO, internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5ug/ml) and D9 (mouse IgG2A-5ug/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.40. Dual labelling of Concanamycin A treated SW480-P6 cells for the transferrin receptor and FMDV at 15 minutes after initiation of virus internalisation. SW480-P6 cells, prepared on coverslips were pre-treated with 100nM Concanamycin A for 30 minutes at 37°C and then incubated with FMDV strain O1Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C in the presence of Concanamycin A internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5µg/ml) and D9 (mouse IgG2A-5µg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.41. Dual labelling of DMSO treated SW480-p6 cells for late endosomes/lysosomes (LAMP-2) and FMDV at 15 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were pre-treated with 0.001% DMSO for 30 minutes at 37 °C, and then incubated with FMDV strain O1Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C in the presence of DMSO, internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies H4B4 (mouse IgG1-0.25µg/ml) and D9 (mouse IgG2A-5µg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 4.42. Dual labelling of Concanamycin A treated SW480-p6 cells for late endosomes/lysosomes (LAMP-2) and FMDV at 15 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were pre-treated with 100nM Concanamycin A for 30 minutes at 37 °C, and then incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C in the presence of Concanamycin A internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
4.4 Summary and Discussion

The data presented in chapter 3 showed that αvβ6-mediated infection of SW80-β6 cells is dependent on clathrin-dependent endocytosis and active endosomal acidification, but not on intact lipid rafts and suggest that FMDV most likely infects its host cell following virus uptake into early- or recycling-endosomes. To confirm these observations the intracellular trafficking of FMDV was determined using immunofluorescence confocal microscopy.

Virus (and fluorescently conjugated ligands where appropriate) was bound to the cell surface at 4°C and then allowed to enter SW480-β6 cells for 5, 10, 15 or 30 minutes at 37°C. The cells were fixed and permeabilised, and labelled for virus alone or both virus and markers of specific cellular compartments. At 5 minutes after the initiation of internalisation the majority of virus remained at or near the cell surface, but some virus could already be observed within punctate intracellular vesicles. By 10 to 15 minutes the majority of virus has been internalised and is present in intracellular vesicle-like structures. By 30 minutes after internalisation virus appeared to accumulate in larger vesicle-like structures located closer to the nucleus. The compartment markers used in this study were EEA-1 (a marker of early endosomes), the transferrin receptor (a marker of early and recycling endosomes), fluorescently labelled transferrin (a marker for the recycling endocytic pathway), LAMP-2 (a lysosomal marker), and fluorescently labelled cholera toxin B (previously reported as a ligand which is internalised predominantly via caveolae-dependent endocytosis).

At 5 minutes post internalisation there was significant colocalisation between internalised virus and EEA-1, as well as with co-internalised transferrin, and to a
lesser extent the transferrin receptor. Little or no colocalisation was observed between virus and LAMP-2. At 10 minutes there is extensive colocalisation of virus with EEA-1 and the transferrin receptor, and with co-internalised transferrin. Again, at this time point, little or no colocalisation with LAMP-2 was seen. At 15 minutes virus colocalises extensively with cointernalised transferrin, and colocalisation can still be seen between virus and EEA-1. Colocalisation between the transferrin receptor and virus is extensive and maximal at this time point. Very little colocalisation can be seen between virus and LAMP-2. By 30 minutes little colocalisation can be observed between virus and EEA-1, the transferrin receptor or LAMP-2. Fluorescently labelled transferrin has recycled out of the cells by this time point and so no signal can be observed.

The above data are consistent with the rapid delivery of FMDV to early endosomes and its trafficking to recycling endosomes as virus colocalises with EEA-1 and transferrin at early time points post uptake. The level of colocalisation of virus with the transferrin receptor (a recycling endosome marker) reaches a maximum at 15 minutes, suggesting that a proportion of virus is also delivered from early- to recycling-endosomes. The observation that virus colocalises with co-internalised transferrin (a ligand which is delivered to early- and subsequently recycling-endosomes) at 5, 10, and 15 minutes supports these data. The results of this study are consistent with the inhibition of virus infection by concanamycin A (see section 3.2.3). In chapter 3, this drug was shown to raise endosomal pH and inhibit infection by FMDV, but not inhibit surface integrin expression, virus binding, delivery of FMDV or intracellular virus replication. The data presented in section 4.3 show that concanamycin A does not prevent delivery of FMDV to acidic early- and recycling-endosomes. Dissipation of the acidic pH within these compartments using
concanamycin A therefore most likely blocks FMDV infection of SW480-β6 cells by preventing virus uncoating and/or delivery of viral RNA across the endosomal membrane. The lack of colocalisation of virus with LAMP-2 at any time point suggests that virus is not delivered to late endosomes or lysosomes. This is consistent with the insensitivity of infection of SW480-β6 by FMDV to agents such as wortmannin and nocodazole which inhibit transport between early and late endosomes (see sections 3.2.4 and 3.2.5.1).

At 30 minutes, little colocalisation was observed between virus and EEA-1, the transferrin receptor or LAMP-2. This data suggests that after delivery to early- and recycling-endosomes virus (or virus protein at least) is transported from these compartments to an unidentified compartment, or alternatively that virus causes exclusion of markers of these compartments. However, it is unlikely that virus transport beyond early- or recycling-endosomes is significant for infection. The FMDV capsid is extremely acid labile, and virus uncoating and the subsequent translocation of the viral RNA to the cytoplasm would be triggered by the mildly acidic pH within early- and recycling-endosomes. Once viral RNA has reached the cytoplasm the subsequent fate of viral protein is therefore most likely to be irrelevant to infection.

Cholera toxin and in particular its B subunit, have been favoured as markers of caveolae-dependent endocytosis following early studies showing labelling of caveolae using Cholera toxin conjugated to gold (211). Therefore CTB was used as a marker for caveolae-dependent endocytosis to investigate entry of FMDV into caveolae. Immunofluorescence experiments revealed partial colocalisation between FMDV and CTB at the 10 and 15 minute timepoints. These observations were unexpected as they appear to suggest that FMDV is taken up by a caveolae-dependent mechanism and
would contradict the observations made in chapter 3, that infection of SW480-β6 cells is insensitive to cholesterol binding agents (filipin, nystatin, and methyl-β-cyclodextrin), which inhibit lipid raft dependent endocytosis pathways, including caveolae-dependent endocytosis.

A possible explanation for the apparently contradictory nature of the above data is the observation that CTB can efficiently enter cells via early endosomes when the cell does not express caveolin-1 and lack caveolae (193, 227, 311). The data presented in section 4.2.8 shows that SW480-β6 cells lack caveolin-1, and therefore would not be expected to possess caveolae. In cells lacking caveolin-1, it has also been shown that CTB can be taken up via a non-clathrin and non-caveolae dependent endocytosis pathway (158). This pathway is dependent on lipid rafts, and displays similar properties to the non-caveolae and non-clathrin dependent pathway utilised by SV40 in cells lacking caveolae (62). Additionally CTB has been shown to be able to enter a number of different cell types in a clathrin-dependent manner (193, 227, 311), resulting in its delivery to early endosomes. CTB can also be delivered to early endosomes via caveola-dependent endocytosis, since a recent study has shown that internalised caveolae are delivered to early endosomes as well as caveosomes, and that delivery of CTB to the golgi is dependent on contact with the low pH within endosomes in the system used (248). It is likely that a similar pathway exists in cells lacking caveolin-1, since toxin internalisation and delivery to the golgi occurs normally in cells lacking caveolin-1. In summary, these studies have shown that CTB internalisation is a complex process and that CTB can be delivered to early endosomes via either clathrin-dependent or lipid raft dependent mechanisms.

Consistent with this, the data shown in section 4.2.7 shows that in SW480-β6 cells, internalised CTB colocalises with EEA-1 (an early endosome marker) and the
transferrin receptor (a marker of early/recycling endosomes), indicating that CTB is internalised via early endosomes in this cell type. On SW480-β6 cells, delivery to early endosomes is likely to be via a lipid raft dependent mechanism rather than a clathrin-dependent mechanism since uptake of CTB was inhibited by methyl-β-cyclodextrin under conditions whereby clathrin-dependent endocytosis (as assayed by transferrin uptake) was not blocked (see section 3.2.2). The observations that both CTB and virus are delivered to early- and recycling-endosomes explains why they show colocalisation inside the cell.

In summary, the data presented here show that FMDV is rapidly taken up into compartments positive for markers of early- and recycling-endosomes, and colocalises with co-internalised CTB and transferrin, both of which are known to be delivered to these compartments. When internalisation was slowed by incubation at 19°C rather than 37°C, enabling an early stage of internalisation to be captured, internalised virus and transferrin remained colocalised, suggesting that the two ligands are delivered to early endosomes by the same mechanism (i.e. clathrin dependent endocytosis). Caveola-dependent endocytosis is not required for infection of SW480-β6 cells since this cell line does not express caveolin-1. Taken together the data presented here is consistent with the inhibitor studies presented in chapter 3, and suggests that the entry pathway used by virus to infect SW480-β6 cells is clathrin-dependent delivery of virus from the cell surface to early and recycling endosomes.
5.1 Introduction

Chapter four investigated the entry of FMDV strain O1Kcad2 into SW480-β6 cells by immunofluorescence confocal microscopy. However, as outlined in section 4.2.8, this cell line does not appear to express Caveolin-1, since four antibodies against this protein failed to produce any immunofluorescence signal, and the cells were negative for Caveolin-1 mRNA by PCR. Cells lacking caveolin-1 expression possess little or no caveolae at their cell surface, and reintroduction of Caveolin-1 into such cells induces the formation of caveolae at the plasma membrane (85). It is possible therefore that the virus can utilise different endocytosis pathways in cell lines which do express caveolin-1. In order to explore this possibility FMDV endocytosis and infection was investigated using a cell line which does express caveolin-1, and therefore possesses caveolae, namely CHO-β6 cells. These cells are CHO cells transfected to permanently express the β6 integrin subunit, and consequently express the integrin αvβ6 at the cell surface. The integrin αvβ6 has been shown to act as the major receptor for virus attachment and infection on this cell line (145). This cell line is therefore a useful tool to investigate the entry pathways utilised by FMDV to infect cells which possess caveolae.

5.2 Low pH Dependence of infection of CHO-β6 Cells

Infection of SW480-β6 cells was shown to be dependent on the acid pH within endosomes at an early stage of infection in section 3.2.3. Therefore it was decided to
establish whether this was also true for CHO-β6 cells using Concanamycin A. CHO-
β6 cells were pre-treated with concanamycin A for 0.5h prior to the 1h incubation
with virus in the presence of drug. Infection was determined by using the ELIspot
infection assay at an m.o.i of 0.3. Figure 5.1 shows the results of these experiments.
Each data point represents the mean of three independent experiments, each of which
was carried out in triplicate. As shown in the figure, infection was inhibited by pre
treatment with 10μM (93% inhibition), 1μM (75% inhibition) and 100nM (74%
inhibition) concentrations of Concanamycin A. This is consistent with the data
obtained for SW480-β6 cells, albeit that the level of inhibition observed at
concentrations of 1μM and 100nM are slightly lower. The effect of concanamycin A
on later stages of infection (i.e. intracellular virus replication) is shown on figure 5.1
by the data points marked RC. These samples were treated with the indicated
concentration of Concanamycin A for 4h following removal of the virus inoculum,
i.e. when the entry stage of the replication cycle is over. These data show that 10μM
and 1μM Concanamycin A appear to inhibit infection when added after cell entry has
finished, indicating that these high concentrations of drug inhibit intracellular virus
replication. However, 100nM Concanamycin A has a minimal effect on infection
when added at this time, indicating that this concentration of drug does not inhibit the
intracellular replication of FMDV. To summarise, 100nM Concanamycin A inhibits
infection of CHO-β6 cells by an average of 74% when cells are pretreated with the
drug, but has a minimal impact on infection when added immediately following the
removal of the virus inoculum. These observations show that raising endosomal pH
using 100nM Concanamycin A inhibits infection of CHO-β6 cells at an early stage in
the infection cycle, most likely at the stage of virus entry/uncoating rather than
intracellular replication. Higher concentrations of Concanamycin A appear to inhibit
virus replication as well as cell entry, perhaps due to inhibition of replication complex formation. The data presented here show that infection of CHO-β6 cells by FMDV requires a low pH step at an early stage, just as is the case for SW480-β6 cells.
Figure 5.1. Concanamycin A inhibits FMDV infection.

CHO-β6 cells were treated with Concanamycin A for 0.5h and infected with FMDV (m.o.i ~0.3) for 1h in the presence of the drug. The mock cells were treated with DMSO. Following removal of virus, samples were incubated for a further 4h at 37°C, and then fixed with 4% paraformaldehyde. Data points marked RC represent samples treated with the indicated concentration (nM) of Concanamycin A (in nM) for 4h following removal of the virus inoculum. Infection was quantified as described in figure 3.1. The number of infected cells in the Concanamycin A-treated samples was expressed as a percentage of the number of infected cells in mock-treated samples. Each data point represents the mean and ±SD of three experiments, each of which was carried out in triplicate.
5.3 Uptake of FMDV by CHO-β6 Cells

The work presented in chapter 4 showed, using immunofluorescence microscopy, that FMDV is taken up rapidly into early and recycling endosomes in SW480-β6 cells, consistent with dependence of infection on a low pH step. However this cell line expresses little or no caveolin-1 (see figure 4.36) and as such does not possess caveolae. It is possible that FMDV can utilise caveolae-dependent endocytosis to enter cells which express caveolin-1. It was therefore decided that the endocytic uptake of FMDV by CHO-β6 cells, which do possess caveolae, should be investigated using the same methods used for SW480-β6 cells. Virus was allowed to bind to the cell surface at 4°C, a temperature which does not allow endocytosis to occur. Internalisation was initiated by a shift to 37°C, before paraformaldehyde fixation after a defined period of time (between 5 and 30 minutes). Cells were then permeabilised, and virus and markers of specific cellular compartments labelled using monoclonal or polyclonal primary antibodies, followed by Alexa Fluor conjugated secondary antibodies. The data is divided up below by marker protein.

