Regulation of FMDV infection by cellular rab GTPases

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(B.Sc. Hons)

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Abstract

FMDV infection is initiated by virus binding to integrin receptors at the cell surface. Virus binding to the integrin triggers internalization of the virus-receptor complex which enters the cell via clathrin-dependent endocytosis. The low pH within endosomes triggers capsid disassembly and translocation of the viral RNA across the endosomal membrane into the cytosol. The precise identity of the endocytic compartment from which infection by FMDV takes place is currently unknown.

Rab GTPases are central regulators of endocytosis. Each rab protein is enriched in one or more specific membrane compartments. In this study dominant-negative versions of a number of rabs are used to investigate the early events in FMDV infection of a pig kidney cell line (IBRS2). Infection is inhibited by expression of dominant-negative rab5 (which inhibits formation of early endosomes) but not by dominant-negative rab4 (which inhibits rapid recycling from early endosomes to the plasma membrane) or by dominant-negative rab9 (which inhibits trafficking from late endosomes to the Golgi). Dominant negative rab11, which inhibits a slower recycling pathway via recycling endosomes inhibits FMDV infection to an extent, although the effect of dominant-negative rab5 on infection is greater.

While a dominant-negative form of rab7 (which regulates trafficking from early- to late endosomes) unable to bind membranes inhibits FMDV infection, a membrane binding but inactive rab7 does not. This inhibition is shown to be at the stage of replication rather than entry. It is suggested that rab7 may be required for intracellular virus replication, possibly anchoring the replication complex to the replication vesicle.
Acknowledgements

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<tr>
<td>ABS</td>
<td>Adult bovine serum</td>
</tr>
<tr>
<td>APS</td>
<td>Ammoniumperoxodisulphate</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>CA</td>
<td>Constitutively active</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CRE</td>
<td>cis-acting replication element</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay accelerating factor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagles medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>2’deoxynucleotide 5’triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees celcius</td>
</tr>
<tr>
<td>ECL</td>
<td>Electrochemiluminescence</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosomal antigen 1</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELIspot</td>
<td>Enzyme-linked immunospot</td>
</tr>
<tr>
<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
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<tr>
<td>FMDV</td>
<td>Foot-and-mouth disease virus</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDF</td>
<td>GDI-displacement factor</td>
</tr>
<tr>
<td>GDI</td>
<td>GDP dissociation inhibitor</td>
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<tr>
<td>GEF</td>
<td>GDP/GTP exchange factor</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HAV</td>
<td>Hepatitis A virus</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HRV</td>
<td>Human rhinovirus</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosome-associated membrane protein</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule associated protein-1 light-chain 3</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamps</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal antibody</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>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PABP</td>
<td>Poly(A) binding protein</td>
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<tr>
<td>PCBP</td>
<td>Poly(C) binding protein</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PK</td>
<td>Pseudoknot</td>
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<tr>
<td>POL</td>
<td>Polymerase</td>
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<tr>
<td>PRO</td>
<td>Protease</td>
</tr>
<tr>
<td>PV</td>
<td>Poliovirus</td>
</tr>
<tr>
<td>REP</td>
<td>Rab escort protein</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>SVDV</td>
<td>Swine vesicular disease virus</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-hydroxymethylaminomethane</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Polyoxyethylene sorbitan monolaurate</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VGM</td>
<td>Viral growth medium</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>VPg</td>
<td>Viral protein, genome linked</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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XIV
Chapter one: Introduction

1.1 Foot-and-mouth disease virus

Foot-and-mouth disease virus (FMDV) is the type species of the *aphthovirus* genus within the *Picornaviridae* family, the only other member of this genus being equine rhinitis A virus (King, 2000). FMDV is the causative agent of foot-and-mouth disease, a highly contagious condition affecting cloven hoofed animals. Due to its highly contagious nature and economic importance for many countries, FMD is the first disease on the Office International des Épizooties (OIE) list of notifiable diseases. Outbreaks in developed countries have a significant economic impact due to loss of animals through culling and restrictions on trade.

The disease causes a high rate of mortality in young animals although it is rarely fatal in the adult. Over 70 mammalian species can be infected, many sub-clinically. This may lead to a wild animal acting as a reservoir for the virus, infecting domestic livestock (Thomson et al., 2003). Infected cattle exhibit the most obvious symptoms (vesicular lesions on the mouth and feet) although these are rarely seen in sheep or goats. Sheep are thought to have been responsible for transmission of the virus during the 2001 outbreak in the UK as the disease is so difficult to diagnose clinically in these animals (Hughes et al., 2002).

The infectious nature of the virus has made it notoriously difficult to control. There are many reasons for this, including the large amount of infectious material produced by infected animals, high susceptibility of host species (particularly cattle) and the short incubation time. Sheep and cows are able to become persistently infected (Burrows, 1966; Salt, 1993) enabling them to act as asymptomatic carriers. The
virus can also survive outside the host for long periods of time, up to several weeks in a sheltered environment. This means that the virus can be passed by contamination of vehicles and clothing of people attending affected livestock. However, due to the narrow pH range tolerated by the virus (6.0-9.0), it can be destroyed by sterilisation with acid.

Transmission from animal to animal is usually via aerosol (Donaldson et al., 1987), although it can also be passed by direct contact through skin or mucosal abrasions. Infected animals excrete large amounts of virus, especially pigs (Alexandersen & Donaldson, 2002), and under favourable conditions, the virus may travel in the air distances up to 10km over land, or 250km over water.

The route of infection is via the respiratory tract, with the initial site of replication thought to be in the epithelial cells of the pharynx and dorsal soft palate (Alexandersen et al., 2001; Brown et al., 1992; Burrows et al., 1981). The virus then spreads throughout the animal, targeting mainly epithelial tissues in the feet and mouth (Alexandersen et al., 2001; Brown et al., 1995; Brown et al., 1996; Burrows et al., 1981). Infected animals often have a fever, and most develop viremia. After infection, a neutralising antibody response can be seen by 4-5 days, peaking at 28 days. The immune response varies according to host species, age and health together with virus dose, strain and route of infection. It is thought that interferons α, β and γ are involved in host defence, along with other cytokines (Brown et al., 2000; Chinsangaram et al., 2001).

Most of the world has seen outbreaks of FMDV, with New Zealand as the only exception (see figure 1.1). It is endemic in Africa, Asia and South America. The EU
Figure 1.1 Geographical distribution of FMDV serotypes during 2001

N.J. Knowles 03 March 2002

This figure shows the distribution of the seven serotypes of FMDV.
is usually FMDV free, except for occasional incursions such as the UK outbreak in 2001 which spread to France, Holland and the Republic of Ireland. There are sporadic outbreaks in Russia. Australia and North America are currently free of FMDV. There are seven serotypes of FMDV: A, O, C, South African Territories (SAT) 1, 2, 3 and Asia 1. These are further sub-divided into strains and grouped by topotype (Bachrach, 1968). The UK outbreak was caused by serotype O.

FMDV diagnosis and control by vaccination are made more difficult as the error-prone nature of the viral RNA dependent RNA polymerase leads to frequent mutations (Drake & Holland, 1999). This means that new variants often appear in the field. The major site of sequence variation is in the P1 region, giving rise to antigenic variation. Diagnosis of FMDV is currently by quantitative real-time RT-PCR and virus isolation on primary cells. RT-PCR methods are hoped to be able to diagnose FMDV in the field (Callahan et al., 2002; Hearps et al., 2002; Reid et al., 2003).