5.3.1 Early Endosomal Antigen 1 (EEA-1)

Early endosomal antigen-1 (EEA-1) is a marker for early endosomes. A timecourse of αvβ6-mediated internalisation of FMDV strain O1Kcad2 by CHO-β6 cells was carried out as described above. At each time point CHO-β6 cells were fixed, permeabilised and Virus and EEA-1 were labelled using monoclonal antibodies. Viral protein (red) was detected using the antibody D9 which recognises a linear epitope within the viral protein VP1, and EEA-1 (green) was detected using the
monoclonal antibody 14 which was recognizes an epitope within human EEA-1, but cross-reacts with the hamster version. The data obtained are shown in figures 5.2-5.5. At 5 minutes following the initiation of virus internalisation (figure 5.2), a large proportion of internalised virus is colocalised with EEA-1. By 10 minutes (figure 5.3) the majority of virus has been internalised, and was colocalised with EEA-1. At 15 and 30 minutes (figures 5.4 and 5.5), FMDV seems to have accumulated in large vesicles in the perinuclear area, but still exhibited significant colocalisation with EEA-1. The data presented here show that virus is delivered to EEA-1 positive early endosomes by 10 minutes after internalisation and a significant proportion seems to remain associated with early endosomes right up to 30 minutes after the initiation of internalisation. This contrasts with SW480-β6 cells where virus is delivered to early endosomes by 10-15 minutes, but no longer colocalises with EEA-1 by 30 minutes. It is possible that this is because traffic of viral protein onwards from early endosomes is either slower in CHO-β6 cells or else it may not occur at all.
Figure 5.2. Dual labelling of CHO-36 cells for EEA-1 and FMDV at 5 minutes after initiation of virus internalisation. CHO-36 cells, prepared on coverslips were incubated with FMDV strain 01Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5µg/ml) and D9 (mouse IgG2A-5µg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.3. Dual labelling of CHO-j36 cells for EEA-1 and FMDV at 10 minutes after initiation of virus internalisation. CHO-j36 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5µg/ml) and D9 (mouse IgG2A-5µg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.4. Dual labelling of CHO-β6 cells for EEA-1 and FMDV at 15 minutes after initiation of virus internalisation. CHO-β6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.5. Dual labelling of CHO-β6 cells for EEA-1 and FMDV at 30 minutes after initiation of virus internalisation. CHO-β6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
5.3.2 Transferrin Receptor

A timecourse of αβ6-mediated internalisation of FMDV strain O1Kcad2 by CHO-β6 cells was carried out as described above. At each time point CHO-β6 cells were fixed, permeabilised and virus and the transferrin receptor (a marker for early and recycling endosomes) were labelled using monoclonal antibodies. Viral protein (red) was detected using the antibody D9, and the transferrin receptor (green) was detected using the monoclonal antibody H68.4 which was raised against the human transferrin receptor but cross-reacts with the hamster version. The results of these experiments are shown in figures 5.6-5.9. At 5 minutes after the initiation of internalisation (figure 5.6) there is some colocalisation between internalised virus and the transferrin receptor. By 10 minutes (figure 5.7), the majority of virus has been internalised and there is partial colocalisation between virus and the transferrin receptor was observed. At 15 and 30 minutes (figures 5.8 and 5.9 respectively), most virus positive structures within the cell also show transferrin receptor labelling. The above data suggests that FMDV is delivered to early and recycling endosomes by 15 minutes after the initiation of internalisation and remains predominantly within these compartments by 30 minutes.
Figure 5.6. Dual labelling of CHO-p6 cells for the transferrin receptor and FMDV at 5 minutes after initiation of virus internalisation. CHO-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5μg/ml) and D8 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.7. Dual labelling of CHO-p6 cells for the transferrin receptor and FMDV at 10 minutes after initiation of virus internalisation. CHO-p6 cells, prepared on coverslips were incubated with FMDV strain 01.Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.8. Dual labelling of CHO-pG cells for the transferrin receptor and FMDV at 15 minutes after initiation of virus internalisation. CHO-pG cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.9. Dual labelling of CHO-P6 cells for the transferrin receptor and FMDV at 30 minutes after initiation of virus internalisation. CHO-P6 cells, prepared on coverslips were incubated with FMDV strain 01Kcad2 (5ug/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and virus were detected using monoclonal antibodies H68.4 (mouse IgG1-5ug/ml) and D9 (mouse IgG2A-5ug/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show transferrin receptor labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
5.3.3 Lysosomal-associated Membrane Glycoprotein-2 (LAMP-2)

A timecourse of ανβ6-mediated internalisation of FMDV strain O1Kcad2 by CHO-β6 cells was carried out as described above. At each time point CHO-β6 cells were fixed, permeabilised and virus and LAMP-2 (a marker for late endosomes and lysosomes) were labelled using monoclonal antibodies. Viral protein (red) was detected using the antibody D9, and LAMP-2 (green) was detected using the monoclonal antibody H4B4 which is raised against human LAMP-2 but cross-reacts with the hamster version. The results of these experiments are shown in figures 5.10-5.12. Little or no colocalisation could be detected between virus and LAMP-2 at 10 (figure 5.10), 15 (figure 5.11), or 30 minutes (figure 5.12) after the initiation of internalisation. This data suggests that infection of CHO-β6 cells by FMDV does not require delivery of virus to late endosomes and lysosomes, and is in agreement with studies carried out using SW480-β6 cells. However, it is possible that virus is delivered to lysosomes but is rapidly degraded and therefore colocalisation is not observed. This scenario seems unlikely however since the level of virus labelling does not decline over the time period studied, and also viral protein remains associated with markers of early and recycling endosomes after 30 minutes.
Figure 5.10. Dual labelling of CHO-p6 cells for LAMP-2 and FMDV at 10 minutes after initiation of virus internalisation. CHO-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies H4B4 (mouse IgG1-0.5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.11. Dual labelling of CHO-pS cells for LAMP-2 and FMDV at 15 minutes after initiation of virus internalisation. CHO-pS cells, prepared on cover slips were incubated with FMDV strain 01Kcad2 (5 μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies H4B4 (mouse IgG1-0.5 μg/ml) and D9 (mouse IgG2A-5 μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.12. Dual labelling of CHO-K1 cells for LAMP-2 and FMDV at 30 minutes after initiation of virus internalisation. CHO-K1 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and virus were detected using monoclonal antibodies H4B4 (mouse IgG1-0.5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show LAMP-2 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
5.3.4 Caveolin-1

The data described above suggests that in CHO-β6 cells, FMDV is delivered via classical endocytic pathways to early- and recycling-endosomes just as is the case in SW480-β6 cells. Caveolin-1 is a major protein constituent of caveolae and acts as a marker protein for a non-classical endocytosis pathway, the caveolae-dependent uptake pathway (see section 1.5.2.1). Caveosomes and internalised caveolar vesicles are negative for classical early endosomal markers such as EEA-1 and transferrin, have a neutral pH, and contain caveolin-1 within their limiting membrane. The neutral pH within the caveosome would not be expected to trigger the acid-mediated uncoating of FMDV thought to be required for infection.

A timecourse of αvβ6-mediated internalisation of FMDV strain O1Kcad2 by CHO-β6 cells was carried out as described above. At each time point CHO-β6 cells were fixed, permeabilised and virus and caveolin-1 (a marker for early and recycling endosomes) were labelled using monoclonal antibodies. Viral protein (red) was detected using the antibody D9, and caveolin-1 (green) was detected using a rabbit polyclonal serum produced using an epitope mapping to the N-terminus of human caveolin-1. The results of these experiments are shown in figures 5.13-5.16. At 5 and 10 minutes (see figures 5.13 and 5.14 respectively) a limited amount of colocalisation between virus and caveolin-1 was observed at the cell surface and in intracellular vesicles. This most likely represents virus that is present in early endosomes, since caveolin-1 partially colocalises with EEA-1(334), as trafficking between caveosomes and early endosomes has been observed in a number of cell types. At 15 and 30 minutes (see figures 5.15 and 5.16 respectively) virtually no colocalisation between
virus and the caveolin-1 protein was seen. This data is not consistent with uptake of FMDV via caveolae-dependent endocytosis.
Figure 5.13. Dual labelling of CHO-P6 cells for Caveolin-1 and FMDV at 5 minutes after initiation of virus internalisation. CHO-P6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 5 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, Caveolin-1 and virus were detected using a rabbit polyclonal serum directed against the N-terminus of human Caveolin-1 (1/250) and the mouse monoclonal antibody D9 (mouse IgG2A 5µg/ml) respectively, followed by Alexa fluor conjugated goat anti-rabbit and goat anti-mouse secondary antibodies. Panels A and D show Caveolin-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.14. Dual labelling of CHO-p6 cells for Caveolin-1 and FMDV at 10 minutes after initiation of virus internalisation. CHO-p6 cells, prepared on coverslips were incubated with FMDV strain 01Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 10 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, Caveolin-1 and virus were detected using a rabbit polyclonal serum directed against the N-terminus of human Caveolin-1 (1/250) and the mouse monoclonal antibody D9 (mouse IgG2A-5µg/ml) respectively, followed by Alexa fluor conjugated goat anti-rabbit and goat anti-mouse secondary antibodies. Panels A and D show Caveolin-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.15. Dual labelling of CHO-p6 cells for Caveolin-1 and FMDV at 15 minutes after initiation of virus internalisation.

CHO-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, Caveolin-1 and virus were detected using a rabbit polyclonal serum directed against the N-terminus of human Caveolin-1 (1/250) and the mouse monoclonal antibody D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated goat anti-rabbit and goat anti-mouse secondary antibodies. Panels A and D show Caveolin-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 5.16. Dual labelling of CHO-p6 cells for Caveolin-1 and FMDV at 30 minutes after initiation of virus internalisation. 
CHO-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5µg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 30 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, Caveolin-1 and virus were detected using a rabbit polyclonal serum directed against the N-terminus of human Caveolin-1 (1/250) and the mouse monoclonal antibody D9 (mouse IgG2A-5µg/ml) respectively, followed by Alexa fluor conjugated goat anti-rabbit and goat anti-mouse secondary antibodies. Panels A and D show Caveolin-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
5.4 Summary and Discussion

The data presented in chapter 4 shows that virus is rapidly taken up by SW480-β6 cells and delivered to early/recycling endosomes, consistent with the biochemical data presented in chapter 3. However, SW480-β6 cells do not express caveolin-1 and therefore do not possess caveolae at their cell surface. It is possible therefore that the virus uses different uptake pathways in cell lines which do express caveolin-1. In order to investigate this possibility we carried out experiments using CHO-β6 cells, a cell line which expresses Caveolin-1 and therefore possesses caveolae.

A key feature of the infection of SW480-β6 cells by FMDV is that it requires a low pH step at an early stage (see section 3.2.3). The experiments outlined in section 5.2 investigate whether the early stages of infection of CHO-β6 cells by FMDV also require a low pH step. CHO-β6 cells were treated with concanamycin A, an agent which specifically inhibits the vacuolar H+-ATPase, and therefore dissipates the acidic pH within endosomes. 100 nM Concanamycin A inhibited infection of CHO-β6 cells by an average of 74% when cells were pretreated with the drug, indicating that infection of CHO-β6 cells by FMDV is dependent on a low pH step. This concentration of drug was shown to inhibit infection at an early stage since the addition of the drug at a later stage (i.e. immediately following removal of the virus inoculum) had virtually no effect on infection. These observations indicate that raising the endosomal pH inhibits infection at a stage, prior to virus RNA translation/replication, and post attachment to the cell surface. In contrast to the situation seen with SW480-β6 cells, higher concentrations of drug inhibited infection (10μM and 1μM) when added prior to the addition of virus and when added at a later stage. This indicates that higher concentrations of drug inhibit replication of virus as
well as cell entry. The data presented in section 5.2 show that infection of CHO-β6 cells by FMDV requires a low pH step at an early stage, just as is the case for SW480-β6 cells.

In chapter 4, FMDV was shown to be delivered to early- and recycling-endosomes in SW480-β6 cells by immunofluorescence confocal microscopy. Section 5.3 shows the results of similar experiments carried out using CHO-β6 cells. FMDV was allowed to enter cells for 5, 10, 15 or 30 minutes and the cells labelled for virus and markers of cellular compartments. The compartment markers used were EEA-1 (a marker of early endosomes), the transferrin receptor (a marker of early and recycling endosomes), LAMP-2 (a marker of lysosomes), and caveolin-1 (a marker of caveolae, caveosomes, and caveolae-dependent uptake pathways). At 5 and 10 minutes post-entry the majority of virus had been internalised into intracellular vesicle-like structures. By 15 and 30 minutes, virtually all virus had been internalised into larger intracellular vesicles. At 5 minutes, there was extensive colocalisation of internalised virus EEA-1, partial colocalisation with the transferrin receptor, and little or no colocalisation with LAMP-2. There was a small amount of colocalisation between virus and caveolin-1 at the cell surface and just below the plasma membrane. At 10 minutes extensive colocalisation between virus and EEA-1, partial colocalisation with the transferrin receptor, and little or no colocalisation with LAMP-2, was observed. Again at this time point a small amount of colocalisation can be observed between virus and caveolin-1 at the cell surface and intracellular vesicles, although the majority of virus was not colocalised with caveolin-1. At 15 minutes colocalisation can still clearly be seen between virus and EEA-1 but to a lesser extent than seen at 10 minutes. Extensive colocalisation between virus and the transferrin receptor, and very little colocalisation between virus and LAMP-2 or caveolin-1, were observed at this
time point. At 30 minutes virus can still clearly be seen to colocalise with EEA-1, but to a lesser extent than observed at the 10 and 15 minute time-points. Extensive colocalisation between virus and the transferrin receptor, and very little colocalisation between virus and LAMP-2 or caveolin-1, were again observed at this time point.

The above data shows that the majority of internalised virus is delivered to EEA-1 positive early-endosomes by 10 minutes after the initiation of virus internalisation. The level of colocalisation with transferrin receptor reaches a maximum at the later timepoints (15 and 30 minutes) presumably due to delivery of a proportion of virus from early to recycling endosomes. Viral protein does not appear to be delivered to lysosomes since no colocalisation was observed with LAMP-2 at any time point. A small amount of colocalisation of FMDV with caveolin-1 was detected at 5 and 10 minutes, and little or no colocalisation at 15 and 30 minutes. The small amount of colocalisation of virus with caveolin-1 presumably represents virus in early endosomes, since caveolin-1 has been shown to colocalise partially with the early endosomal marker EEA-1 in a number of cell types (334).

The above observations suggest that FMDV is delivered from the surface of CHO-β6 cells to early- and recycling-endosomes, but not late endosomes and lysosomes, just as is the case in SW480-β6 cells. This entry pathway is consistent with the low pH dependence of infection shown in section 5.2. These data are inconsistent however with virus uptake via caveolae-dependent endocytosis.

The virus SV40 is taken up by caveolae-dependent endocytosis and delivered to the neutral pH caveosome, and so is not exposed to acidic pH. Caveolae-dependent endocytosis is also a relatively slow process in comparison to the clathrin-dependent pathway and SV40 has a half time for internalisation of around 60 minutes (251). FMDV however is almost completely internalised within 15 minutes, a rapid
timescale which is not consistent with uptake via caveolae-dependent endocytosis. FMDV also displayed very little colocalisation with caveolin-1, a marker of caveolae-dependent endocytosis, yet colocalised extensively with markers of early and recycling endosomes. Again this is not consistent with uptake of virus by caveolae-dependent endocytosis.

FMDV is rapidly delivered to early/recycling endosomes in both SW480-β6 and CHO-β6 cells. In CHO-β6 cells, virus still colocalises with early and recycling endosome markers after 30 minutes, whereas in SW480-β6 cells virus no longer colocalised with these markers, or the lysosome marker LAMP-2, at this time point. It is possible that transfer of viral protein onwards from early and recycling endosomes may occur more slowly in CHO-β6 cells or it may not occur at all. It is unlikely that this difference between the two cell lines has any bearing on infection however, as the extreme sensitivity of the FMDV capsid to low pH makes it highly probable that the viral RNA has already been released into the cytoplasm before the virus reaches recycling endosomes. Human rhinovirus 2 (HRV-2) undergoes a conformational change triggered by pH values less than 5.6, which are present in late endosomal compartments (261) (endosomal carrier vesicles and late endosomes). These compartments are the first compartments the virus enters with sufficiently low pH to trigger this conformational change. This low pH-triggered conformational change leads to viral uncoating and delivery of viral RNA to the cytoplasm from late endosomes (262). Uncoating of the FMDV capsid occurs at pH values just below neutrality, meaning that the first compartment reached with a pH low enough to trigger this event is the early endosome. Transfer of viral RNA to the cytoplasm across the endosomal membrane would be expected to occur soon afterwards, as is the case for HRV-2. Since the transfer of viral RNA to the cytoplasm is all that is
required to initiate infection of tissue culture cells by FMDV, the fate of internalised
viral capsid protein after it has left early and recycling endosomes and parted ways
with viral RNA is unlikely to make a difference to infection.

Taken together, the data presented in chapters 3, 4, and 5 show that infection of both
SW480-β6 cells and CHO-β6 cells requires an acid pH step at an early stage, and also
delivery of virus from the cell surface to early- and recycling-endosomes. The data is
inconsistent with a requirement for caveolae-dependent endocytosis for infection,
since FMDV still infects cells lacking caveolae (SW480-β6), and does not appear to
utilise this pathway in cells which possess caveolae (CHO-β6). The data presented
here suggest that FMDV utilises a very similar entry pathway both in cells which lack
caveolae and cells which possess caveolae, namely clathrin-dependent delivery of
virus from the cell surface to acidic early and recycling endosomes.
Chapter Six: The role of the Integrin αvβ6 in infection of SW480-β6 cells by FMDV

6.1 Introduction

The integrin αvβ6 has been shown to act as an attachment receptor for FMDV on the surface of SW480-β6 and CHO-β6 cells (145, 147). Virus binding (and infection) on these cells is blocked by a function blocking antibody directed against αvβ6, and by peptides containing the RGD integrin-binding motif. The parent cell lines (SW480 and CHO cells) are not able to bind virus or to support FMDV infection since they do not express the β6 integrin subunit and therefore do not express the integrin αvβ6 at their cell surface. However, despite its role as an attachment receptor, little is known of the role of the integrin αvβ6 in subsequent virus internalisation and infection. Deletion of virtually the entire cytoplasmic domain of the β6 integrin subunit does not prevent virus binding to αvβ6 when this integrin is expressed at the surface of SW480-β6 cells, yet virtually abolishes infection (204). This shows that the integrin is involved in post-attachment events, but does not provide evidence as to its role. Therefore the role of the integrin in the events following the initiation of virus internalisation was investigated using immunofluorescence confocal microscopy.

6.2 The Role of the Integrin αvβ6 in FMDV infection of SW480-β6 cells

6.2.1 FMDV Binding to SW480-β6 Cells
The integrin αvβ6 has been previously shown to act as the major receptor for virus attachment to SW480-β6 cells using flow cytometry (147). To further investigate this observation, attachment of virus to the surface of SW480-β6 cells was investigated by immunofluorescence microscopy. Virus was allowed to bind to the cell surface for 45 minutes at 4°C, and excess virus was removed by washing. Cell surface virus and the integrin αvβ6 were labelled using monoclonal antibodies D9 and E7P6 respectively on unpermeabilised cells. Figure 6.1 shows that virus bound to the surface of SW480-β6 cells (red – panels A and D), and cell surface αvβ6 (green – panels B and E) both adopt a very similar punctate labelling pattern, which is evenly distributed over the plasma membrane. When the two channels are overlayed (panels C and F) it can be seen that virus almost completely colocalises with integrin at the cell surface, consistent with the role of αvβ6 as the primary attachment receptor for FMDV on SW480-β6 cells.

The distribution of the integrins αvβ6 and αvβ5 at the surface of SW480-β6 cells were also compared. αvβ5 is an RGD-dependent integrin, but does not act as a receptor for FMDV, since wild-type SW480 cells (i.e. not transfected with the β6 subunit and therefore not expressing αvβ6) express this molecule but do not support FMDV binding or infection. Unpermeabilised SW480-β6 cells were labelled for cell surface αvβ6 (green) and αvβ5 (red) using the function-blocking monoclonal antibodies 10D5 and P1F6 respectively. Figure 6.2 shows this data. Both integrins display a punctate labelling pattern, but there is not extensive colocalisation between αvβ6 and αvβ5 at the cell surface, although the distribution of the two integrins does overlap. This experiment shows that αv integrins are not all associated with one another in discrete membrane domains, but instead show differing locations at the cell
surface. Since αvβ6 and αvβ5 do not colocalise extensively, it is unlikely that the
colocalisation observed between FMDV and αvβ6 occurs by chance.

Experiments were carried out to further explore the FMDV-attachment receptors on
SW480-β6 cells. FMDV was prebound to SW480-β6 cells and the integrins αvβ6
and αvβ5 were detected at the cell surface using monoclonal antibodies (MAbs) 10D5
and P1F6 respectively. These MAbs are function blocking antibodies, i.e. they block
the binding of natural ligands to their integrin receptors. It was reasoned that binding
of a large multivalent ligand, such as FMDV, to the RGD-binding site on the integrin
would be expected to block binding of the MAb. Figure 6.3 shows a number of cells
which have been labelled for αvβ6 and αvβ5 in the absence (Panel A) and presence
(Panel B) of prebound virus. In the absence of prebound virus (Panel A) cell surface
labelling for both integrins (αvβ6 (green) and αvβ5 (red)) can be seen. However in
the presence of prebound virus (Panel B), the αvβ6 labelling (green) is reduced,
whilst the αvβ5 labelling is similar to that seen in panel A. These observations are
consistent with the role of αvβ6 (but not αvβ5) as the major attachment receptor for
FMDV on this cell line.
Figure 6.1. FMDV colocalises with the integrin αvβ6 at the surface of SW480-P6 cells. FMDV strain O1Kcad2 (5μg/ml) was bound to the surface of SW480-P6 cells for 45 minutes at 4°C. Cells were not permeabilised and all subsequent steps were carried out on ice. Virus and the integrin αvβ6 were detected using monoclonal antibodies D9 (mouse IgG2A-5μg/ml) and E7P6 (mouse IgG1-20μg/ml), followed by Alexa fluor conjugated goat-anti mouse isotype specific secondary antibodies. Panels A and D show αvβ6 at the cell surface (red) and panels B and E show FMDV (green) at the cell surface. Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 6.2. The integrin αvβ6 and αvβ5 partially colocalise at the surface of SW480-P6 cells. Integrins αvβ6 and αvβ5 were detected using monoclonal antibodies 10D6 (mouse IgG2A-20µg/ml) and P1F6 (mouse IgG1-20µg/ml), followed by Alexa fluor conjugated goat-anti mouse isotype specific secondary antibodies. Cells were not permeabilised and all steps were carried out on ice. Panels A and D show αvβ6 at the cell surface (red) and panels B and E show αvβ5 (green) at the cell surface. Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 6.3. FMDV blocks the binding of a function blocking antibody against the integrin αvβ5 but not αvβ15 at the surface of SW480-p6 cells. SW480-p6 cells were incubated with FMDV strain O1Kcad2 in DMEM (5μg/ml) (Panel B) or DMEM alone (Panel A) for 45 minutes at 4°C. Cells were not permeabilised and all subsequent steps were carried out on ice. The integrins αvβ6 (green) and αvβ15 (red) at the cell surface were detected using function blocking monoclonal antibodies 10D5 (mouse IgG2A-20μg/ml) and P1F6 (mouse IgG1-20μg/ml) respectively, followed by Alexa fluor conjugated goat-anti mouse isotype specific secondary antibodies. DAPI stained nuclei are shown as blue. Bars = 20μm. In the absence of surface bound FMDV (Panel A) both αvβ6 (green) and αvβ15 (red) can be detected at the cell surface. In the presence of prebound FMDV (Panel B), only αvβ15 labelling is still observed but αvβ6 labelling is virtually absent. This indicates that FMDV binds αvβ6 but not αvβ15 at the surface of SW480-p6 cells, since virus bound to its cell surface receptor has blocked the binding of the anti-αvβ6 function blocking antibody 10D5, but not the anti-αvβ15 function blocking antibody P1F6.
6.2.2 The cell surface distribution of ανβ6

As shown in figure 6.1, ανβ6 labelling at the surface of SW480-β6 cells shows a punctate distribution. The plasma membrane also contains cholesterol and sphingolipid rich lipid rafts, which are specialised membrane domains that act as platforms for cell signalling and endocytic uptake pathways. The experiments described in chapters 3, 4, and 5 show that infection by FMDV does not require intact lipid rafts or caveolae-dependent endocytosis. To investigate whether ανβ6 is associated with lipid rafts at the cell surface the B subunit of cholera toxin (CTB) was utilised. CTB binds the ganglioside GM1 which is located predominantly within lipid rafts. Fluorescently conjugated CTB can therefore be used as a marker for lipid rafts at the cell surface. CTB was allowed to bind SW480-β6 cells for 45 minutes at 4°C, before being crosslinked with a rabbit anti-cholera toxin polyclonal serum. Janes et al. (1999) have utilised this method of lipid raft visualisation previously and showed that CTB labelled membrane patches display characteristics consistent with biochemically isolated lipid rafts (149). Following paraformaldehyde fixation, ανβ6 at the cell surface was labelled using the monoclonal antibody E7P6. Figure 6.4 shows that ανβ6 (red) and CTB (green) display minimal colocalisation at the cell surface indicating that ανβ6 is predominantly not located within lipid rafts, at least in the absence of ligand. A number of cell surface receptors have been shown to only partition preferentially to lipid rafts following crosslinking by antibody or multivalent ligand. Examples include the folate receptor and the integrin α6β4 (89). It is therefore possible that binding and crosslinking of ανβ6 by ligand could cause a redistribution of ανβ6 such that it colocalises with lipid raft markers to a greater extent. In order to test this hypothesis the experiment shown in figure 6.4 was
repeated except that FMDV was added to the cells at the same time as fluorescently labelled CTB in order to crosslink surface integrin. As shown in figure 6.5, crosslinking of αvβ6 by virus did not increase the degree of colocalisation of αvβ6 (red) with crosslinked CTB (green) at the surface of SW480-β6 cells. This indicates that αvβ6 does not partition preferentially to lipid raft microdomains at the cell surface when crosslinked by ligand.

In order to investigate whether virus bound to the cell surface is associated with lipid rafts, CTB and virus were prebound to SW480-β6 cells for 45 minutes at 4°C. CTB at the plasma membrane was then crosslinked with a rabbit anti-cholera toxin polyclonal serum (in order to visualise lipid rafts). Following paraformaldehyde fixation, FMDV bound to the cell surface was visualised using the monoclonal antibody D9 and an Alexa-568 conjugated goat anti-mouse IgG2A secondary antibody. As shown in figure 6.6, FMDV (red) and lipid rafts (green) did not colocalise at the surface of SW480-β6 cells. The degree of colocalisation is if anything less than that observed between αvβ6 and crosslinked CTB. These experiments show that neither αvβ6 nor virus at the cell surface is associated with lipid rafts, and hence would not be expected to be internalised by lipid raft dependent endocytic uptake pathways. These observations are consistent with the insensitivity of infection of SW480-β6 cells to the disruption of lipid rafts and lipid raft dependent endocytosis by cholesterol depletion (see section 3.2.2.1).
Figure 6.4. Colocalisation of the integrin αvβ6 with a marker for lipid rafts. Alexa-488 conjugated cholera toxin B (CTB-0.2μg/ml) was bound to the surface of SW480-16 cells for 45 minutes at 4°C. CTB was crosslinked using an anti cholera toxin polyclonal rabbit anti serum. Following fixation with 4% paraformaldehyde, the integrin αvβ6 was detected using the monoclonal antibody E7P6 (mouse IgG1-20μg/ml), followed by an Alexa fluor-668 conjugated goat-anti mouse secondary antibody. Panels A and D show αvβ6 at the cell surface (red) and panels B and E show crosslinked CTB (green) at the cell surface. Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. αvβ6 at the cell surface does not show extensive colocalisation with a marker of lipid rafts (crosslinked CTB).
Figure 6.5. Colocalisation of the integrin αvβ6 with a marker for lipid rafts following binding of virus FMDV strain O1Kcad2 (5μg/ml) and Alexa-488 conjugated cholera toxin B (CTB-0.2μg/ml) were bound to the surface of SW480-αv cells for 45 minutes at 4°C. CTB was crosslinked using an anti cholera toxin polyclonal rabbit anti serum. Following fixation with 4% paraformaldehyde, the integrin αvβ6 was detected using the monoclonal antibody E7P6 (mouse IgG1-20μg/ml), followed by an Alexa fluor-568 conjugated goat-anti mouse secondary antibody. Panel A and D show αvβ6 at the cell surface (red) and panels B and E show crosslinked CTB (green) at the cell surface. Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. αvβ6 at the cell surface does not show extensive colocalisation with a marker of lipid rafts (crosslinked CTB) following binding of FMDV.
Figure 6.6. FMDV does not colocalise with a marker for lipid rafts. FMDV strain O1Kcad2 (5μg/ml) and Alexa-488 conjugated cholera toxin B (CTB-0.2μg/ml) were bound to the surface of SW480-j6 cells for 45 minutes at 4°C. CTB was crosslinked using an anti cholera toxin polyclonal rabbit anti serum. Following fixation with 4% paraformaldehyde, surface bound virus was detected using the monoclonal antibody D9 (mouse IgG2A-5μg/ml), followed by an Alexa fluor-568 conjugated goat-anti mouse isotype specific secondary antibody. Panels A and D show FMDV at the cell surface (red) and panels B and E show crosslinked CTB (green) at the cell surface. Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. FMDV at the cell surface does not colocalise with a marker of lipid rafts (crosslinked CTB), indicating that FMDV endocytosis is not dependent on lipid rafts.
6.2.3 The Role of αvβ6 in Post Attachment Events

From the data presented above and previous reports, it is clear that αvβ6 acts as the primary attachment receptor on the surface of SW480-β6 cells. The role of this integrin in post-attachment events is however less clear. Deletion mutations within the cytoplasmic domain of the β6 integrin subunit which eliminate the NPXY putative endocytosis motif, produce receptors that still support FMDV binding but no longer mediate infection (204). This implies that αvβ6 has a role in post-attachment events, but does not give an indication as to which stage the block in infection occurs. Therefore experiments were carried out to determine whether the integrin αvβ6 can mediate virus internalisation as well as binding at the cell surface.

Virus bound to the cell surface at 4°C colocalises with αvβ6 (see figure 6.1). If αvβ6 acts to internalise virus particles, then virus and αvβ6 would be expected to colocalise with one another inside cells at early times following the initiation of virus internalisation. FMDV was allowed to bind SW480-β6 cells at 4°C, and internalisation was initiated by shifting the temperature to 37°C. After 5 or 10 minutes at 37°C internalisation was halted by shifting the temperature back to 4°C and paraformaldehyde fixation. Following Triton permeabilisation, virus and αvβ6 were labelled using the monoclonal antibodies D9 and 4B5 respectively (131). At 5 minutes (figure 6.7) virus (green) and the integrin αvβ6 (red) were colocalised inside the cell. At 10 minutes (figure 6.8) virus was still colocalised with αvβ6. These observations strongly suggest that αvβ6 serves not only as an attachment receptor, but also to deliver the virus to early endosomes.