Vaccines are available against FMDV based on inactivated virus. Vaccination programmes control the virus in many countries, but vaccination is unpopular in an emergency outbreak situation. When vaccination is used, disease-free status is lost and hence trade with disease free countries is restricted. It can be difficult to distinguish between vaccinated and recovering animals. Vaccinated animals may also carry the virus and potentially infect other animals although this is as yet unproven. Further problems with vaccination are that the vaccine virus must be matched precisely with the outbreak strain and the high rate of antigenic variation caused by frequent mutations means that viruses may evolve to evade vaccines.
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1.2 The picornaviruses

Picornaviruses are small non-enveloped RNA viruses. There are nine genera in the Picornavirus family, containing a number of important human and animal pathogens (See Table 1.1). Apart from FMDV, notable members of the picornavirus family include poliovirus (PV), human rhinovirus (HRV), hepatitis A virus (HAV) and swine vesicular disease virus (SVDV).

Poliovirus is the causative agent of poliomyelitis (Popper, 1909), a contagious disease of the nervous system with few obvious symptoms other than paralysis in less than 1% of cases, leading to death. It affects mainly children under the age of five and can spread rapidly through populations. A global vaccination programme begun in the 1950s has all but eradicated poliovirus, but some reservoirs exist where populations cannot be reached to be vaccinated, especially in war-torn areas. Two vaccines have been developed, the inactivated vaccine (IPV) by Jonas Salk (Salk et al., 1954) and the live attenuated oral vaccine (OPV) by Albert Sabin (Sabin, 1957).

Human rhinovirus is the main cause of the common cold, thought to account for up to 80% of infections during peak cold season (Turner, 2001). It is estimated that time off work due to infection with human rhinovirus accounts for millions of work days in the USA alone (Monto et al., 2001), and the market for over-the-counter remedies is worth billions of dollars (Bertino, 2002). The virus is also associated with other more serious conditions including sinusitis and pneumonia and exacerbation of asthma, chronic bronchitis and cystic fibrosis (Abzug et al., 1990; Couch, 2001; Ghosh et al., 1999; Johnston et al., 1995; McMillan et al., 1993).
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### Introduction

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<tr>
<td><strong>Unassigned species</strong></td>
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Table 1.1 Picornavirus classification
Proposed in 8th ICTV Report (Stanway, 2005). The Rhinovirus genus was suggested to merge with the Enterovirus genus.
Hepatitis A virus (HAV) is the causative agent of type A viral hepatitis with the virus targeting the liver. Recovery can be complete in 4-6 weeks (Ross & Anderson, 1991), but occasional fatalities do occur. Increasing standards in public health and sanitation have lead to a decrease in the number of cases in the developed world, with most being contracted while travelling abroad.

All picornaviruses have certain features in common. They are all single stranded RNA viruses (Drake & Holland, 1999), with positive sense genomes between 7 and 8.5kb in length. The genome organisation of the picornavirus is shown in figure 1.2. Instead of a 7-methyl guanine ($m^7$G) cap as in cellular mRNAs, the 5' end of the picornavirus genome is covalently linked to a small viral encoded protein known as viral genome-linked protein (VPg) (Flanegan et al., 1977; Lee et al., 1977). The 5'UTR of the picornavirus genome is highly structured with a number of notable features. The 5'UTR is followed by a single open reading frame. This can be divided into three regions: P1 which encodes the structural proteins, plus P2 and P3 which yield the non-structural proteins (Rueckert & Wimmer, 1984).

1.3 FMDV genome organisation

1.3.1 The 5'UTR

The 5' UTR (untranslated region) of FMDV is shown in figure 1.3. It begins with the S-fragment, a 350-380nt region which is predicted to form a long hairpin (Clarke et al., 1987). There is no known function for this structure, but it has been suggested that the equivalent element in poliovirus (the cloverleaf) aids genome stability (Barton et al., 2001), and that proteins involved in genome replication bind to it, circularising the genome (see section 1.4.7 ‘Genome replication’).
Figure 1.2 Picornavirus genome organisation

The 5' end of the picornavirus genome is uncapped but has a small viral encoded protein (VPg) covalently attached. The 5' UTR is highly structured and contains a number of notable features. P1 encodes the viral structural proteins and P2 and P3 encode the non-structural proteins. The 3' UTR is followed by a poly(A) tail of variable size.
Figure 1.3 The FMDV 5'UTR

The 5'UTR of FMDV contains many notable features, including regions of high secondary structure.

- VPg/3B-viral genome-linked protein
- S-fragment-equivalent to poliovirus cloverleaf
- polyC tract of variable length
- PK-a number of pseudoknots
- *cis*-acting replication element (*cre*)
- internal ribosome entry site (IRES)

This is followed by the open reading frame (ORF) and the 3'UTR and polyA tail. These features are discussed in section 1.3.1.
Some picornavirus genomes contain a polyC tract of varying length, longest in the cardio- and aphthoviruses. It is composed of over 90% C-residues and its function is unknown. However, viruses engineered to have a shortened polyC tract revert to a longer version on passaging in cell culture (Rieder et al., 1993). After the polyC tract are three or four tandemly repeated pseudoknots with unknown function (Clarke et al., 1987; Escarmis et al., 1995).

The FMDV 5'UTR contains a cis-acting replication element (cre), a hairpin structure required for genome replication (Mason et al., 2002). In other picornaviruses, the cre is in the coding region, but its location has little effect on its function (Goodfellow et al., 2000). This element will be further discussed in section 1.4.7 'Genome replication'.

The cre is followed by the highly structured internal ribosome entry site (IRES), whose function is to enable translation of picornavirus proteins to proceed while host cell mRNA translation is inhibited (Jang et al., 1988; Pelletier et al., 1988). This element will be discussed in section 1.4.5 'Polyprotein translation'.

1.3.2 The polyprotein

The FMDV RNA genome is infectious (Belsham & Bostock, 1988). On virus uncoating and release of the genetic material into the cytoplasm, translation of the open reading frame is the first step and the viral proteins are derived from a single polyprotein (Summers & Maizel, 1968). This is cleaved during synthesis by viral proteases into the viral structural and non-structural proteins (Clarke & Sangar, 1988). The cleavage reactions occur as shown in figure 1.4. Some precursors have their own distinct functions, which will be discussed later. The majority of cleavage
Genome organisation and polyprotein cleavage for FMDV. Only erbo-and aphthoviruses encode the L protease and only FMDV has 3 copies of 3B. All cleavage reactions are mediated by 3C, except cleavage of the leader and 2A proteins. Functions of proteins (structural and non-structural) including precursors is discussed in sections 1.3.3 and 1.3.4.

Figure 1.4 Processing pathway of FMDV polyprotein
reactions are mediated by the 3C protease. Cardio-, aphtho- and teschoviruses encode an additional protein, the leader (L) protein at the 5’ end of the genome. In FMDV and erboviruses, the L protein is an active protease and mediates its own cleavage from the polyprotein (Strebel & Beck, 1986) along with cleavage of the eukaryotic translation initiation factor eIF4G (Devaney et al., 1988; Hinton et al., 2002). This activity is directed by the 2A protease in some other picornaviruses. This will be further discussed in section 1.4.5 ‘Polyprotein translation’. The leader protein from cardioviruses is released by 3C cleavage and its function is unknown, but it is not an active protease.