If virus internalisation is mediated by αvβ6, then initiation of virus uptake would be expected to cause a change in the distribution of this integrin. Experiments designed
to examine whether this is the case were therefore carried out. SW480-β6 cells were double labelled for αvβ6 and markers of intracellular compartments (i.e. EEA-1, the transferring receptor and LAMP-2) in the presence or absence of virus internalisation. One group of coverslips had no virus added prior to fixation and double labelling. The other group of coverslips were incubated with virus at 4°C to allow virus binding to the cells, before initiation of virus internalisation for 10 minutes at 37°C. The cells were then fixed and double labelled for αvβ6 and cellular compartment markers as above. In this way, the distribution of αvβ6, and the level of colocalisation of αvβ6 with compartment markers at steady state, could be compared with that observed fixed after 10 minutes of virus internalisation. In the absence of virus (see figures 6.9, 6.11, and 6.13 – panels B and E), the integrin labelling is relatively diffuse, and is present at the cell surface and in small intracellular vesicles. Following virus internalisation however, the integrin labelling appears more intense, and is predominantly found in larger intracellular vesicular structures (see figures 6.10, 6.12, and 6.14 – panels B and E). Internalisation of virus therefore seems to be concomitant with an alteration in the distribution of αvβ6. SW480-β6 cells fixed after incubation in the absence of virus, or after 10 minutes of virus internalisation, were double labelled for αvβ6 and EEA-1 (a marker of early endosomes) and are shown in figure 6.9 and 6.10 respectively. In the absence of virus, αvβ6 (red) colocalises little with EEA-1 (green) (see figure 6.9 panels C and F). Following 10 minutes of virus internalisation, αvβ6 is found in larger intracellular vesicle-like structures and colocalises with EEA-1 to a greater extent (see figure 6.10 panels C and F). SW480-β6 cells double labelled for αvβ6 and the Transferrin Receptor (a marker of early and recycling endosomes) in the absence of virus and following 10 minutes virus internalisation are shown in figure 6.12 and 6.11 respectively. In the
absence of virus the integrin $\alpha v\beta 6$ (red) is found at the cell surface and in small intracellular vesicles and colocalises little with the transferrin receptor (green) (see figure 6.11 panels C and F). Following 10 minutes virus internalisation $\alpha v\beta 6$ labelling is more intense, is found in larger intracellular vesicle-like structures, and colocalises with the transferrin receptor to a much greater extent (see figure 6.12 panels C and F). When similar experiments were carried out involving the marker LAMP-2 (a lysosomal marker protein), the distribution of integrin changed as described above following virus internalisation, but there was little colocalisation of $\alpha v\beta 6$ and LAMP-2 either in the absence of virus (figure 6.13 panels C and F) or following 10 minutes virus internalisation (figure 6.14 Panels C and F). The above data indicates that internalisation of FMDV bound to the surface of SW480-$\beta 6$ cells causes a change in the distribution of the integrin $\alpha v\beta 6$, and greater colocalisation with markers of early- and recycling-endosomes (the very compartments to which virus is delivered). These observations are consistent with a model whereby FMDV binding to the cell surface and delivery to early- and recycling-endosomes are both mediated by the RGD-dependent integrin $\alpha v\beta 6$. 
Figure 6.7. FMDV colocalises with the integrin αvβ6 at 5 minutes after the initiation of virus internalisation. FMDV strain O1Kcad2 (5 μg/ml) was bound to the surface of SW480-P6 cells for 45 minutes at 4°C. Internalisation of bound virus was initiated by incubation at 37°C. After 5 minutes at 37°C virus internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation using 0.1% Triton, virus and the integrin αvβ6 were detected using monoclonal antibodies D9 (mouse IgG2A-5 μg/ml) and 4B5 (Rabbit IgG), followed by Alexa fluor conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. Panels A and D show αvβ6 labelling (red) and panels B and E show FMDV (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 6.8. FMDV colocalises with the integrin αvβ6 at 10 minutes after the initiation of virus internalisation. FMDV strain O1Kcad2 (5μg/ml) was bound to the surface of SW480-β6 cells for 45 minutes at 4°C. Internalisation of bound virus was initiated by incubation at 37°C. After 10 minutes at 37°C virus internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation using 0.1% Triton, virus and the integrin αvβ6 were detected using monoclonal antibodies D9 (mouse IgG2A-5μg/ml) and 4B5 (Rabbit IgG), followed by Alexa fluor conjugated goat-anti mouse and goat anti rabbit secondary antibodies. Panels A and D show αvβ6 labelling (red) and panels B and E show FMDV (green). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 6.9. The integrin αvβ6 colocalises little with EEA-1 in the absence of FMDV. SW480-β6 cells, prepared on coverslips were fixed using 4% paraformaldehyde and permeabilised using 0.1% Triton. A marker of early endosomes (EEA-1) and the integrin αvβ6 were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and 4B5 (Rabbit IgG), followed by Alexa fluor conjugated goat-anti mouse and goat anti-rabbit secondary antibodies. Panels A and D show EEA-1 (green) and panels B and E show integrin αvβ6 labelling (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 6.10. The integrin αvβ6 colocalises to a greater extent with EEA-1 following FMDV internalisation. SW480-86 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by incubation for 10 minutes at 37°C. Internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, a marker of early endosomes (EEA-1) and the integrin αvβ6 were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and 4B5 (Rabbit IgG-Neat) respectively, followed by Alexa fluor conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show labelling for αvβ6 (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. Comparison of panels C and F with those same panels in figure 6.9 shows that virus internalisation causes a change in the distribution of the integrin αvβ6 and greater colocalisation of virus with EEA-1.
Figure 6.11. The integrin αvβ6 colocalises little with the transferrin receptor in the absence of FMDV. SW480-β6 cells, prepared on coverslips were fixed using 4% paraformaldehyde and permeabilised using 0.1% Triton. The transferrin receptor and the integrin αvβ6 were detected using monoclonal antibodies H68.4 (mouse IgG1-5µg/ml) and 4B5 (Rabbit IgG), followed by Alexa fluor conjugated goat-anti mouse and goat anti rabbit secondary antibodies. Panels A and D show the transferrin receptor (green) and panels B and E show integrin αvβ6 labelling (red). Panels C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 6.12. The integrin $\alpha v\beta 6$ colocalises to a greater extent with the transferrin receptor following FMDV internalisation. SW480-$\beta 6$ cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5 $\mu$g/ml) for 45 minutes at 4°C. Virus internalisation was initiated by incubation for 10 minutes at 37°C. Internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the transferrin receptor and the integrin $\alpha v\beta 6$ were detected using monoclonal antibodies H68.4 (mouse IgG1-5$\mu$g/ml) and 4B5 (Rabbit IgG- neat) respectively, followed by Alexa fluor conjugated goat-anti mouse and goat anti rabbit secondary antibodies. Panels A and D show transferrin receptor labelling (green) and panels B and E show labelling for $\alpha v\beta 6$ (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. Comparison of panels C and F with those same panels in figure 6.11 shows that virus internalisation causes a change in the distribution of the integrin $\alpha v\beta 6$ and greater colocalisation of virus with the transferrin receptor.
Figure 6.13. The integrin αvβ6 colocalises little with LAMP-2 in the absence of FMDV. SW480 β6 cells, prepared on coverslips were fixed using 4% paraformaldehyde and permeabilised using 0.1% Triton. A marker of lysosomes (LAMP-2) and the integrin αvβ6 were detected using monoclonal antibodies H4B4 (mouse IgG1-0.5μg/ml) and 4B5 (Rabbit IgG), followed by Alexa fluor conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. Panels A and D show LAMP-2 (green) and panels B and E show integrin αvβ6 labelling (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 6.14. The integrin αvβ6 colocalises little with LAMP-2 following FMDV internalisation. SW480-β6 cells, prepared on coverslips were incubated with FMDV strain O1Kcard2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by incubation for 10 minutes at 37 °C. Internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, LAMP-2 and the integrin αvβ6 were detected using monoclonal antibodies H4B4 (mouse IgG1-0.5μg/ml) and 4B5 (Rabbit IgG) respectively, followed by Alexa fluor conjugated goat-anti mouse and goat anti rabbit secondary antibodies. Panels A and D show LAMP-2 (green) and panels B and E show labelling for αvβ6 (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. Comparison of panels C and F with those same panels in figure 6.13 shows that virus internalisation causes a change in the distribution of the integrin αvβ6 but integrin does not colocalise with LAMP-2 in the presence or absence of virus.
6.2.4 The Trigger for \( \alpha\nu\beta6 \) Internalisation

The evidence presented above suggests that FMDV requires only the integrin \( \alpha\nu\beta6 \) for binding to and internalisation by SW480-\( \beta6 \) cells. The rate of internalisation of FMDV is consistent with uptake of the virus:integrin complex being triggered by the virus rather than simply mediated by membrane turnover. If this is the case, the trigger could be occupation of the ligand binding site on \( \alpha\nu\beta6 \), \( \alpha\nu\beta6 \) cross linking, or a combination of both ligand-binding and integrin cross-linking. In order to investigate these possibilities, integrin-binding peptides, a ligand mimetic function-blocking anti-\( \alpha\nu\beta6 \) antibody, and a non-ligand mimetic function-blocking anti-\( \alpha\nu\beta6 \) antibody were utilised. The change in \( \alpha\nu\beta6 \) distribution observed when virus internalisation is allowed to occur was used to indicate that internalisation of \( \alpha\nu\beta6 \) had been triggered.

The first experiment was designed to assess whether occupation of the RGD-binding site of \( \alpha\nu\beta6 \) was sufficient to initiate its internalisation. A 12-mer RGD-containing peptide whose sequence corresponds to the GH loop of type O FMDV, and the equivalent RGE peptide were utilised. At the concentrations used (10\( \mu \)M) virus binding to SW480-\( \beta6 \) cells is completely blocked by the RGD but not the RGE peptide (147), indicating that this concentration of the former peptide results in the occupation of virtually all available virus binding sites. The peptides were allowed to bind at 4\( ^\circ \)C, before incubation at 37\( ^\circ \)C for 10 minutes in order to allow any integrin internalisation to occur. One group of coverslips had no peptide added prior to fixation. The cells were then double labelled for \( \alpha\nu\beta6 \) (red) and EEA-1 (green) using monoclonal antibodies 4B5 and 14 respectively. The distribution of integrin observed
was no different when SW480-β6 cells were treated with the RGD peptide (figure 6.17), RGE peptide (figure 6.18), or with no peptide at all (figure 6.15). In all cases integrin was primarily found at the cell surface and in small intracellular vesicles, and colocalised little with EEA-1. In contrast, when SW480-β6 cells were treated with virus (figure 6.16) in place of peptide, αvβ6 was found primarily in larger intracellular vesicles and colocalised with EEA-1 to a greater extent. This indicates that, unlike virus, the RGD-peptide was unable to trigger internalisation of αvβ6, and hence that ligand binding alone is insufficient to trigger internalisation of αvβ6.

The second experiment was designed to investigate whether αvβ6 crosslinking acts as a trigger for its internalisation. For these studies two anti-αvβ6 function blocking antibodies were utilised. The antibody 6.8G6 contains the integrin binding RGD-motif, and so is classified as a "ligand mimetic" anti-αvβ6 function-blocking antibody (329). The antibody 6.3G9 is an anti-αvβ6 function-blocking antibody but contains no such RGD-like motif and so is classed as non-ligand mimetic (329). Consistent with this, binding of 6.8G6 but not 6.3G9 to αvβ6 is inhibited by an RGD peptide (329). Antibody 6.8G6 therefore crosslinks αvβ6 and occupies the ligand binding site, whereas antibody 6.3G9 merely causes αvβ6 crosslinking. Experiments using these antibodies were carried out in the same way as those using RGD and RGE peptides. Cells were either not treated with antibody, or were treated with antibodies 6.8G6 or 6.3G9 (at a concentration of 10μg/ml). The antibodies were bound to the cell surface at 4°C followed by a 10 minute incubation at 37°C. Following paraformaldehyde fixation, labelling for αvβ6 was carried out using the rabbit monoclonal antibody 4B5. It was not possible to also carry out labelling for EEA-1 since 6.8G6, 6.3G9 and the anti-EEA-1 antibody are all mouse isotype IgG1. As
shown in figure 6.19, the distribution of integrin following treatment with the non-
ligand mimetic monoclonal antibody 6.3G9 (Panels C and F) was virtually the same
as that observed when no antibody was added prior to fixation (Panels A and D).
However following treatment with the ligand mimetic monoclonal antibody 6.8G6
(Panels B and E), the distribution of αvβ6 was altered compared to that observed in
the absence of crosslinking antibody, and appeared similar to that observed following
10 minutes of virus internalisation (figure 6.8). It therefore appears that antibody
6.8G6, but not 6.3G9, is capable of triggering internalisation of αvβ6. This data
suggests that crosslinking of αvβ6 alone is insufficient to trigger αvβ6 internalisation.
Instead αvβ6 must be both crosslinked, and the RGD binding site occupied, in order
to trigger αvβ6 internalisation. This is consistent with a model whereby RGD-
dependent binding and crosslinking of αvβ6 by multivalent FMDV particles at the
cell surface triggers endocytic uptake of the integrin:virion complex.
Figure 6.15. The integrin αvβ6 colocalises little with EEA-1 in the absence of Peptide. SW480-16 cells, prepared on coverslips were fixed using 4% paraformaldehyde and permeabilised using 0.1% Triton. A marker of early endosomes (EEA-1) and the integrin αvβ6 were detected using monoclonal antibodies 14 (mouse IgGl-5μg/ml) and 4B5 (Rabbit IgG), followed by Alexa fluor conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. Panels A and D show EEA-1 (green) and panels B and E show integrin αvβ6 labelling (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 6.16. The integrin αvβ6 colocalises to a greater extent with EEA-1 following FMDV internalisation. SW480-β6 cells, prepared on coverslips were incubated with FMDV strain O1Kac2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by incubation for 10 minutes at 37°C. Internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, a marker of early endosomes (EEA-1) and the integrin αvβ6 were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and 4B5 (Rabbit IgG) respectively, followed by Alexafluor conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show labelling for αvβ6 (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. Comparison of panels C and F with those same panels in figure 6.15 shows that virus internalisation causes a change in the distribution of the integrin αvβ6 and greater colocalisation of virus with EEA-1.
Figure 6.17. A 17-mer RGD peptide does not alter the distribution of the integrin αvβ6. SW480-β6 cells, prepared on coverslips were incubated with a 17-mer RGD peptide corresponding to the GH loop of FMDV (10μM) for 45 minutes at 4°C. Coverslips were then incubated for 10 minutes at 37°C in order to allow any integrin internalisation to occur. Internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, a marker of early endosomes (EEA-1) and the integrin αvβ6 were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and 485 (Rabbit IgG) respectively, followed by Alexa fluor conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show labelling for αvβ6 (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. Comparison of panels C and F with those same panels in figure 6.15 shows that RGD peptide does not alter the distribution of the integrin αvβ6, unlike whole virus particles (see figure 6.16), hence occupation of the RGD binding site of αvβ6 is insufficient to trigger its internalisation.
Figure 6.18. A 17-mer RGE peptide does not alter the distribution of the integrin αvβ6. SW480-β6 cells, prepared on coverslips were incubated with a 17-mer RGE peptide corresponding to the GH loop of FMDV (10μM) for 45 minutes at 4°C. This peptide does not block virus binding. Coverslips were then incubated for 10 minutes at 37°C in order to allow any integrin internalisation to occur. Internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, a marker of early endosomes (EEA-1) and the integrin αvβ6 were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and 4B5 (Rabbit IgG-Neat) respectively, followed by Alexa fluor conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show labelling for αvβ6 (red). Panel C shows an overlay of panels A and B, Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue. Comparison of panels C and F with those same panels in figure 6.15 shows that the RGE peptide does not alter the distribution of the integrin αvβ6, unlike whole virus particles (see figure 6.16), as expected since this peptide does not block virus binding.
Figure 6.19. The ligand mimetic function-blocking anti-αvβ6 antibody 6.8G6 triggers internalisation of αvβ6. SW480-αvβ6 cells, prepared on coverslips, were incubated with a ligand mimetic function blocking anti-αvβ6 antibody 6.8G6 (Panels B and E), the non-ligand mimetic function-blocking anti-αvβ6 antibody 6.3G9 (Panels C and F), or with DMEM (Panels A and D) for 1 hour at 4°C. Coverslips were then incubated for 10 minutes at 37°C in order to allow any integrin internalisation to occur. Internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, the integrin αvβ6 was detected using the monoclonal antibody 4B5 (Rabbit IgG), followed by an Alexa fluor goat anti-rabbit secondary antibody. DAPI stained nuclei are shown as blue. Incubation with a non-ligand mimetic function blocking anti-αvβ6 antibody 6.3G9 prior to fixation (Panels C and F) did not alter the distribution of integrin observed in the absence of antibody (Panels A and D). In contrast, the ligand mimetic function-blocking anti-αvβ6 antibody 6.8G6 (Panels B and E) caused a redistribution of integrin similar to that observed upon FMDV internalisation (see figures 6.9-6.14).
6.3 Summary and Discussion

The RGD-dependent integrin αvβ6 has been reported previously to act as the major attachment receptor for FMDV on SW480-β6 cells (147). Consistent with this αvβ6 colocalises extensively with virus at the surface of SW480-β6 cells (figure 6.1). Also, virus binding to the cell surface prevents binding of a function blocking antibody against αvβ6, but not a function blocking antibody against αvβ5 which does not act as a receptor for FMDV. These data confirm previous reports that αvβ6 is the major FMDV attachment receptor in this cell line. αvβ6 was shown to colocalise with a marker of lipid rafts (crosslinked CTB) at the cell surface in the presence or absence of virus. Similarly the virus did not colocalise with lipid rafts. These observations are consistent with the insensitivity of infection of SW480-β6 cells by FMDV to the disruption of lipid rafts and lipid raft dependent endocytosis by cholesterol depletion (see section 3.2.2.1).

The role of αvβ6 in post-attachment events has been less well defined. Certain deletion mutations within the cytoplasmic domain of the β6 integrin subunit prevent FMDV infection of, but not binding to, SW480-β6 cells, suggesting that αvβ6 plays a role in post-attachment events. The role of αvβ6 in post-attachment events was further investigated as part of this study (section 6.2.2). Internalised virus remained colocalised with its integrin receptor αvβ6 at early times after the initiation of internalisation (5 and 10 minutes). This is consistent with a model whereby virus internalisation is also mediated by αvβ6. Additionally, the distribution of integrin was altered following internalisation of virus. In the absence of virus, αvβ6 is primarily located at the cell surface and in small intracellular vesicles. Following virus
internalisation αvβ6 labelling is less diffuse and is primarily located in larger intracellular vesicles. Following virus internalisation the level of colocalisation of αvβ6 with markers of early and recycling endosomes (EEA-1 and the Transferrin Receptor) is also increased. The initiation of virus internalisation therefore seems to result in a recruitment of αvβ6 to early and recycling endosomes, which are the very compartments to which virus is recruited (see chapter 4).

Occupation of the RGD binding site and crosslinking are both required to trigger internalisation of αvβ6, since a reagent capable of achieving both these things (monoclonal antibody 6.8G6) was able to trigger αvβ6 internalisation, whereas reagents capable of achieving only one of these things (RGD peptide, monoclonal antibody 6.3G9) could not. This is consistent with the findings of Weinreb et. al. (329), who found that non ligand mimetic anti-αvβ6 function blocking antibodies were not able to induce internalisation of this integrin, whereas ligand mimetic anti-αvβ6 function blocking antibodies were able to do so. Interestingly the complementarity determining region of the antibody 6.8G6 contains the integrin-binding motif RGDRXXL (in CDR H3), which is also found in the majority of strain SAT-2 isolates of FMDV.

In summary, evidence has been presented to show that FMDV is delivered to early endosomes whilst still bound to αvβ6, and therefore that αvβ6 serves not only as an attachment receptor, but also to internalise virus. The data presented here therefore support the following model: Virus attaches to αvβ6 at the cell surface, triggering endocytosis of the integrin:virus complex, and its delivery to early- and hence recycling-endosomes.
Chapter Seven: The role of the cytoplasmic domain of the β6 integrin subunit in FMDV infection

7.1 Introduction

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. The ability of the integrin αvβ6 to mediate infection of SW480-β6 cells by FMDV has also been shown to be dependent on the cytoplasmic domain of the β6 subunit by Miller et al. (204).

This study used SW480 cell lines transfected with β6 subunits containing cytoplasmic domain deletions, and thus expressing mutant versions of the integrin αvβ6. These cell lines were designated T1-T5 (204). Cell lines T4-T1 express integrin containing progressively greater deletions of the β6 subunit cytoplasmic domain, and the T5 cell line has an internal deletion within this domain (residues 746-764). The T1-T5 cell lines were all able to support virus binding. However, only those cell lines which still possessed an NPXY motif (T4 and T3) were able to support infection, albeit at reduced levels. The NPXY motif is highly conserved among integrin β-subunits and has been shown to mediate the clathrin-mediated endocytosis of receptors, such as the LDL receptor (49). It was therefore hypothesised that this motif mediates virus induced clathrin-dependent uptake of αvβ6. The block of infection observed in cell lines T1, T2, and T5 could therefore be due to a block in αvβ6-mediated entry of the virus. However neither the precise role of the NPXY motif, nor the stage at which infection was blocked in the T1, T4, or T5 cell lines was investigated (204).
Therefore the role of the cytoplasmic domain of the β6 subunit was further investigated, using cell lines containing mutations in the NPXY motif itself.

### 7.2 The role of tyrosine based motifs within the cytoplasmic domain of the β6 integrin subunit

#### 7.2.1 Mutagenesis of the β6 Cytoplasmic Domain NPXY and NXXY Motifs

As outlined above, previous studies have shown that the cytoplasmic domain of the β6 integrin subunit is essential for αvβ6-mediated infection of tissue culture cells by FMDV, and point to a potential role for a conserved NPXY motif in this process (204). The β6 subunit also contains a similar conserved NXXY motif in its cytoplasmic domain. In order to investigate the role of these sequences in FMDV infection, cell lines expressing versions of αvβ6 containing mutations within these motifs were utilised. Our collaborating laboratory in the USA (headed by Dean Sheppard) had previously made mutations within the β6 NPXY and NXXY motifs to study the affect of tyrosine phosphorylation on αvβ6 activity in the context of its "natural" ligands (154). In order to do this the two tyrosine residues found within the conserved NPXY and NXXY motifs were mutated to phenylalanine, both individually and together, to give the F1, F2 and F1F2 mutant versions of the β6 subunit.

Figure 7.1 shows the sequences of the wild type cytoplasmic domain of the human β6 integrin subunit, as well as the mutant versions used. Highlighted in blue are the two tyrosine based motifs (NPXY and NXXY) found of the β6 subunit cytoplasmic domain, and mutated residues are highlighted in red. The F1 β6 subunit contains an
NPXF sequence instead of the wild type NPXY motif. The F2 β6 subunit contains an NXXF sequence instead of the wild type NXXY motif. The F1F2 subunit contains both mutations.

Mouse embryonic fibroblasts (MEF) expressing αvβ6 containing the F1, F2, or F1F2 β6 cytoplasmic domains were obtained from D. Sheppard and were designated MEF-F1, MEF-F2, and MEF-F1F2 respectively. Mock transfected cells were designated MEF-MK and cells expressing wild type αvβ6 were designated MEF-WT.

The level of expression of αvβ6 and virus binding by the MEF-F1, MEF-F2, and MEF-F1F2 cell lines was investigated using FACS analysis. The integrin αvβ6 or virus at the cell surface were labelled using monoclonal antibodies 10D5 and D9 respectively, followed by a PE-conjugated goat anti-mouse secondary antibody. The level of fluorescent labelling at the cell surface represents the degree of surface αvβ6 expression or virus binding and was quantified by flow cytometry counting 6000 cells per sample. The resulting data is summarised in figure 7.2. The level of integrin expression and virus binding for each cell line are expressed as a percentage of the equivalent value obtained using the MEF-WT cell line (which expresses wild type αvβ6) after correction for background. Representative histograms for this experiment are shown in figure 7.3; the figures above each curve represent the geographic mean of that curve. The MEF cell lines transfected with wild type or a mutant form of β6 all express αvβ6 at the cell surface (figure 7.2 - panel B), and bind virus (figure 7.2 - panel A), whereas the mock transfected cell line neither expresses αvβ6 nor binds virus. This shows that transfection of MEF's with the β6 integrin subunit greatly enhances expression of αvβ6 and virus binding at the cell surface, consistent with the role of αvβ6 as an attachment receptor for FMDV (147, 204). The cell lines
expressing mutant integrins (MEF-F1, MEF-F2, MEF-F1F2) all bind virus, although less well than the MEF-WT cell line. However the lower level of virus binding can be explained by correspondingly lower levels of surface αvβ6 expression. In conclusion mutation of the NPXY motif to NPXF (MEF-F1, MEF-F1F2), and the NXXY motif to NXXF (MEF-F2, MEF-F1F2) did not appear to inhibit virus binding to αvβ6.

The ability of each cell line to support FMDV infection was assessed using an infectious centre assay. In this assay, 1 million cells in suspension are infected with FMDV O1Kcad2 at an m.o.i of ~0.25 for 1 hour at 37°C. After a 2 minute acid wash to remove any virus left at the cell surface, the infected cells are serially diluted and overlaid on to BHK cells. After two days plaques are visualised by methylene blue staining. This data is shown in Table 7.1 and is expressed as the number of infectious centres per million cells. Infection is clearly dependent on the integrin αvβ6 since transfection with the human β6 subunit increases the level of infection from less than 50 infectious centres per million cells (MEF-MK cell line) to over 14000 (MEF-WT cell line). When the MEF-WT cell line was incubated at 4°C (rather than 37°C) prior to the acid wash step in order to prevent virus internalisation, no infection was observed, confirming that the acid wash step is effective in destroying virus left at the cell surface. The cell lines which express mutant integrin are all clearly able to support high levels of infection (greater than 13500 infectious centres per million cells on average), similar to that supported by the wild type integrin. In conclusion, mutation of the tyrosine residues of the NPXY and NXXY motifs found in the cytoplasmic domain to phenyalanine appears to have little or no impact on infection.

The data above show that like SW480 cells, mouse embryonic fibroblasts do not bind FMDV or mediate FMDV infection unless transfected with the β6 integrin.
subunit such that αυβ6 is expressed at the cell surface. However, mutation of the
tyrosine residue within the NPXY and NXXY motifs to phenylalanine did not inhibit
virus binding or infection mediated by the integrin αυβ6. Similarly, mutation of both
tyrosine residues had no effect. This shows that the tyrosine residues found within the
cytoplasmic domain of the β6 subunit (as well as any related phosphorylation and
signalling events) are not essential for infection of β6 transfected MEF cell lines.
However, these data do not conclusively prove that the NPXY motif is not involved in
post attachment events in FMDV infection. This is because mutation to
phenylalanine may not completely inhibit endocytosis of receptor from the cell
surface. For example CD18 is internalised by means of a related NPXF motif upon
ligand binding (265) rather than the NPXY type internalisation motif seen in the LDL
receptor (49). It has also been shown that the tyrosine residue of the NPXY motif in
the LDL receptor can be substituted for phenylalanine without completely abolishing
receptor uptake (101). In order to define clearly the role of this motif, more severe
mutations of the NPXY sequence were required.
**Figure 7.1.** Schematic diagram showing the amino acid sequences of the different cytoplasmic domains of the β6 integrin subunits used in this study. The sequence of wild type human β6 is shown topmost using the single-letter amino acid code, with amino acids thought to make up the cytoplasmic domain capitalised. The last four residues of the transmembrane domain are also shown in lower case. The two tyrosine-based motifs (NPXY and NXXY) are picked out in blue type. The sequences of the mutant β6 cytoplasmic domains are shown below with residues differing from the wild type picked out in red.
Figure 7.2. FACS analysis of virus binding and αvβ6 expression by mouse embryonic fibroblast (MEF) cell lines transfected with wild type or mutant β6 integrin subunits. αvβ6 integrin and surface bound FMDV were labelled using monoclonal antibodies 10D5 (10μg/ml) and B2 (10μg/ml) respectively, followed by a PE-conjugated goat anti mouse secondary antibody. Surface fluorescence was detected by flow cytometry counting 6000 cells per sample. Integrin expression (Panel B) and virus binding (Panel A) for each cell line are expressed as a percentage of the equivalent value obtained using the MEF-WT cell line which expresses the wild type human β6 integrin subunit. Each value represents the mean of duplicate samples. αvβ6 expression and virus binding were detected on all cell lines transfected with the β6 integrin subunit (MEF-WT, MEF-F1, MEF-F2, MEF-F1F2) but not on the mock transfected cell line (MEF-MK).
Figure 7.3. FACS analysis of virus binding and αvβ6 expression by mouse embryonic fibroblast (MEF) cell lines transfected with wild type or mutant β6 integrin subunits. For each cell line αvβ6 integrin expressed at the cell surface and surface bound FMDV (20 μg/ml) were labelled using monoclonal antibodies 10D5 (10 μg/ml) and B2 (10 μg/ml) respectively, followed by a PE-conjugated goat anti mouse secondary antibody. Surface fluorescence was detected by flow cytometry counting 6000 cells per sample. Representative histograms are shown depicting virus binding and αvβ6 expression by MEF-WT cells (panels A and B respectively), MEF-MK cells (panels C and D), MEF-F1 cells (panels E and F), MEF-F2 (panels G and H), and MEF-F1F2 cells (panels I and J). In each case the blue filled curve represents background fluorescence assessed in the absence of virus or the primary integrin detection antibody. The unfilled curve represents the level of virus binding or integrin expression. The figure above each curve represents the mean fluorescent intensity of the curve. αvβ6 expression and virus binding were detected on all cell lines transfected with the β6 integrin subunit (MEF-WT, MEF-F1, MEF-F2, MEF-F1F2) but not on the mock transfected cell line (MEF-MK).
<table>
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<th>Experiment 3</th>
<th>Experiment 4</th>
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<td>2250</td>
<td>15000</td>
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<tr>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>MEF- F2</td>
<td>-</td>
<td>-</td>
<td>9600</td>
<td>27000</td>
<td>18300</td>
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<tr>
<td>MEF- F1F2</td>
<td>-</td>
<td>-</td>
<td>14250</td>
<td>13500</td>
<td>13875</td>
</tr>
</tbody>
</table>

Table 7.1. A table detailing the ability of virus(type O1KCad2) to infect MEF cell lines expressing wild type and mutant β6 integrin subunits by means of an infectious centre assay. The figures represent the number of infectious centres obtained per million cells, infected at an MOI of -0.25. Each experiment was carried out in duplicate.
7.2.2 Alanine substitution within the β6 Cytoplasmic Domain NPXY motif

In order to disrupt the NPXY motif of the β6 cytoplasmic domain more severely, I constructed a mutant form of the β6 subunit in which both the asparagine (N) and tyrosine (Y) residues of the NPXY motif were changed to alanine. This mutant subunit was termed the APLA subunit, and the sequence of its cytoplasmic domain is shown in figure 7.1. The work described in the following paragraph was carried out by a research assistant (Sarah Gold).

The APLA mutant subunit was transfected into SW480 cells to produce the SW480-APLA cell line. This cell line was found to bind virus to a similar extent to the cell line expressing wild type β6 (SW480-β6 cells). To determine whether the integrin was correctly assembled at the cell surface the ectodomain of the mutant αvβ6 integrin was subjected to epitope mapping using a number of monoclonal antibodies to αvβ6 or the β6 subunit. These studies showed that no epitopes were lost due to the mutations within the cytoplasmic domain. In further experiments this cell line was shown not to be infected by FMDV strain O1Kcad2. Mutation of the NPXY motif to APLA therefore did not prevent virus binding or alter the gross conformation of the β6 ectodomain, but did abolish the ability of αvβ6 to mediate infection of β6-transfected SW480 cells. This strongly suggests that the NPXY motif is required for a post-attachment stage of infection of SW480 cells by FMDV.

Internalisation of the LDL receptor is blocked by mutation of the NPXY motif within its cytoplasmic domain to APLA. I therefore investigated whether mutation of the NPXY motif within the cytoplasmic domain of the β6-integrin subunit prevents internalisation of αvβ6 (and therefore virus), using immunofluorescence microscopy.
Virus was allowed to bind to the cell surface at 4°C, a temperature which does not allow endocytosis to occur. Internalisation was initiated by a shift to 37°C, before being halted by paraformaldehyde fixation. Virus was detected using the monoclonal antibody D9. Figure 7.4 shows SW480-β6 cells (Panels A) and SW480-APLA cells (Panel B) labelled for virus at 15 minutes after the initiation of virus internalisation. It appears that virus has been internalised in both cell lines. However, the pattern of virus labelling differs between the two cell lines. In SW480-APLA cells, virus appears to be present in vesicle like structures around the periphery of the cell, whereas virus appears to have migrated further towards the centre of the cell in SW480-β6 cells.

Further experiments were carried out in which virus was allowed to enter both cell lines for 15 minutes, before fixation and labelling for virus and the early endosome marker EEA-1. This data shows that virus colocalises with EEA-1 at this time point both in SW480-β6 cells (figure 7.5) and SW480-APLA cells (figure 7.6), despite the fact that virus is more restricted to the periphery in SW480-APLA cells.