1.3.3 Structural proteins

During FMDV infection, P1-2A is cleaved from 2BC and the P3 region, followed by release of the 2A protease from P1. It is thought that this is not a proteolytic event, but a ribosomal ‘skip’ which releases the P1-2A peptide and then re-initiates translation of the downstream product without forming a peptide bond (Donnelly et al., 2001). P1 is cleaved by 3C into VP1, VP3 and VP0. VP0 is cleaved on encapsidation by an unknown mechanism to give the two remaining structural proteins, VP2 and VP4. VP1-VP4 form the viral capsid, with 60 copies of each protein present. VP1, VP2 and VP3 combine to form the outer face of the capsid structure, with VP4 on the inside. This will be further discussed in section 1.4.1 ‘FMDV capsid structure’.

1.3.4 Non-structural proteins

There are 14 mature FMDV proteins, along with a number of precursors with their own distinct functions. The P2 proteins (2B and 2C) are released by 3C protease
cleavage of the 2BC precursor. This precursor has its own function in FMDV, inhibition of the secretory pathway (Moffat et al., 2005). This is similar to the function of 3A in poliovirus (Doedens & Kirkegaard, 1995). Expression of 2BC is sufficient to block trafficking of the VSV G protein from the ER to the Golgi (Moffat et al., 2005). This may be advantageous to the virus in many ways. Such inhibition may downregulate secretion of interferons and proinflammatory cytokines or may prevent cell surface MHC class 1 expression and antigen presentation. This may help to shield the infected cell from the host immune response. It has also been shown that poliovirus 3A reduces expression of the TNF receptor at the cell surface thus protecting cells from TNFa-induced apoptosis (Neznanov et al., 2001).

The P3 region is composed of 3A, 3B, 3C and 3D. Protein 3A is believed to be involved in formation of the replication complex, along with 2B and 2C. These proteins are also implicated in formation of the vesicles required for viral RNA replication. 2B has no assigned activity and 2C has both helicase and nucleotide binding motifs. Although helicase activity has yet to be shown, some picornavirus 2C proteins do have NTPase activity (Klein et al., 1999; Rodriguez & Carrasco, 1993). 3A contains hydrophobic sequences that may facilitate membrane association of 3B. 3B is the viral genome-linked protein, also known as VPg. In its uridylylated form, 3B acts as the primer for viral RNA synthesis (see section 1.4.7 'Genome replication'). There are three distinct copies of this protein in FMDV, in contrast to the single copy in other picornaviruses. Each of these is functional (King et al., 1980) and deletion of any one of the three impairs virus replication (Falk et al., 1992).
3CD is cleaved into 3C and 3D. The precursor 3CD is needed for proteolytic activity in poliovirus infection (Leong, 2002), and in human rhinovirus (HRV), PV and FMDV it is required for the uridylylation of VPg (Nayak et al., 2005). During infection by FMDV, 3CD is rapidly cleaved to 3Cpro and 3Dpol (Grubman et al., 1984). 3D is the RNA dependent RNA polymerase (RdRP) responsible for replication of the viral RNA. It is used for initial negative strand synthesis, creating a template for the subsequent rounds of positive strand synthesis, also catalysed by 3Dpol (Paul, 2002).

The protease 3C is a member of the trypsin-like family of serine proteases and is responsible for the cleavage reactions within P1, P2 and P3. It also has protease activity towards a number of cellular proteins. In FMDV, it has been shown to cleave histone H3 in the nucleus, presumably to inhibit host cell transcription (Falk et al., 1990). It also cleaves eIF4A and eIF4GI, but at a slower rate than the leader protease (Belsham et al., 2000). This will be further discussed in section 1.4.5 ‘Polyprotein translation’.

1.3.5 The 3'UTR

The 3'UTR is specific to each picornavirus. FMDV has a stem-loop structure followed by a poly(A) region of varied length. This region stimulates translation in FMDV (Lopez de Quinto et al., 2002) and replication in poliovirus (Herold & Andino, 2001).
1.4 The FMDV life cycle

The life cycle of FMDV is shown in figure 1.5. The whole process from infection to virion release can take as little as 4-6 hours in cultured cells (Rueckert, 1996). FMDV binds to the cell surface via integrin receptors and is internalised by clathrin-dependent endocytosis. The virus enters endosomes where the prevailing low pH causes capsid disassembly and the viral RNA genome is translocated into the cytoplasm. In the cytoplasm, the viral RNA is translated via an internal ribosome entry site as a single polyprotein, which is processed into structural and non-structural proteins. This translation is allowed to proceed while host cell mRNA translation is inhibited. Non-structural proteins cause rearrangement of membranes to form vesicles that are believed to serve as ‘platforms’ for formation of the replication complex and subsequent virus replication. The genome is then packaged and mature virions are released from the cell.

1.4.1 FMDV capsid structure

The FMDV capsid is 30nm in diameter and the crystal structure was first solved at 2.9Å resolution in 1989 (Acharya et al., 1989). Since this work carried out on a type O virus, the structures of type A and type C viruses have also been published (Curry et al., 1996; Lea et al., 1995, 1994). The sequences for these serotypes are around 86% identical, with most differences being in the side chains (Lea et al., 1995). The FMDV capsid is made up of four structural proteins, VP1-VP4, with 60 copies of each. The VP1 proteins are located around the 5-fold axis, with VP2 and VP3 alternating around the 2-and 3-fold axes (see figure 1.6). This gives the virus capsid
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Figure 1.5 The FMDV life cycle

FMDV enters the cell through integrin-mediated endocytosis. The virus enters endosomes where the prevailing low pH causes the capsid to dissociate. The RNA genome is translocated into the cytoplasm where translation of the viral polyprotein occurs, followed by processing by 3Cpro into structural and non-structural proteins. Non-structural proteins cause rearrangement of membranes to form replication vesicles. Positive strands are copied into negative strands by 3Dpol. These are used as a template for multiple rounds of positive strand synthesis. The genome is then packaged and mature virions are released from the cell. This is discussed in section 1.5.
Figure 1.6 Structure of a Picornavirus capsid

Shown above is a representation of a picornavirus capsid such as FMDV. VP1 is in blue, VP2 is in green and VP3 is in red. VP4 is not shown as it is on the inside of the capsid.
a pseudo T=3 icosahedral structure. VP4 is found exclusively on the inside of the capsid. VP1-VP3 adopt the RNA virus fold (RVF) common to the majority of RNA viruses, a wedge-shaped eight stranded β-barrel. VP1 is the most variable of the structural proteins in terms of sequence, and contributes most to antigenicity and receptor binding (Cavanagh et al., 1977) (Liebermann et al., 1991).

VP1 contains a loop structure known as the GH loop, from residue 140-160 and named for the two β-strands it joins together. The FMDV structural proteins are smaller than those in other picornaviruses, leading to the formation of a much smoother capsid. FMDV also lacks the pit and canyon structures seen in other picornaviruses, which leads to a difference in the way FMDV interacts with its receptor. In some picornaviruses, receptor binding occurs at the canyon (Hogle et al., 1985) (Luo et al., 1987) (Rossmann et al., 1985). In FMDV, receptor binding is via the previously mentioned GH loop (Jackson et al., 1997). This will be discussed in section 1.4.2 ‘Receptors’.