The above data shows that virus is internalised and delivered to EEA-1 positive early endosomes in both SW480-β6 and SW480-APLA cells. This data would appear to suggest that mutation of the NPXY motif of the β6 integrin subunit to APLA abolishes infection, but not αvβ6-mediated delivery of virus to early endosomes. This was unexpected since one would hypothesise that mutation of the NPXY putative endocytosis motif would inhibit infection by preventing αvβ6-mediated virus internalisation.
Figure 7.4. Uptake of FMDV by SW480-p6 and SW480-APLA cells. SW480-p6 (Panel A) and SW480-APLA (Panel B) cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was allowed to proceed for 15 minutes at 37°C. Internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, virus (red) was detected using the monoclonal antibody D9 (mouse IgG2A-5μg/ml), followed by an Alexa fluor conjugated isotype specific goat-anti mouse secondary antibody. Bars = 10μm. Both SW480-p6 (Panel A) and SW480-APLA cells (Panel B) have internalised virus at this time point. However some virus remained at the surface of the SW480-APLA cells.
Figure 7.5. Dual labelling of SW480-p6 cells for EEA-1 and FMDV at 15 minutes after initiation of virus internalisation. SW480-p6 cells, prepared on coverslips were incubated with FMDV strain O1Kcad2 (5μg/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5μg/ml) and D9 (mouse IgG2A-5μg/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
Figure 7.6. Dual labelling of SW480-APLA cells for EEA-1 and FMDV at 15 minutes after initiation of virus internalisation. SW480-APLA cells, prepared on coverslips were incubated with FMDV strain 01Kcad2 (5ug/ml) for 45 minutes at 4°C. Virus internalisation was initiated by shifting the temperature to 37°C. Following 15 minutes incubation at 37°C internalisation was halted by fixation on ice using 4% paraformaldehyde. Following permeabilisation with 0.1% Triton, EEA-1 and virus were detected using monoclonal antibodies 14 (mouse IgG1-5ug/ml) and D9 (mouse IgG2A-5ug/ml) respectively, followed by Alexa fluor conjugated isotype specific goat-anti mouse secondary antibodies. Panels A and D show EEA-1 labelling (green) and panels B and E show virus (red). Panel C shows an overlay of panels A and B. Panel F shows an overlay of panels D and E. DAPI stained nuclei are shown as blue.
7.3 Summary and Discussion

Previous studies have shown that the cytoplasmic domain of the β6 integrin subunit is required for αvβ6-mediated infection of tissue culture cells by FMDV, and point to a potential role for a conserved NPXY motif in this process (204). In order to explore this possibility we utilised cell lines transfected with β6 subunits carrying mutations within the NPXY (and related NXXY) motif of the cytoplasmic domain (see figure 7.1).

The β6 cytoplasmic domain contains two tyrosine residues, one of which forms part of the conserved NPXY motif, and one of which forms part of a similar conserved NXXY motif. We obtained mouse embryonic fibroblasts cell lines transfected with the wild type β6 integrin subunit (MEF-WT), a subunit in which the NPXY motif was mutated to NPXF (MEF-F1), a subunit in which the NXXY motif was mutated to NXXF (MEF-F2), and a subunit containing both mutations (MEF-F1F2). We also obtained a MEF cell line transfected with the expression plasmid alone containing no insert (MEF-MK). Infection of MEF's was found to be dependent on the β6 integrin subunit since MEF's transfected with the wild type β6 integrin subunit were able to bind virus and support FMDV infection, whereas mock transfected MEF's were unable to support virus binding or infection. The cell lines expressing mutant αvβ6 (MEF-F1, MEF-F2, MEF-F1F2) were still able to support virus binding and infection. Since the F1F2 mutant does not contain any tyrosine residues in its cytoplasmic domain, this indicates that tyrosine phosphorylation and/or tyrosine mediated signalling mediated by the β6 cytoplasmic domain are not essential for infection of MEF's by FMDV. However these data do not clearly prove that the NPXY motif is not involved in post attachment events in FMDV infection. This is because mutation
of tyrosine to phenylalanine may not completely inhibit uptake of receptor from the cell surface. For example CD18 is internalised by means of an NPXF motif upon ligand binding (265) rather than the NPXY type internalisation motif seen in the LDL receptor (49). It has also been shown that the tyrosine residue of the NPXY motif in the LDL receptor can be substituted for phenylalanine without completely abolishing receptor uptake (101).

In order to investigate the role of the NPXY motif further, a cell line expressing αvβ6 containing the sequence APLA in place of the β6 cytoplasmic domain NPXY motif integrin subunit was generated. Replacement with alanine of either the asparagine (N) residue or tyrosine residue (Y) of the NPXY motif found in the cytoplasmic domain of the LDL receptor reduces its clathrin-dependent internalisation to around 10% of control values (49). If internalisation of αvβ6 is mediated by the NPXY motif found in the β6 cytoplasmic domain, this double mutation would be expected to block uptake of this integrin and therefore FMDV uptake and infection.

In our laboratory, SW480-APLA cells were found to bind FMDV to a similar extent to the cell line expressing wild type β6 (SW480-β6), and in an RGD-dependent fashion. Epitope mapping of the αvβ6 ectodomain using monoclonal antibodies revealed that no epitopes were lost due to the mutations within the cytoplasmic domain, indicating correct assembly of αvβ6 at the cell surface. However, infection of SW480-APLA cells was greatly reduced in comparison to SW480-β6 cells. This strongly suggests that mutation of the NPLY motif to APLA inhibits αvβ6-mediated infection of β6 transfected SW480 cells at a post attachment stage.

In order to try to identify the stage at which infection of SW480-APLA cells is blocked, uptake of virus was studied by immunofluorescence microscopy. The data is shown in figures 7.4-7.6. Contrary to expectations, virus was found to have entered
both SW480-β6 and SW480-APLA at 15 minutes after the initiation of internalisation, and also to colocalise with EEA-1 in both cell lines at this time point.

This data would appear to show that the inhibition of infection observed in SW480-APLA cells is not due to inhibition of clathrin-dependent delivery of virus to early endosomes, but at a different stage. However, these studies use saturating quantities of virus and may not reveal differences in the rate of virus uptake by the wild type and mutant forms of αvβ6. Although not conclusive, the studies shown in figures 7.4-7.6 suggest that: 1) less virus may have entered the cells expressing the APLA mutant compared with the cells expressing the wild type integrin, and 2) the internalised virus had a more peripheral location in the cells expressing the APLA mutant.

One possible explanation is that mutation of the NPXY motif within the β6 cytoplasmic domain causes subtle changes within the β6-ectodomain which result in a reduced affinity of αvβ6 for FMDV. It has been reported previously that changes made within the cytoplasmic domain of the β6 subunit can result in 1) changes in the conformation of the ectodomain of the integrin αvβ6, 2) altered specificity for RGD peptides, and 3) a reduction in the concentration of RGD peptide required to prevent FMDV binding (142). These observations imply that such alterations result in a reduced affinity of FMDV for αvβ6. Such a reduction in affinity would not necessarily be detected using the FACS virus-binding assay since this assay uses saturating concentrations of virus.

Why should a reduced affinity of αvβ6 for FMDV result in a slower rate of virus internalisation? The work described in chapter 6 suggests that internalisation of integrin:FMDV complexes at the cell surface requires both occupation of the integrin RGD-binding site and cross-linking of αvβ6. It may be that a lower affinity of αvβ6
for virus results in less receptor cross linking, and therefore reduced infection as a result of a slower rate or lesser extent of FMDV internalisation.

A reduced affinity of FMDV for αvβ6 could also inhibit infection by reducing the delivery of viral RNA to the host cell cytoplasm. Poliovirus undergoes a conformational change upon binding its receptor, resulting in the externalisation of VP4 and the N-terminus of VP1. This altered conformation of poliovirus (the A-particle) is able to interact directly with host cell membranes, an interaction which is thought to involve both the N-terminus of VP1 and VP4 (63). FMDV however does not possess the N-terminal hydrophobic extension of VP1 which is thought to mediate attachment of poliovirus to host cell membranes. Instead, FMDV most likely remains close to the cell membrane by prolonged interaction with its receptor, until uncoating occurs within acidic endosomal compartments. A reduction in affinity of αvβ6 for FMDV could mean the virus dissociates from its receptor at the cell surface or within endosomes before acid dependent capsid uncoating takes place. As a result the viral RNA would no longer be locally concentrated in the vicinity of the target membrane, inhibiting its transfer across the endosomal membrane to the cytoplasm. In order to test this hypothesis, a biochemical analysis of the affinity and kinetics of binding of FMDV to both wild type and mutant αvβ6 would need to be carried out. Analysis of ion channel formation triggered by FMDV in liposomes containing either wild type or mutant αvβ6 could also be carried out.

Another possible explanation for the data presented in this chapter is that the cytoplasmic domain of the β6 integrin subunit is required for translocation of the viral RNA into the cytoplasm, and that this process is prevented by mutation of the NPLY motif to APLA. This is not without precedent since αvβ5-mediated translocation of adenovirus 2 particles across endosomal membranes has been shown to be dependent
upon the cytoplasmic domain of the β5 integrin subunit (328, 330). However, this scenario is unlikely for FMDV as virus has been shown to be able to use a variety of non-integrin receptors in tissue culture, including heperan sulphate proteoglycans (143), the Fc receptor (when virus is coated with antibodies) (189, 192), and a single chain antibody fused to intercellular adhesion molecule-1 (269). Also, the mechanisms of host membrane permeabilisation utilised by adenoviruses and picornaviruses are thought to differ. Adenovirus is thought to penetrate host cell membranes by causing local membrane disruption (262), whereas human rhinovirus-2 (262) and poliovirus (63) are thought to form pores in the host membrane through which the viral RNA is extruded. Since FMDV can utilise non-integrin receptors in tissue culture and picornaviruses are thought to permeabilise host membrane by a different mechanism to adenovirus, it would seem unlikely that the cytoplasmic domain of the β6 integrin subunit (or specifically the NPXY motif) is essential for release of viral RNA to the cytosol.
Chapter Eight: The role of VP4 in FMDV Cell Entry

8.1 Introduction

FMDV particles are formed when twelve 14S pentameric intermediates associate to form either 140S RNA containing viral particles, or 75S empty capsids. Each pentamer is formed from five protomers, each of which contains a single copy of VP0, VP1, and VP3. The final step in the assembly process occurs when VP0 in the provirion is cleaved into VP2 and VP4, in a process known as maturation cleavage. VP4 is effectively an N-terminal extension of VP2, which, when released by maturation cleavage, forms a small relatively disordered protein chain, which is located on the inner surface of the capsid.

The precise role of VP4 in the infectious process is not well understood. However, for many picornaviruses, cleavage of VP0 is required for infectivity. This has been shown for poliovirus (53), rhinovirus-14 (170), and hepatitis A virus (34), and more recently for FMDV (160). It should be noted that VP0 cleavage is not necessary for all picornaviruses because VP0 of some of the kobu- and parechoviruses remains uncleaved in the mature virion. Recombinant FMDV particles carrying uncleavable VP0 are able to assemble and bind to cells normally, and have a similar degree of acid sensitivity to wild-type virions (160). The only major difference observed was that the acid breakdown products of the VP0-containing particles were considerably more hydrophobic than the wild type virus, presumably due to a failure to release myristoylated VP4. This evidence shows that VP0 cleavage is not needed for normal RNA replication, viral protein expression, virus assembly, receptor-binding, or acid-mediated capsid dissociation. These data appear to indicate that the maturation cleavage is required for the translocation of the viral RNA into the cytoplasm. It is
unclear from this evidence whether VP4 itself is involved in this process directly, or whether the cleavage and subsequent release of VP4 simply allows the viral pentamers to perform this task in some way.

Evidence from studies with poliovirus suggests that VP4 could be involved in RNA translocation into the cytosol. Poliovirus undergoes a conformational change upon receptor binding resulting in the externalisation of both VP4 and the N-terminus of VP1. This altered form of the poliovirus particle (or "A-particle") has been shown to be capable of forming ion channels within the artificial lipid bilayers (312, 313). It has been hypothesised that these channels serve to deliver the viral RNA across host cell membranes to the cytoplasm. Mutation of threonine-28 of VP4 (which abolishes a threonine-myristate hydrogen bond but does not prevent VP0 cleavage) abolishes the infectivity of the viral particle, without affecting receptor binding characteristics (214). Furthermore, mutations at this position have been shown to alter both the characteristics of the ion channels formed by poliovirus following receptor binding, and the rate of delivery of viral RNA to the cytoplasm (63, 313). Taken together these observations indicate that VP4 plays a role in ion channel formation and RNA escape in poliovirus.

FMDV differs from poliovirus in that it does not form A-particles or possess the hydrophobic N-terminal extension of VP1 thought to be partly responsible for binding of poliovirus to the host cell membrane (63, 86). Instead FMDV is uncoated in response to the low pH within endosomes rather than by receptor binding. In FMDV the role of VP4 in infection is less clear, although as mentioned above, it is thought that the maturation cleavage is necessary to allow RNA to enter the cytoplasm (160). The aim of the work described in this chapter was to shed further light on the role of VP4 in FMDV infection. To determine the regions and sequences of VP4 that are
essential for infection, recombinant viruses were constructed in which all or part of VP4 were swapped for the equivalent sequences from equine rhinitis A virus (the other member of the genus *aphthovirus*). The results obtained from this work are described below.

### 8.2 The Role of VP4 in FMDV cell entry

#### 8.2.1 Creation of Recombinant Viruses containing mutations in VP4.

In our laboratory we possess a reverse genetics system enabling the manipulation of the FMDV genome. This consists of a plasmid containing a full-length infectious cDNA clone of the O1Kaufbeuren strain of FMDV (pT7S3) downstream of a T7 polymerase promoter site (78). RNA copies of the FMDV cDNA can be synthesised *in vitro* using T7 polymerase. Since FMDV RNA is infectious, virus particles can be produced by transfection of the viral RNA into susceptible cells. This system was utilised to investigate the role of VP4 in FMDV infection.

The mutations made to VP4 are described in figure 8.1 panel A. The amino acid sequence of the mutant forms of VP4 are aligned with that of the wild type virus in panel B. The plasmids pT7S3-ER-VP4, pT7S3-F/E-VP4 and pT7S3-E/F-VP4 all contain copies of VP4 in which all or part of the FMDV sequence has been replaced by the equivalent sequence from Equine Rhinitis A Virus (ERAV).