While VP1 contributes most towards antigenicity, VP3 contributes the most towards capsid stability. The N-termini of VP3 knit together at the 5-fold axis to form pentamers of VP1-VP4, leaving a pore or channel in the centre. The FMDV capsid is very sensitive to acid, dissociating from the intact 160S particle into 12S pentameric subunits on exposure to pH lower than 6.8 (Brown & Cartwright, 1961). The Entero- and rhinoviruses have an additional 135S intermediate known as the altered or ‘A’ particle which FMDV does not (see section 1.4.3 ‘Virus entry by formation of ‘A’ particles’).
1.4.2 Receptors

The first clue as to the identity of the cellular receptor for FMDV began with the observation that the GH loop of VP1 contained a highly conserved RGD motif, which is also found on the ligands for integrin receptors (Fox et al., 1989; Pfaff et al., 1988). Integrins are type I transmembrane glycoproteins. They are made up of two subunits, α and β, which are noncovalently linked. There are 18α and 8β subunits that interact to form 24 integrin heterodimers (Hynes et al., 1987). Some α subunits contain an inserted (I) domain, which is a major ligand-binding site (Tagaki et al., 2002). Integrins are composed of a long extracellular domain, a transmembrane domain and a short cytoplasmic domain (Hynes et al., 1992; Sastry and Horwitz, 1993.) One exception is the β4 subunit, whose cytoplasmic domain is 1000 amino acids in length as compared to around 50 in other subunits. Whereas the shorter subunits target actin and therefore microfilaments, the longer β4 subunit contacts with the intermediate filaments of the cytoskeleton.

Integrins are involved in both cell-cell adhesion and in interactions between cells and the extracellular matrix (ECM). There are two forms of signalling mediated by integrins, the classic "outside-in" as a consequence of ligand binding, which leads to remodelling of the cytoskeleton and cell differentiation. Other effects of ligand binding to integrins include cell movement, survival, growth and proliferation. There is also "inside-out" signalling, where signals from inside the cell can affect the conformation of the integrin's extracellular ligand-binding domain and thus its activation state (Hynes et al., 1992; Schwartz et al., 1995). Known ligands for integrins are numerous, but include fibronectin, vitronectin, laminin and collagen (Plow et al., 2000). Some integrins are widely expressed, for example αVβ3, which
is found on many cell types. In contrast, $\beta_1$ and $\beta_2$ integrins are found only on leukocytes (Gahmberg et al., 1997).

The first evidence for the involvement of the RGD in receptor recognition came from a number of studies using RGD containing peptides (Baxt & Becker, 1990; Fox et al., 1989; Surovoi et al., 1988). The first integrin receptor implicated in FMDV entry was $\alpha V\beta 3$, the vitronectin receptor (Berinstein et al., 1995). This was previously shown to be recognised by coxsackie A9 (Roivainen et al., 1994). Three other integrins have since been identified as receptors for FMDV: $\alpha V\beta 1$, $\alpha V\beta 6$ and $\alpha V\beta 8$ (Jackson et al., 2000; Jackson et al., 2002; Jackson et al., 2004). A recent study has shown that the integrin binding loop of FMDV is more highly adapted to binding to $\alpha V\beta 6$ than $\alpha V\beta 3$ or $\alpha V\beta 8$ (Burman et al., 2006). The $\alpha V\beta 6$ integrin is the most likely candidate for FMDV cell entry in vivo as it is expressed on epithelial cells, the target tissue for FMDV (Monaghan et al., 2005). A recent study showed expression of $\alpha V\beta 6$ but not $\alpha V\beta 3$ on epithelial tissue targeted by FMDV infected cattle (Monaghan et al., 2005). Heparan sulphate proteoglycans have also been identified as FMDV receptors (Jackson et al., 1996), but this is thought to be a tissue culture adaptation and is unlikely to be significant in vivo. Viruses adapted to using heparan sulphate were found to be attenuated in cattle (Sa-Carvalho et al., 1997).

On binding to its receptor, FMDV does not form ‘A’ particles (see below). Instead, capsid uncoating is triggered by the low pH within endosomes. Other examples of picornaviruses that do not form ‘A’ particles include the human rhinovirus (HRV) minor receptor group, which use the non-immunoglobulin superfamily (IgSF) receptor very-low-density lipoprotein receptor (VLDL-R) which does not bind in the canyon (Hofer et al., 1994). A number of studies have shown a requirement for low
pH during infection by these minor receptor group human rhinoviruses (Brabec et al., 2003; Schober et al., 1998).

Like FMDV, human parechovirus-1 (HPEV-1) also uses integrins (αVβ3 and αVβ1) as receptors (Triantafilou et al., 2000). The virus is thought to be taken up into endosomes, as it co-localises with a marker for early endosomes, although a requirement for low pH has not yet been demonstrated (Joki-Korpela et al., 2001).

1.4.3 Virus cell entry by formation of ‘A’ particles

For several picornaviruses, binding to their receptor not only attaches the virus to its host cell, but also triggers formation of the altered, or ‘A’ particle and release of the viral RNA. ‘A’ particle formation is dependent on how the virus capsid interacts with the receptor. Receptors that bind in the canyon of the virus capsid, a depression in the surface of the virus particle that encircles the fivefold axes of symmetry, generally trigger formation of ‘A’ particles. The ‘A’ particle has a sedimentation coefficient of 135S as opposed to 160S for the intact virion and their formation is believed to be a prerequisite for release of the RNA genome. The conformational change involved in ‘A’ particle formation leads to the externalization of both VP4 and the amino terminus of VP1 (Chow et al., 1987; Fricks & Hogle, 1990).

Poliovirus forms ‘A’ particles on binding to its receptor. All three PV serotypes utilise the same receptor, termed the poliovirus receptor (PVR), or CD155 (Mendelsohn et al., 1989). This receptor belongs to the immunoglobulin superfamily (IGSF), a family of receptors which are known to be able to bind in the canyon of the picornavirus capsid. Poliovirus has been seen in endosome-like
structures (Kronenberger et al., 1998), but does not seem to require exposure to low pH for infection (Perez & Carrasco, 1993).

Coxsackie B viruses (CVB) share a receptor with adenoviruses, known as the coxsackie-adenovirus receptor (CAR) (Bergelson et al., 1997). CAR is thought to have a role in cell adhesion (Hondo, 2000). Although in CVB infection, CAR is needed for both attachment and infection (Martino et al., 2000), it is needed only for attachment in adenoviruses, with αV integrins required for cell entry (Bai et al., 1993; Nemerow, 2000). CVB uses decay accelerating factor (DAF) as a co-receptor, but ‘A’ particle formation can only be triggered by CAR (Goodfellow et al., 2005; Milstone et al., 2005). A recent study suggests that CVB binds to DAF on the surface of polarised epithelial cells where rac signalling is activated and actin remodelling allows the virus to travel to the tight junction, where it binds to CAR and enters the cell. ‘A’ particle formation can then be induced inside cellular compartments (Coyne & Bergelson, 2006).

Coxsackie A9 virus uses the integrin αVβ3 as a receptor, but may also require interaction with GRP78 and MHC-I molecules for targeting of the virus to its internalisation pathway (Triantafilou et al., 2002). It has recently been shown that coxsackie A9 can also use integrin αVβ6 as a receptor (Williams et al., 2004). Coxsackievirus A21 binds to both intercellular adhesion molecule-1 (ICAM-1) and DAF, but DAF expression alone is insufficient for infection (Shafien et al., 1997). This is because ICAM-1 is needed to induce ‘A’ particle formation and the role of DAF is thought to be concentration of virus particles at the cell surface (Newcombe et al., 2004).
The major receptor group human rhinoviruses (HRV) use ICAM-1 as their receptor (Greve et al., 1989; Staunton et al., 1989), and receptor binding triggers ‘A’ particle formation (Hoover-Litty & Greve, 1993). The HRV3 and -14 serotypes can convert to ‘A’ particles at neutral pH, whereas HRV16 requires a more acidic environment. This is thought to be due to the presence of a ‘pocket factor’, a fatty acid-like molecule in a hydrophobic cavity of VP1 in HRV16 (Zhao et al., 1996). The acidic environment in endosomes may enhance major group rhinovirus infection (Nurani et al., 2003).

1.4.4 Internalisation via clathrin-dependent endocytosis

After attachment, virus particles can enter the cell via direct penetration at the plasma membrane or through endocytosis. Endocytosis can be through clathrin coated pits, caveolae or other pathways. Viruses requiring low pH for infection usually enter cells via clathrin-dependent endocytosis. The first acidic compartment encountered by viruses using this pathway is the early endosome. The pH in early endosomes is 6.0-6.5, sufficient for several viruses to uncoat. Others must traffic to late endosomes which are more acidic, pH 5.5-6.0. Other viruses entering cells by cholesterol dependent pathways are targeted to the caveosome, a compartment comparable to the early endosome but with a neutral pH. The goal for the virus on entering the cell is to deliver its genome to the site of replication, which in the case of FMDV is the cytoplasm. To do this, the virus must cross one or more membranes. Enveloped viruses such as influenza, VSV and SFV fuse their outer membranes with the endosomal or plasma membrane to release their genome, but non-enveloped viruses must adopt other strategies. Adenovirus is a non-enveloped
virus which has been shown to completely disrupt the endosomal membrane (Oliver Meier, 2003).

The FMDV capsid does not have a canyon and does not form ‘A’ particles. Instead, it binds to its integrin receptors via the GH loop of VP1. The FMDV capsid is extremely sensitive to acid and rapidly dissociates at less than neutral pH, 6.8-6.5 (Curry et al., 1995). Capsid disassembly is thought to be triggered by exposure to acidic conditions in the endosome as agents raising endosomal pH such as monensin and concanamycin A can inhibit FMDV infection (Baxt, 1987; Berryman et al., 2005; Miller et al., 2001). FMDV has been shown by a variety of methods to enter cells via clathrin-dependent endocytosis, and not to require caveolae (Berryman et al., 2005). These studies were carried out in cells expressing the integrin receptor αVβ6, a primary candidate for FMDV binding in vivo. It is not known how the viral RNA crosses the endosomal membrane, however it is likely to occur by a different mechanism to poliovirus (see below) as the N-terminal region of VP1 externalised on poliovirus binding to it receptor is absent from FMDV VP1. At early times during infection, FMDV has been co-localised with markers for early- and recycling endosomes (Berryman et al., 2005), making these candidates for penetration by the viral RNA. Virus did not co-localise with LAMP-2, a marker for lysosomes, and treatment of cells with nocodazole has no effect on FMDV infection, suggesting that late endosomes are not a site of viral RNA release (Berryman et al., 2005).

Human parechovirus-1 (HPEV) is also taken up into endosomes by clathrin-dependent endocytosis. Treatment of cells with the drug chlorpromazine, which is an inhibitor of clathrin-dependent endocytosis inhibited infection, and virus was seen to co-localise with markers of both early and late endosomes (Joki-Korpela et al., 2000).
However, treatment with nocodazole to inhibit trafficking from early to late endosomes did not affect infection (Joki-Korpela et al., 2001). This implies that release of the viral RNA can occur at the early endosome, although the mechanism is unknown.

Infection by human rhinovirus-2 (HRV-2), a minor receptor group rhinovirus also requires low pH, with capsid disassembly occurring at pH 5.5. This is lower than the pH found in the early endosome, suggesting that uncoating takes place in the late endosome. Raising the endosomal pH using bafilomycin inhibits infection, with the virus particles seemingly trapped in the early endosome and unable to access the lower pH in the late endosome (Prchla et al., 1994). A requirement for clathrin dependent endocytosis was shown by multiple methods, including drug treatments and expression of dominant-negative proteins that inhibit endocytosis (Snyers et al., 2003). HRV-2 infection occurs from late endosomes. At low pH, the N-terminus of VP1 and VP4 become externalised and may interact with the endosomal membrane to form a pore of defined size which is thought to allow the viral RNA to pass through (Prchla et al., 1995). A study comparing HRV-2 and adenovirus using single organelle flow analysis (SOFA) confirmed that HRV-2 makes small pores in the membrane whereas adenovirus disrupts the endosome completely (Brabec et al., 2005).

The poliovirus capsid is stable when exposed to acid and does not appear to require exposure to low pH for infection (Perez & Carrasco, 1993). However, poliovirus has been seen to be taken up into endosome-like structures (Kronenberger et al., 1998) and the endosomal route for infection has been reported by other studies (Ohka et al., 2004; Zeichhardt et al., 1985). In contrast, infection by poliovirus is not dependent
on dynamin, a molecule required for formation of both clathrin-coated pits and caveolae (DeTulleo & Kirchhausen, 1998). Taken together, these results suggest that poliovirus may use multiple pathways to enter cells (Arita et al., 1999). Poliovirus is also thought to form pores via the externalisation of VP1 and VP4, a consequence of ‘A’ particle formation, as mutations in these proteins abolish RNA release (Danthi et al., 2003; Kirkegaard, 1990). VP1 and VP4 can integrate into membranes and form pores, presumably to allow the RNA genome to pass into the cytoplasm (Danthi et al., 2003; Tosteson & Chow, 1997). It is not known which membrane (plasma membrane or internal vesicles) the viral RNA crosses. The N-terminus of VP1 has been shown to bind membrane in systems using artificial membrane vesicles (Bubeck et al., 2005; Tuthill et al., 2006).

1.4.5 Polyprotein translation

Once inside the cytosol the viral genome is translated to produce the viral proteins. The FMDV 5'UTR contains a type II internal ribosome entry site, or IRES. Its function is to enable translation of FMDV proteins to proceed while host cell mRNA translation is inhibited (Belsham & Jackson, 2000). Cellular mRNAs require a 7-methyl guanine (m7G) 5' cap and an intact eIF4F complex for translation initiation (Shatkin, 1976). The eIF4F complex is composed of 3 proteins; eIF4E which binds to the 5' cap structure, eIF4A which binds to the viral RNA and has helicase activity and eIF4G which acts as a scaffold protein between these two factors (see figure 1.6a). The eIF4F complex also recruits eIF3, which binds to the 40S ribosomal subunit. The methionyl-primed tRNA then binds the 40S subunit via interaction
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Figure 1.7(a) Cellular (cap-dependent) mRNA translation

The eIF4F complex associates with the 5' cap (via eIF4E) and the mRNA (via eIF4A). The 40S ribosomal subunit binds via eIF3 and the methionyl-primed tRNA binds to the complex via eIF2. This 48S pre-initiation complex scans down to the AUG initiation codon where the translation factors dissociate, the 60S ribosomal subunit associates, and translation begins.