In order to construct these plasmids it was first necessary to introduce restriction enzyme sites at either end of the region coding for VP4 in the FMDV infectious copy plasmid. The strategy for achieving this is shown in figure 8.3. First, a KpnI subclone of the plasmid pT7S3 was cloned into pGEM7Zf and used as a template for
overlap PCR. To introduce a HindIII site at the 5' end of VP4, primer P1 and the
mutagenic primer P3 (for primer sequences see figure 8.2) were used to create PCR
product PCR1, and primer P2 and the mutagenic primer P4 were used to give PCR
product PCR2. PCR product PCR1 contains the HindIII site introduced by primer P3
at its 3' end, and PCR product PCR2 contains the HindIII site introduced by primer
P4 at its 5' end. The two PCR products (PCR1 and PCR2) contain a region of overlap
corresponding to the junction between L and VP4 and containing the introduced
HindIII site. PCR products 1 and 2 were mixed and amplified using the outside
primers P1 and P2 to give PCR product 3. PCR product 3 consists of a 600bp region
of the infectious copy plasmid covering the whole of VP4 and parts of the L protease
and VP2, in which a HindIII site has been introduced at the 5' end of VP4 by means
of a silent mutation (aagctc to aagctt). The final PCR step was carried out using Taq
polymerase, thus allowing the PCR product to be cloned in to pGemT for sequencing.
This construct was then used as a template for a further round of overlap PCR as
shown in figure 8.3, in order to introduce an Nhel site at the 3' end of VP4 by means
of a silent mutation (tctcgc to gctacg). The resulting construct was cloned in to
pGemT and sequenced to confirm that the Nhel site had been introduced and that no
unforeseen changes had been introduced by PCR. The resulting construct (pGemT-
HVP4N) contains a 600bp region of the infectious copy plasmid encompassing all of
VP4 and parts of L and VP2, with a HindIII site and an Nhel site at the 5' and 3' ends
of VP4 respectively. The location of the introduced restriction sites, as well as the
sequence data confirming that both sites had been successfully introduced, are shown
in figure 8.4. DNA sequencing showed that no other changes had been introduced by
the PCR reactions.
The introduced HindIII and NheI sites were subsequently utilised to swap FMDV VP4 for altered forms containing all or part of VP4 from ERAV. Figure 8.5 shows the process by which non-chimeric and chimeric forms of VP4 bounded by HindIII and NheI sites were produced. Wild type ERAV VP4 was amplified from ERAV cDNA by PCR with HindIII and NheI sites being introduced at either end by the PCR primers. As a control wild type FMDV was amplified from pT7S3 and HindIII and NheI sites were added in the same way. In order to form F/E VP4 (N-terminus from FMDV, C-terminus from ERAV) and E/F VP4 (N-terminus from ERAV, C-terminus from FMDV) overlap PCR was required. Figure 8.5 shows the primers and strategy utilised to achieve this. All final PCR products were cloned into pGemT and sequenced (see figure 8.6). The process by which these altered forms of VP4 were incorporated into the infectious copy plasmid is shown in figure 8.7. Firstly the wild type form of VP4 found in the pGemT-HVP4N construct was replaced by the mutant forms utilising the introduced HindIII and NheI sites. Unique SmaI and SalI sites were then utilised to reconstitute this altered form of VP4 and surrounding sequences into the KpnI subclone of pT7S3. The KpnI fragment of pT7S3 was then replaced by the KpnI fragments containing the altered forms of VP4 to give full length pT7S3 in which FMDV VP4 had either been wholly or partially replaced by the equivalent sequences from ERAV. The resulting plasmids were pT7S3-ER-VP4, pT7S3-F/E-VP4 and pT7S3-E/F-VP4.

A previous study has shown that mutations at the boundary between VP4 and VP2 (AD to FK) results in the production of a non-infectious virus in which cleavage of VP0 does not occur (160). These mutations were recreated in our system using overlap PCR as shown in figure 8.8. The initial template used for overlap PCR was PCR3 from figure 8.1. This construct contains a 600bp region of the infectious copy
plasmid spanning all of VP4, and parts of L and VP2, and contains a HindIII site at the 5' end of VP4. The mutagenic primers P9 and P10 were used in the first step of overlap PCR in order to alter the sequence at the VP4-VP2 boundary from gccgac (codes for the amino acid sequence AD) to tcaaa (equivalent to the sequence FK at the amino acid level). Two bases were altered within each codon in order to minimise the possibility of reversion to the wild type sequence. The final PCR product was cloned into pGemiT and sequenced in order to confirm the presence of the desired mutation and to check for any errors introduced by PCR. The location of the mutations made, together with the sequence data confirming that the desired mutation had indeed been introduced are shown in figure 8.9. The sequence coding for the mutated VP4-VP2 boundary was reconstituted into the full-length infectious copy plasmid as shown in figure 8.7 (from the second stage onwards) to give the plasmid pT7S3-VP0*. 
Figure 8.1. VP4 mutant viruses constructed and utilised in this study. Panel A shows a list of mutant viruses utilised, together with a description of the changes made relative to wild type FMDV expressed from the pT7S3 infectious copy plasmid. Panel B shows a lineup of the amino acid sequences of VP4 (plus the first amino acid of VP2) from wild type and each of the mutant viruses using the single letter amino acid code. Residues which are identical to wild type FMDV (top row) are identified by a dot (.). Gaps are represented by a dash (-) The boundary between VP4 and VP2 is denoted by a black vertical line. The central serine residue which represents the boundary of the N-terminal and C-terminal domains of VP4 is boxed.

(A)

<table>
<thead>
<tr>
<th>Name of Virus</th>
<th>Changes Made</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>None</td>
</tr>
<tr>
<td>rWT</td>
<td>HindIII and NheI sites introduced at 5' and 3' end of VP4 by silent mutation</td>
</tr>
<tr>
<td>ER VP4</td>
<td>VP4 swapped for that from Equine Rhinitis A Virus</td>
</tr>
<tr>
<td>F/E VP4</td>
<td>C-terminus of VP4 swapped for that from ERAV</td>
</tr>
<tr>
<td>E/F VP4</td>
<td>N-terminus of VP4 swapped for that from ERAV</td>
</tr>
<tr>
<td>VP0*</td>
<td>Junction of VP4 and VP2 made uncleavable by mutation from AD to KF</td>
</tr>
</tbody>
</table>

(B)

<table>
<thead>
<tr>
<th>WT and rWT</th>
<th>VP0*</th>
<th>ER VP4</th>
<th>F/E VP4</th>
<th>E/F VP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>GAGQSSPATGSQNSGNTGSIIINNYMQQY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>................................</td>
<td></td>
<td></td>
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<tr>
<td>*</td>
<td>................................</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT and rWT</td>
<td>QNSMDTQLGDNAILGSAGSNEGSTDSTTSTH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP0*</td>
<td>................................</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>I.ADV ................................</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>................................</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT and rWT</td>
<td>NTQNNNDWFSKLASSAFSLGFAKALLAD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP0*</td>
<td>................................</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*</td>
<td>................................</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>VP4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP2</td>
<td>................................</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Figure 8.1. VP4 mutant viruses constructed and utilised in this study. Panel A shows a list of mutant viruses utilised, together with a description of the changes made relative to wild type FMDV expressed from the pT7S3 infectious copy plasmid. Panel B shows a lineup of the amino acid sequences of VP4 (plus the first amino acid of VP2) from wild type and each of the mutant viruses using the single letter amino acid code. Residues which are identical to wild type FMDV (top row) are identified by a dot (.). Gaps are represented by a dash (-) The boundary between VP4 and VP2 is denoted by a black vertical line. The central serine residue which represents the boundary of the N-terminal and C-terminal domains of VP4 is boxed.
Figure 8.2. A figure showing the sequences of the primers utilised in VP4 mutagenesis.
KpnI subclone of Infectious Copy Plasmid in pGEM 7ZF

KpnI Subclone used as initial template for two rounds of overlap PCR in order to introduce HindIII and NheI sites at boundaries of VP4

Round 1

P1  P3

P4

P2

PCR 1  PCR 2

PCR 1 and 2 mixed and used as template for PCR 3

P1

P2

HindIII

Round 2

P1  P5

P6

P2

PCR 4  PCR 5

PCR 4 and 5 mixed and used as template for PCR 6

P1

P2

HindIII  NheI

Final Product

600 bp PCR product containing altered VP4 region.

Figure 8.3. A schematic diagram showing the strategy for the introduction of HindIII and NheI sites at the 5' and 3' ends of FMDV VP4 respectively. PCR primers are depicted as unbroken arrows lying alongside DNA sequence. Non-mutagenic primers are depicted as straight arrows, whereas mutagenic primers are depicted as kinked arrows. See figure 8.2 for the sequences of the primers depicted in this diagram. The labels in orange (P1-P6) at either end of each PCR product represent the primers used to produce that PCR product. Naturally occurring restriction sites are highlighted by black broken arrows, and those introduced by PCR are highlighted by blue arrows. HindIII and NheI sites were introduced at the indicated locations by two rounds of overlap PCR as shown.
Figure 8.4. Sequence data for the introduction of HindIII and Nhel sites at the 5' and 3' ends of FMDV VP4 respectively. Panels A and B show sequence data for the 5' and 3' ends respectively of VP4 from the wild type infectious copy plasmid. Panels C and D show sequence data for the 5' and 3' ends respectively of VP4 following the introduction of silent mutations by overlap PCR as shown in figure 8.3. In each case the equivalent amino acid sequence is written below the nucleotide sequence, with each amino acid being placed beneath the first residue of the codon which encoded it. The junction between VP4 and the L protease (panels A and C) or VP4 and VP2 (panels B and D) are shown by a vertical line. Residues mutated by overlap PCR are boxed and the introduced restriction enzyme sites highlighted. All sequencing shown is of the coding strand sequenced in the 5' to 3' direction using primer P1 (see figure 8.2) and a Beckman automatic sequencer.
Figure 8.5. A schematic diagram showing the strategy for production of altered forms of VP4. Wild-type ERAV VP4 and FMDV VP4 were amplified by a single round of PCR using primers containing HindIII or Nhel sites. The VP4 chimeras F/E-VP4 and E/F-VP4 were produced by overlap PCR as shown utilising primers complementary to the join between the N- and C-terminal domains of the final product. Regions of nucleotide sequence derived from ERAV VP4 are coloured blue and sequence derived from FMDV VP4 are coloured red. PCR primers are depicted as arrows lying alongside DNA sequence, and are also colour coded according to whether they are complementary to ERAV VP4 (blue) or FMDV VP4 (red). See figure 8.2 for the sequences of the primers depicted in this diagram. The labels in green at either end of each PCR product represent the names of the primers used to produce that PCR product. Restriction enzyme sites introduced by PCR are highlighted by blue arrows.
Figure 8.6. Sequence data for the production of VP4 chimeras. Panels A and B show sequence data for the boundary between the N- and C-terminal domains of VP4 from wild type FMDV VP4 and ERAV VP4 respectively (PCR 14 and 13 from figure 8.5). Panels C and D show sequence data for the same region of the F/E and E/F chimeric versions of VP4 respectively (PCR 12 and 9 from figure 8.5). In each case the equivalent amino acid sequence is written below the nucleotide sequence, with each amino acid being placed beneath the first residue of the codon which encoded it. The serine residue (and the codon which encodes it) common to both FMDV and ERAV VP4 which marks the boundary between the N- and C-terminal domains is boxed (see figure 8.1). All sequencing shown is of the coding strand sequenced in the 5' to 3' direction using primer P1 (see figure 8.2) and a Beckman automatic sequencer.
600 bp PCR product containing altered VP4 region:

FMDV VP4 swapped for mutant version using HindIII and Nhel sites

Reconstitute in to Kpnl subclone of pT7S3 using SunI and Sail sites.

Kpnl subclone of pT7S3 containing E/F VP4.

Ligate Kpnl subclone of pT7S3 (containing mutant VP4) back in to resealed vector

Infectious Copy Plasmid Containing E/F VP4.

Figure 8.7. A schematic diagram showing the strategy for the reconstitution of VP4 mutants produced by PCR into the FMDV full length infectious copy plasmid (pT7S3). Mutant forms of VP4 produced as shown in figure 8.5 were introduced into pT7S3 as shown above using the E/F VP4 chimera as an example. Sequence derived from ERAV VP4 is coloured blue and that derived from FMDV VP4 is coloured red. VP2 is shaded purple, L protease pale green, and the remainder of the coding region of the FMDV genome is shaded a darker green. Naturally occurring restriction sites are highlighted by black broken arrows, and those introduced by PCR are highlighted by blue arrows.
PCR 3 (see figure 8.1) used as template for overlap PCR in order to introduce mutations at the VP4-VP2 junction

Overlap PCR

PCR 15 and 16 mixed and used as template for PCR 17

Final Product

600 bp PCR product containing altered VP4 region.

Figure 8.8. A schematic diagram showing the strategy for the creation of the VP0* Mutant. The junction between VP4 and VP2 was altered by overlap PCR in order to prevent VP0 cleavage (see figure 8.9 for the specific mutations made) as reported previously for type A viruses. PCR primers are depicted as arrows lying alongside DNA sequence. Non-mutagenic primers are depicted as straight arrows, whereas mutagenic primers are depicted as kinked arrows. See figure 8.1 for the sequences of the primers depicted in this diagram. The labels in green at either end of each PCR product represent the names of the primers used to produce that PCR product. Naturally occurring restriction sites are highlighted by black broken arrows, and sequences introduced by PCR are highlighted by blue arrows.
Introduce mutations by overlap PCR at VP4/VP2 boundary to prevent maturation cleavage of VP0

Figure 8.9. Sequence data for the creation of the VP0* mutant. Panel A shows sequence data for the junction between VP4 and VP2 from the wild type infectious copy plasmid. Panel B shows sequence data for the same region following overlap PCR as shown in figure 8.8. The amino acid sequence at the VP4-VP2 boundary was changed from AD to FK as shown by mutation of the boxed residues in panel A to the boxed residues shown in panel B by overlap PCR. In each case the equivalent amino acid sequence is written below the nucleotide sequence, with each amino acid being placed beneath the first residue of the codon which encoded it. The junction between VP4 and VP2 is shown using a vertical line. All sequencing shown is of the coding strand sequenced in the 5' to 3' direction using primer P1 (see figure 8.2) and a Beckman automatic sequencer.
8.2.2 Infectivity of VP4 Mutant Viruses

Described above is the construction of infectious copy plasmids containing mutant versions of VP4. The names of the mutant plasmids along with a description of the mutations made, as well as the amino acid sequence of VP4 coded by each plasmid are shown in figure 8.1. Next, T7 polymerase was used to produce full length genomic RNA from each plasmid, which was processed using the RNAeasy kit from AMBION. The viral RNA was electroporated into BHK cells using two charges of 750V at a capacitance of 25μFD, the cells were then incubated for 2 days at 37°C. A lysate (derived by freeze-thawing the cells) prepared from the electroporated cells was subsequently passed four times on BHK cells. A control sample of cells was electroporated in the absence of viral RNA and handled as above. At each passage the development of cytopathic effect, was observed over a period of 2 days.

The results of these observations are set out in figure 8.10. At passage 1, samples derived from both the original infectious copy plasmid (pT7S3-WT) and the infectious copy plasmid with HindIII and NheI sites introduced at either end of VP4 (pT7S3-rWT), rapidly exhibited signs of cytopathic effect. This shows that the introduction of HindIII and NheI sites does not appear to inhibit virus infectivity. All other mutants including the “No RNA” control displayed no cytopathic effect at passage 1. This indicates that mutation of the VP4-VP2 junction to prevent VP0 cleavage (pT7S3-VP0*), as well as replacement of all of VP4 (pT7S3-ER-VP4), the N-terminus of VP4 (pT7S3-E/F-VP4) or the C-terminus of VP4 (pT7S3-F/E-VP4) with the equivalent sequence from ERAV does not result in the production of viable virus.