Figure 1.7(b) Translation initiation by internal ribosome entry

The C-terminal fragment of the cleaved eIF4G protein, bound to eIF4A, is recruited directly to the highly structured IRES. This can then recruit the 48S pre-initiation complex, and translation of the FMDV polyprotein proceeds. This is discussed in section 1.4.5 'Polyprotein translation'
with eIF2. This completes the formation of the 48S pre-initiation complex. This complex scans along the mRNA until it reaches an AUG initiation codon in a good context (Kozak, 1989). The initiation factors then dissociate, the 60S ribosomal subunit joins to form the 80S complex, and translation begins (Hershey, 2000). Translation of the picornavirus genome is initiated in a different way. As it does not have a 5' cap structure, it cannot initiate translation in the same way as cellular mRNAs (Jackson et al., 2005). A viral protease (L or 2A) modifies the eIF4F complex by cleaving the bridging protein, eIF4G. The N-terminal fragment bound to eIF4E, the cap binding protein, dissociates from the complex. The C-terminal fragment of eIF4G remains bound to eIF4A, and targets the cleaved eIF4F complex to the highly structured IRES (see figure 1.6b). These factors are sufficient to initiate translation of the genome (Ohlmann et al., 1995). Cellular mRNAs (with a few exceptions) do not contain IRES elements and therefore cleavage of the eIF4F complex is sufficient to inhibit their translation. IRES elements can be divided into multiple types, based on sequence and secondary structure. Type I IRES elements are found in entero- and rhinoviruses, type II elements in cardio-, aphtho- and parechoviruses and type III elements in hepatoviruses. A new class of IRES element has been proposed for picornaviruses including sapelo- and teschoviruses as they more closely resemble the IRES element of hepatitis C virus (a flavivirus) in sequence, secondary structure and requirements for host translation initiation factors (Chard et al., 2006). In entero- and rhinovirus infection, the 2A protease is responsible for cleavage of eIF4G, whereas in FMDV infection it is the leader, or L protease. The leader protease is a papain-like cysteine protease (Roberts & Belsham, 1995) which can be translated from two different start sites to give two forms, Lab and Lb (Sangar et al., 1987) both of which are functional (Medina et al., 1993).
1.4.6 Replication membranes

Infection by most positive-strand RNA viruses causes extensive host cell membrane rearrangements and vesiculation. The replication complex is stabilised on these membranes, which allows the viral replication proteins to be concentrated in one place and/or to protect them from host antiviral responses. The vesicles that form during infection can, in some cases, be induced by expression of one or more viral non-structural proteins. The virus induced vesicles are thought to form from existing membranes, although the precise cellular origin of these vesicles cannot be agreed on as they appear to exclude most markers of cellular compartments. For poliovirus, the vesicles may originate from the ER, but other organelles implicated include early and late endosomes (Kujala et al., 2001), mitochondria (Miller & Ahlquist, 2002), and the Golgi apparatus (Mackenzie et al., 1999).

The membranes induced on viral infection vary between viruses. In the case of poliovirus, the vesicles are double membraned and around 200-400nm in diameter (Dales et al., 1965). Poliovirus genome replication takes place on the cytoplasmic face of these vesicles (Egger et al., 2000). Expression of non-structural proteins 3A and 2BC are sufficient to induce membrane rearrangements similar to those seen in poliovirus infection (Suhy et al., 2000). These proteins are localised to the ER (Doedens et al., 1997), and vesicles can be seen budding from the ER in infected cells (Bienz et al., 1987; Rust et al., 2001). The usual pathway involving vesicles budding from the ER is COPII dependent retrograde transport. Poliovirus vesicles have been shown to contain COPII proteins Sec13p and Sec31p; although poliovirus sensitivity to brefeldin A also suggests a role for COPI vesicles (Gazina et al., 2002). PV-induced vesicles have also been reported to acquire LC3 and LAMP1 later in
infection, suggesting an autophagosome-like origin (Suhy et al., 2000). The observation that the vesicles are double membraned also supports this view. Co-localisation of poliovirus non-structural proteins with a marker for cellular autophagy has been seen in infected cells (Jackson et al., 2005).

In the case of human parechovirus 1 (HPEV-1), replication membranes label for 2C and a trans-Golgi marker, GalT. Some unusual elongated, branching membranous structures can be seen, in addition to the typical vesicles. Although these structures contain 2C, no associated vRNA was seen, suggesting that these are not a site of replication (Krogerus et al., 2003). HRV2 infected cells contain vesicles that appear to be single rather than double membraned (Harris & Racaniello, 2005). A number of other positive-strand RNA viruses such as nidoviruses also replicate on double membraned vesicles similar in appearance to autophagosomes (Gosert et al., 2002; Pedersen et al., 1999; Prentice et al., 2004).

The vesicles induced on infection with FMDV were first described in 1983 and found to be associated with viral RNA and the RNA dependent RNA polymerase (RdRP) (Polatnick & Wool, 1983a; Polatnick & Wool, 1983b). They most closely resemble those of HPEV-1, being less numerous than those seen in PV infected cells, and not clustered (Monaghan et al., 2004). The vesicles are sometimes double, but more often single membraned (see figure 1.7). Some vesicles have been seen associated with ER-like structures and have a granular appearance (Monaghan et al., 2004). The most striking feature of FMDV infected cells is the accumulation of virtually all cytoplasmic organelles in a perinuclear area of the cell, described as the replication site (Monaghan et al., 2004).
Figure 1.8 Comparison of vesicles formed during FMDV and BEV infection
(Monaghan et al., 2004)

A) Vesicles formed during FMDV infection
B) Vesicles formed during BEV infection.
The vesicles induced on infection with FMDV are less numerous than those seen in BEV infected cells and are not clustered. The vesicles are sometimes double, but more often single membraned. They can be seen associated with ER-like structures and have a granular appearance. Both pictures from infection in baby hamster kidney (BHK) cells. This is discussed in section 1.8.1.
There are differences in sensitivity to the fungal metabolite brefeldinA in picornaviruses, with FMDV and EMCV being resistant (Barton et al., 2001; Gazina et al., 2002), HPEV-1 partially resistant (Gazina et al., 2002) and most rhino- and enteroviruses, including PV and BEV being sensitive. This is possibly related to the origin of the vesicles, with COPI vesicle formation being inhibited in brefeldinA treated cells (Rothman, 1994), although the picture is likely to be much more complicated than this.

1.4.7 Genome replication

The first stage of FMDV genome replication is synthesis of negative strand copies of the viral genome which then serve as templates for further rounds of positive strand synthesis. The positive strand RNA genome of picornaviruses must function as a template for both translation and replication. It is assumed there must be a 'switch' between the two processes as they cannot take place simultaneously on the same molecule (Gamarnik & Andino, 1998).

Initiation and elongation of the negative strand is catalysed by 3Dpol (Flanagan et al., 1977). The 3D polymerase requires a protein primer, provided by 3B (or VPg) (Paul, 2002). In order to act as a primer, VPg must first be modified by the addition of one or two uridyl residues. It was not known how this was achieved until a sequence in HRV-14 was found to be required for genome replication (McKnight & Lemon, 1996). This was traced to a stem-loop structure now termed the cis-acting replication element, or cre, which is now known to act as a template for uridylylation of VPg through a conserved AAACA motif (McKnight & Lemon, 1998). The viral 3Dpol catalyses the addition of the uridyl residues to VPg in PV (Paul et al., 2000).
and HRV (Gerber et al., 2001) using a “slide-back” mechanism (Paul et al., 2003).

Equivalent cre elements have now been identified in a number of picornaviruses (Goodfellow et al., 2000; Lobert et al., 1999), and interestingly, are not found in the same region of the genome and can even be moved to other sites without destroying their activity (Goodfellow et al., 2000). In fact, it has been found that they can act in trans, making the name cre misleading and leading to the suggestion of an alternative name-3B uridylylation site, or bus (Tiley et al., 2003). The FMDV cre is situated in the 5’ UTR rather than the coding region as in other picornaviruses (Mason et al., 2002). FMDV requires uridylylated VPg for both positive- and negative strand synthesis and the uridylylation reaction requires the cre as a template and unlike poliovirus, cannot use the polyA tail (Nayak et al., 2005). The factors required for uridylylation of FMDV VPg are UTP, 3B peptide (each of the three copies is functional but VPg3 has the highest activity), RNA transcripts containing the cre/bus, 3Dpol, and the 3CD precursor, although this is known to be rapidly cleaved in FMDV infected cells (Nayak et al., 2005). The uridylylated VPg is proposed to translocate to the 3’UTR in order to initiate negative strand synthesis (Paul et al., 2000).

Once the negative strand has been made, multiple rounds of positive strand synthesis follow. Free single-stranded, negative sense RNA is not detectable in the cell, but exists as the double stranded ‘replicative form’ (RF) (Agol et al., 1999). This must be unwound prior to positive strand synthesis, possibly by 2C. The ratio of positive to negative strands in poliovirus infected cells is 50:1 (Novak & Kirkegaard, 1991), suggesting that one negative strand acts as a template for multiple positive strands.
1.4.8 Genome packaging and virus release

Once genome replication has occurred, the final stages of picornavirus infection are packaging of newly generated positive-strand RNA genomes and release of virus particles from the cell. These processes are not well understood.

Encapsidation of the viral RNA begins with association of VP0, VP2 and VP3. Five of these associate to form a pentamer. Twelve pentamers then associate to form the viral capsid. The RNA is encapsidated and the final maturation is achieved by cleavage of VP0 into VP2 and VP4. This step is required in order for virus to be infectious (Lee et al., 1993).

It is assumed that there must be a way in which the viral RNA is selected for encapsidation rather than cellular RNA molecules. Poliovirus positive-strand RNA is specifically packaged with the exclusion of negative strands (Novak & Kirkegaard, 1991). Coxsackievirus A21, coxsackievirus B3 or enterovirus 70 capsids would not encapsidate poliovirus RNA when supplied in trans (Porter et al., 1998). It has been suggested that RNA packaging is coupled to replication in poliovirus (Nugent et al., 1999), and that there may be a physical interaction between the replication complex and the capsid, possibly through 3D or 2C (Diamond & Kirkegaard, 1994; Vance et al., 1997). Aichi virus was found to have an encapsidation signal in the 5'UTR, the first discovered in a picornavirus (Sasaki & Taniguchi, 2003).

Release of picornavirus particles has always been thought to be via cell lysis, except in the case of hepatitis A virus (Vallbracht et al., 1984). Cell lysis in poliovirus has been suggested to be triggered by 3A or 3AB, through disruption of membrane
potential (Lama et al., 1998), although a theory has been put forward that poliovirus can release particles without lysis by fusion of the outer membrane of its double-membraned replication vesicles with the plasma membrane (Jackson et al., 2005)-see above, section 1.4.7 ‘FMDV replication membranes’. This view is supported by previous work showing vectorial release of poliovirus in polarised epithelial cells (Tucker et al., 1993).

1.5 Rab GTPases

Dominant-negative rab GTPases are increasingly being used to dissect vesicular trafficking through the endocytic pathway. Rab GTPases are members of the ras superfamily (ras-related in brain (Touchot et al., 1987)), and are homologous to the YP7Y/SEC4 family of GTPases which regulate membrane trafficking in yeast. The human genome encodes over 70 different rab proteins (Bock et al., 2001) and at least 13 have been identified as regulators of the endocytic pathway (Harrison et al., 2003). As with all GTPases, they cycle between active, GTP-bound and inactive, GDP-bound forms. They are involved in formation, movement and fusion of endocytic vesicles. Each rab GTPase is specific to a particular stage, or stages of endocytosis (Seabra et al., 2002)(Lombardi et al., 1993). They attach to the target membrane and the active form recruits effector proteins for specific downstream functions.

Figure 1.8 shows the stage or stages at which individual rab GTPases act during endocytosis. Rab5 is involved in formation of the early endosome. Rab4 and rab11 are involved in recycling of receptors back to the plasma membrane. Rab4 controls a rapid recycling pathway directly from the early endosome to the plasma membrane.
and rab11 controls a slower recycling pathway via the perinuclear recycling endosome. Rab7 is involved in trafficking from early- to late endosomes and from late endosomes to lysosomes. Rab9 controls trafficking from the late endosome to the Golgi. There is evidence that different rab GTPases can exist on the same vesicle, occupying separate domains on the membrane. In this way, rab4, rab5 and rab11 can all exist on the same early endosome, as shown by three colour imaging (Sonnichsen et al., 2000). Rab7 and rab9 have also been shown to exist on the same late endosome (Barbero et al., 2002).

Rab proteins cycle between active and inactive forms, and are at different stages either membrane bound or cytosolic. The state of activation and localisation of rab proteins are regulated by a number of factors (see figure 1.9). Rabs are synthesised as soluble proteins in the cytosol, and are sequestered by rab escort proteins (REPs), which act as chaperones. These factors deliver the rab protein to a rab geranylgeranyl transferase enzyme for prenylation. One (or sometimes two), geranylgeranyl groups are added (Anant et al., 1998). REPs then target the rab protein to the appropriate membrane where these lipid attachments allow the rab protein to integrate into the lipid bilayer (Alexandrov et al., 1994).

Guanine nucleotide exchange factors (GEFs) facilitate the activation of membrane bound rab proteins by exchanging GDP for GTP. The effector can then bind to the activated rab protein. After vesicle movement or fusion, GTP hydrolysis occurs, restoring the rab protein to its inactive state. The GDP dissociation inhibitor (GDI), then removes the inactive rab protein from the membrane back into the cytosol (Ullrich et al., 1993). The dissociation of the REP (in the case of newly synthesised
Figure 1.9 Localisation of rabGTPases in the endocytic pathway

Rab5 is involved in formation of the early endosome.

Rab4 controls a rapid recycling directly from the early endosome to the plasma membrane.

Rab11 controls a slower recycling via the perinuclear recycling endosome and movement from the Golgi to the cell surface.

Rab7 is involved in trafficking from early to late endosomes and from late endosomes to lysosomes.