It is possible that some of the mutant viruses which displayed no cytopathic effect after the first passage underwent limited replication, due to the introduction of
compensatory mutations. These mutations may enhance virus replication on a second
passage and provide information on the residues within VP4 which are essential for
its function. On subsequent passage in BHK cells the samples derived from plasmids
pT7S3-F/E-VP4, pT7S3-E/F-VP4, and pT7S3-ER-VP4 continued to display no
detectable cytopathic effect. However, the VP0* mutant, which did not cause
cytopathic effect at passages 1 and 2, caused extensive cytopathic effect after
passages 3 and 4. In order to establish the sequence changes that caused this
transition, viral RNA extracted from passage 4 was reverse transcribed and
sequenced. For comparison purposes, passage 4 of the WT and rWT virus was also
sequenced. Figure 8.11 shows an alignment of the nucleotide sequence of VP4 and
10 codons either side derived from pT7S3-WT prior to electroporation and passage
(FMDV WT P0), with the sequence derived from virus after 4 passages (FMDV WT
P4). These sequences are identical showing that there is minimal selection pressure
against the wild type sequence in this region during tissue culture passage. Figure
8.12 shows an alignment of the same region derived from pT7S3-rWT before
electroporation (FMDV-rWT P0), and after 4 passages (FMDV-rWT P4), with the
wild type sequence. Both sequences only differ from wild type at the sites where
HindIII and NheI sites were introduced by silent mutation. After 4 passages the
sequences corresponding to these two restriction sites are still present indicating that
there is no selection pressure against these sequences. Figure 8.13 shows an
alignment of the nucleotide sequences of VP4 and 10 codons either side derived from
pT7S3-VP0* prior to electroporation (VP0* P0), and after 4 passages (VP0* P4), with
the wild type sequence. VP0* P0 differs from the wild type sequence at both the
junction between VP4 and VP2, due to mutations designed to prevent VP0 cleavage,
and at the 5' end of VP4, due to the presence of the same HindIII sequence introduced
in to the pT7S3-rWT sequence. This site was included to act as a marker both during cloning and passage. After 4 passages of the VP0* virus, the sequence of VP4 is identical to the wild type version, having lost the introduced mutations at the VP4/VP2 boundary and also the HindIII site at the 5' end of VP4. It is possible that the cytopathic effect that appeared at passage 3 occurred due to the reversion to the wild type sequence following limited replication. This is unlikely however, since the aagctt "HindIII" sequence was maintained during passage of the rWT virus, indicating that there is little or no selection pressure against this sequence. Also the fact that the two codons either side of the VP2-VP4 junction each contain two nucleotide changes rather than one makes reversion unlikely. Unfortunately it is more likely that the appearance of cytopathic effect two passages downstream of electroporation with RNA derived from the pT7S3-VP0* plasmid is simply due to cross contamination with wild type virus.
Figure 8.10. Infectivity of VP4 mutant viruses as judged by the development of cytopathic effect. BHK cells were electroporated with RNA derived from infectious copy plasmids carrying mutant versions of VP4 (see figure 8.1 to see the amino acid sequence of each mutant). Any virus produced was passaged onto fresh BHK cells four times. After each passage the development of cytopathic effect, characterised by cell rounding and detachment, was observed for 48 hours at 37°C. A plus sign (+) indicates that cytopathic effect was observed, whilst a minus sign (-) indicates that no cytopathic effect was observed.
Figure 8.11. Sequence alignment of FMDV WT VP4 before and after tissue culture passage, with the wild type sequence. T7 polymerase was used to create full genome length RNA from the wild type infectious copy plasmid pT7S3 WT. The RNA was then electroporated into BHK cells and the mutant virus formed passed four times in tissue culture.

The above figure shows an alignment of the nucleotide sequences of VP4 derived from the infectious copy plasmid (FMDV WT P0), with that obtained from viral RNA following four passages in BHK cells (FMDV WT P4). 10 codons either side of VP4 are also shown. Residues which are identical to the wild type sequence are identified by a dot (.). The boundaries between VP4 and VP2, and between VP4 and the L protease are each denoted by a black vertical line.

No differences were observed between the sequences.
Figure 8.12. Sequence alignment of FMDV rWT VP4 before and after tissue culture passage, with the wild type sequence. T7 polymerase was used to create full genome length RNA from the mutant infectious copy plasmid pT7S3 rWT. The RNA produced carries sequences corresponding to HindIII and NheI sites introduced at either end of VP4 by silent mutation. The RNA was then electroporated into BHK cells and the mutant virus formed passed four times in tissue culture.

The above figure shows an alignment of the nucleotide sequences of VP4 derived from the mutant infectious copy plasmid (FMDV rWT P0), and from viral RNA following four passages in BHK cells (FMDV rWT P4) with the wild type sequence (FMDV WT). 10 codons either side of VP4 are also shown. Residues which are identical to the wild type sequence are identified by a dot (•). The boundaries between VP4 and VP2, and between VP4 and the L protease are each denoted by a black vertical line. The HindIII and NheI sequences introduced by silent mutation are boxed and remained present after passage through BHK cells.
Figure 8.13. Sequence alignment of FMDV VP0* before and after tissue culture passage, with the wild type sequence. T7 polymerase was used to create full genome length RNA from the mutant infectious copy plasmid pT7S3 VP0*. The RNA produced carries mutations at the boundary of VP4 and VP2 which prevent the maturation cleavage of VP0. The RNA was then electroporated into BHK cells and the mutant virus formed passed four times in tissue culture.

The above figure shows an alignment of the nucleotide sequences of VP4 derived from the mutant infectious copy plasmid (VP0* P0), and from viral RNA following four passages (VP0* P4) with the wild type sequence (FMDV WT). 10 codons either side of VP4 are also shown. Residues which are identical to the wild type sequence are identified by a dot (.) The boundaries between VP4 and VP2, and between VP4 and the L protease are each denoted by a black vertical line. The mutated codons at the VP4/VP2 boundary and the HindIII sequence are boxed. The sequence of VP0* P4 at the HindIII site and the VP4/VP2 boundary are the same as the wild type virus.
8.3 Summary and Discussion

The above work forms the starting point for further study of the role of VP4 in infection of tissue culture cells by FMDV. Section 8.2.1 covers the process by which a system for the creation of VP4 mutant viruses was set up, and infectious copy plasmids containing mutant versions of VP4 in place of the wild type sequence were created. Infectious copy plasmids were constructed in which the N-terminal domain (E/F VP4), the C-terminal domain (F/E VP4), or all of VP4 (ER VP4) were replaced by the same region from ERAV. Each plasmid also has HindIII and Nhel sites at either end of VP4 introduced by silent mutation, since these sites were required during plasmid construction. As a control an infectious copy plasmid was also constructed in which wild type FMDV VP4 is bounded by the same HindIII and Nhel sites (rWT VP4). Finally an infectious copy plasmid was constructed in which the boundary between VP4 and VP2 was mutated such that the maturation cleavage cannot occur (VP0*).

T7 polymerase was used to create full-length positive-sense RNA genome copies from each of the wild type and mutant infectious copy plasmids. The RNA produced was electroporated into BHK cells and virus produced passaged four times in tissue culture. At each tissue culture passage the development of cytopathic effect was observed (figure 8.10). Virus derived from the plasmids pT7S3-WT and pT7S3-rWT caused cytopathic effect at each passage. This indicates that the introduction of HindIII and Nhel sites into the infectious copy plasmid does not inhibit the infectivity of FMDV. No infectious virus was produced following electroporation of BHK cells with RNA derived from the pT7S3-ER-VP4, F/E-VP4, or E/F-VP4 infectious copy
plasmids. However, from this work it is not possible to tell whether the introduced mutations prevent particle assembly.

Following electroporation of BHK cells with RNA derived from infectious copy plasmid pT7S3-VP0*, no cytopathic effect was observed after passage 1 or 2, but cytopathic effect was observed at passage 3 and 4. One possible explanation for the increased virulence in BKH cells is that during passage 1 and 2 low level replication occurs, which is insufficient to produce cytopathic effect, but leads to the introduction of mutations which increase virulence resulting in the production of cytopathic effect on subsequent passage. In order to investigate this possibility, virus derived from the pT7S3-VP0* infectious copy plasmid was sequenced after four passages, and the sequence aligned with the equivalent sequence prior to passage and with the wild type sequence. The sequence of the VP4 region of the virus produced after four passages was identical to the wild type sequence, i.e. the HindIII site had been lost and the sequence at the VP4/VP2 boundary restored to wild type. One possible explanation is that the mutant virus reverted to the wild type sequence. However, passage of the rWT virus shows that the sequence corresponding to the HindIII site is maintained during passage, and is not selected against. Also, the codons either side of the VP4-VP2 boundary were each mutated at two positions making reversion less likely. Unfortunately the most likely explanation for the observed cytopathic effect at passages 3 and 4 of the VP0 virus is contamination by the wild type virus.

All of the changes made to VP4 (other than the introduction of HindIII and NheI sites) prevented the production of infectious virus. It is unclear whether electroporation of cells with RNA containing mutations within VP4 results in the production of virus particles which are nonviable, or whether these mutations prevent the assembly of virus particles. It is possible that VP4 tolerates little sequence
variation, either because this region is important for particle assembly, or because conserved sequences within VP4 are required for an important aspect of FMDV infection.

From this work, the next step would be to investigate whether electroporation of susceptible cells with RNA from each mutant infectious copy plasmid results in the assembly of virus particles. This could be achieved by monitoring the presence or absence of 140S particles (virions) using sucrose density gradient analysis following electroporation. If non-infectious particles are produced, these particles could be purified and characterised in terms of their ability to bind and enter cells, to uncoat in response to acid, and to deliver the viral RNA genome to the cell cytoplasm.

Three infectious copy plasmids were produced in which particular regions of VP4 were swapped for the equivalent sequence from ERAV. No infectious virus was obtained using any of these mutant infectious copy plasmids. These constructs could be utilised as a starting point for more targeted mutagenesis of VP4. ERAV VP4 shows 48% identity and 65% similarity to FMDV VP4. As shown in figure 8.1, the N-terminal domain of ERAV VP4 is more similar in sequence to that of FMDV than the C-terminal domain. Consequently E/F VP4 shows greater sequence identity and similarity to FMDV VP4 than F/E VP4. E/F VP4 contains 85 residues, 72 of which are identical and 6 similar to the equivalent residue in wild type FMDV VP4. This means that there are only 7 positions at which the amino acid residue found in E/F VP4 is significantly different from that found in wild type FMDV VP4. These sequence differences would appear to be sufficient to render infectious copy plasmids carrying E/F VP4 rather than wild type FMDV VP4 unable to produce infectious virus. Production and characterisation of seven mutant viruses in which each of these
seven residue changes are introduced individually in future could prove very beneficial in the investigation of the function of FMDV VP4.
Chapter Nine: Summary and Future

The work presented in this thesis sheds light on the early events in αvβ6-mediated entry to, and infection of, tissue culture cells by FMDV. However, αvβ6 is just one of several RGD-dependent integrins reported to act as receptors for FMDV. A number of lines of evidence would suggest that αvβ6 is the most important receptor in the natural hosts of foot-and-mouth disease virus. In particular, the expression pattern of the integrins reported to act as receptors for FMDV supports this conclusion. FMDV shows a strong tropism for epithelial cells and, following a natural infection, initial virus uptake and replication is thought to take place in the pharynx and soft palate (5). Subsequently, the virus becomes widely disseminated and infectious lesions develop on the feet and mouth of the infected animal (5). Work published during the preparation of this thesis compared the expression of αvβ6 and αvβ3 in tissue sections taken from uninfected and infected cattle (209). These studies showed that αvβ6, but not αvβ3, is expressed on the surface of epithelial cells in the tongue and interdigital skin of uninfected cattle, both of which are sites where FMDV replicates to high levels and lesions normally form. In addition, αvβ6 was also detected on the surface of epithelial cells within the coronary band and ventral soft palate. In these tissues αvβ3 expression was low and was limited to the vasculature. In infected cattle, αvβ6 (but not αvβ3) was detected on the surface of infected cells in tissue sections taken from early stage lesions located on the interdigital skin. The expression of αvβ1 and αvβ8 have not been assessed in a natural host of FMDV, however β1 integrins are ubiquitously expressed in mammalian cells, and although αvβ8 has been detected in human airway epithelia it is largely associated with the basal cells rather than the spinosum layer targeted by FMDV (46). Therefore the
expression pattern of \( \alpha\nu\beta 6 \) within a natural host animal (the cow) matches the cells infected by FMDV much more closely than the expression of \( \alpha\nu\beta 3 \), or that of \( \alpha\nu\beta 1 \) and \( \alpha\nu\beta 8 \) inferred from other mammalian species. In this context, the insights into the process by which \( \alpha\nu\beta 6 \)-mediated uptake of FMDV occurs described in this thesis are of key physiological relevance.

The data presented in this thesis are consistent with a model for FMDV cell-entry involving virus-induced, clathrin-dependent endocytosis of a FMDV-\( \alpha\nu\beta 6 \) complex into early- and recycling-endosomes, where the prevailing low pH triggers capsid disassembly and the translocation of the vRNA into the cytosol. The microtubule network and actin cytoskeleton also did not appear to play an essential role in \( \alpha\nu\beta 6 \)-mediated infection by FMDV. Experiments with \( \beta 6 \)-transfected SW480 and CHO cells showed that lipid raft dependent endocytosis pathways, including the caveolae-dependent uptake pathway, are not essential for FMDV infection.

Although \( \alpha\nu\beta 6 \) has been shown previously to act as an attachment receptor for FMDV, it was not known whether this receptor also mediated virus internalisation. This question was addressed using immunofluorescence microscopy. The studies presented here show that FMDV colocalises with \( \alpha\nu\beta 6 \) both at the cell surface and also inside the cell at early times after the initiation of virus internalisation. In addition, \( \alpha\nu\beta 6 \) was shown to be delivered to the same compartments as FMDV and accumulated in early- and recycling-endosomes on virus internalisation. These observations strongly suggest that \( \alpha\nu\beta 6 \) serves to mediate both the attachment and uptake of FMDV. Internalisation of \( \alpha\nu\beta 6 \) appears to be triggered by a combination of the occupation of the integrins' RGD-binding site, and receptor cross-linking by a multivalent ligand (e.g. FMDV).
The cytoplasmic domain of the β6-integrin subunit contains a conserved NPXY putative endocytosis motif. Alanine substitutions within this motif led to the production of receptors that were able to mediate virus binding but not infection, and virus internalisation appeared to be slowed by these mutations. The stage at which infection is blocked however remains unclear, and this issue requires further study.

The precise compartment in which FMDV uncoating and RNA transfer to the cytosol occur, remains to be determined. Given the extreme acid lability of the FMDV capsid, it would be assumed that particle uncoating and RNA delivery would be triggered in early endosomes, since this is the first acidic compartment to which FMDV is delivered. One possible approach to address this issue would be to investigate the effect of dominant negative Rab proteins on FMDV endocytosis.

Transport steps inside cells are co-ordinated by Rab GTPases, and each Rab protein is thought to act at different stages in the endocytosis pathway (297). Rab5 GTPase for example controls clathrin-dependent delivery to early endosomes, Rab 7 controls traffic from early to late endosomes, and rab 4 and 11 control traffic from early to recycling endosomes. It should therefore be possible, using dominant negative versions of these proteins, to establish whether transport of viral particles to early endosomes is sufficient to initiate infection by FMDV, or whether transport beyond this compartment is necessary. This question could also be addressed by attempting to investigate uncoating more directly. For example, the structural protein VP4 could be used as a marker for uncoating, since this protein is not surface exposed until the virus particle dissociates into its constituent pentamers. It may also be possible to label the viral RNA with fluorescent intercalating dyes, since FMDV particles are permeant to such molecules.
During the preparation of this thesis, O'Donnell et. al. (Plum Island, USA) published work examining FMDV internalisation in cultured cells (232). This work was published back-to-back with my own publication (30) in the journal of virology. The studies by O'Donnell et. al. concentrated primarily on the use of confocal microscopy to study virus internalisation by MCF-10A cells (a human breast cancer line), with only limited biochemical data. Nonetheless, the authors of this work concluded that FMDV was internalised by clathrin-dependent endocytosis in to early endosomes, before passing to a transferrin receptor positive compartment.

Immunofluorescence experiments involving early endosome markers were carried out in αvβ6 transfected COS cells rather than MCF-10A cells, since surprisingly no EEA-1 labelling could be detected within the latter. They also concluded that virus was not transported to late endosomes, since no colocalisation was detected with the mannose-6-phosphate receptor (a late endosome marker). These conclusions are in line with my own findings as outlined above. The authors identified clathrin-dependent endocytosis as the uptake pathway for FMDV because of apparent colocalisation between FMDV and the clathrin light chain, and also the inhibition of infection by chlorpromazine, a drug reported to inhibit this endocytosis pathway. These techniques are different to those used in my own study, and therefore serve to reinforce the findings presented here, namely that the virus is taken up by the clathrin-dependent endocytosis pathway.

The results presented in this thesis will pave the way for future studies to identify the compartment in which FMDV uncoating occurs, and to elucidate the mechanism of viral RNA transfer to the cytosol.
Publications


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