Rab9 controls movement from the late endosome to the Golgi. This is discussed in section 1.6.
proteins), or GDI (in the case of existing rab proteins) when the rab-GDP binds to
the membrane is aided by a GDI displacement factor (GDF) intrinsic in the
membrane (Dirac-Svejstrup et al., 1997). Only one such factor has been identified
so far, Yip3 (Sivars et al., 2005).

Rab proteins have a low intrinsic GTPase activity and rely on a variety of GTPase-
activating proteins (GAPs) to facilitate GTP hydrolysis. Each rab protein may be
regulated by a number of different GAPs and GEFs. GAPs are thought to function
by allowing a catalytically important glutamine residue to align properly, inserting
an ‘arginine finger residue’ into the active site. This stabilises the transition state of
the hydrolysis reaction. This glutamine residue can be replaced with a leucine to
block this GTP hydrolysis, locking the rab protein in its active state. All rab GAPs
have a conserved TBC (Tre-2/Bub2/Cdc16) domain, enabling their identification in
BLAST searches. A total of 51 putative GAPs have been identified (Bernards, 2003)
but most have not been assigned their interacting rab partner.

Another group of interacting proteins are the effectors. These interact with active,
GTP-bound rab proteins on membranes (Harrison et al., 2003). Rabs can interact
with multiple effectors, and some effectors can bind more than one rab protein (de
Renzis et al., 2002; Segev, 2001). The downstream events resulting from effector
binding include vesicle fusion and movement.

The effectors involved in membrane fusion events are known as soluble N-
ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs)
(Brodsky et al., 2001; Jahn & Grubmuller, 2002; Sollner, 2004). SNARE proteins
interact with one another to form extremely stable complexes. It is thought that two
Figure 1.10 Membrane recycling of rabGTPases. (Seabra & Wasmeier, 2004)

(a) GDP-bound rab proteins in the cytosol are bound to chaperone proteins, REP (when newly synthesised) and GDI. (b) Bound rab-GDP is delivered to the target membrane where GDF causes displacement of GDI. Other factors may be involved. (c) Nucleotide exchange catalysed by GEF leading to rab activation. (d) Effectors bind to activated rab-GTP. (e) GTP hydrolysis catalysed by GAP causing rab to return to its inactive state. rab-GDP extracted from membrane by GDI. This is discussed in section 1.6.
SNARE proteins on different membranes may ‘zipper up’ with one another, and this interaction is sufficient to drive fusion. SNAREs are specific to certain compartments and pathways, in the same way that rabs are. The pairing of two similar SNAREs will lead to homotypic endosome fusion, as in the case of two early endosomes fusing with one another. The fusion of different vesicles, for example early and late endosomes, would require two different SNAREs to interact.

Another function of rab proteins is vesicle movement. There are two transport networks in cells, microtubule-based and actin-based. Microtubules facilitate long range, rapid movement of cargo and the actin network aids slower movement over shorter distances. Effector proteins bound to active, GTP-bound rab proteins can recruit molecular motors such as either myosin (for actin) or dynein and kinesin (for microtubules). Rab GTPases recruiting myosins include rab11 (Hales et al., 2001), rab27 (Fukuda et al., 2002) and possibly rab8 (Seabra & Coudrier, 2004). Rab7 can recruit dynamin (Jordens et al., 2001), as can rab6, a Golgi-associated rab GTPase (Short et al., 2002).

1.5.1 Rab5

Rab5 is the rab GTPase involved in the biogenesis of the early (or sorting) endosome (Bucci et al., 1992; Gorvel et al., 1991). It is found on the sorting endosome and also on clathrin coated vesicles. It interacts with up to 30 different effector proteins to facilitate endosome-endosome docking and fusion (Christoforidis et al., 1999). In addition to regulating endosome-endosome fusion, rab5 is also thought to have roles in formation of vesicles at the cell surface (McLauchlan et al., 1998), and movement
of vesicles within the cell via interaction with molecular motors (Nielsen et al., 1999).

One of the effector proteins first identified for rab5 was rabaptin-5, thought to have a role in membrane docking (Mu et al., 1995). This protein also binds to rab4, which is involved in receptor recycling from the sorting endosome back to the cell surface. Rabaptin-5 downregulates rab5 activity through both direct inhibition and by association with rabex-5, a rab5-specific GEF (Alexandrov et al., 1994). In this way, it is thought to create areas of active, membrane-associated rab5.

Another rab5 effector is early endosomal antigen-1 (EEA-1) (Simonsen et al., 1998). This protein is used as a marker specific for early endosomes and has two binding sites for rab5. It is closely related to the FYVE zinc finger protein family. These proteins bind the lipid phosphatidylinositol-3-phosphate (PI(3)P) and are all localised to endosomes (Simonsen et al., 1998). Levels of PI(3)P are controlled by signalling cascades. EEA-1 is thought to act as a tethering protein, and brings together rab5 and SNAREs (Christoforidis et al., 1999). There is also cross-linking evidence for a high molecular weight complex containing rab5 and both EEA-1 and rabaptin-5, prior to endosome fusion. A further rab5 effector protein, rabankyrin5 was found to function in macropinocytosis, but does not co-localise with EEA-1. Therefore there must exist in cells a population of rab5-positive, EEA-1 negative compartments (Schnatwinkel et al., 2004).

A genome screen for the TBC (Tre-2/Bub2/Cdc16) containing motifs found in all rab proteins identified two GAPs thought to act on rab5: RN-Tre and PRC17 (Bernards, 2003). However, a further study showed RN-Tre to be a GAP for rab41 but
identified a further GAP for rab5, known as RabGAP-5 (Haas et al., 2005). The p85 alpha subunit of PI3K has also been identified as having GAP activity towards rab5 (Anderson et al., 2005). Another rab5 GEF has been identified as RAP6 (Rab5-activating protein 6) (Hunker et al., 2006).

1.5.2 Rab4/11

Rab4 and rab11 are involved in recycling of receptors to the plasma membrane. There are two recycling pathways with different kinetics; a rapid recycling process from the early endosome directly to the plasma membrane as well as a slower recycling activity via the perinuclear recycling endosome. There appears to be some overlap in the functions of the two factors, as rab11 controls movement from the early endosome to the perinuclear recycling endosome (Ullrich et al., 1996) and rab4 regulates the more rapid pathway, but both are responsible for trafficking from the perinuclear recycling endosome to the plasma membrane (Sonnichsen et al., 2000). Rab11 is also involved in trafficking from the trans-Golgi network (TGN) to the plasma membrane (Chen et al., 1998).

A role for rab4 in receptor recycling was first identified by co-fractionation with the transferrin receptor, a well known marker for endocytic recycling (van der Sluijs et al., 1991). It was later shown that overexpression of wild-type or dominant-negative rab4 could perturb transferrin recycling (van der Sluijs et al., 1992). Some effector proteins have been identified for rab4, many also being effectors for rab5. These include Rabaptin 5 (Vitale et al., 1998), Rabip4 (Cormont et al., 2001), Rabaptin4 (Nagelkerken et al., 2000) and Rab coupling protein (RCP) (Lindsay et al., 2002). Many of these have been shown to affect recycling of cargoes such as transferrin and
GLUT4. A GTPase activating protein termed rab4-GAP has been identified on membranes in 3T3 cells (Bortoluzzi et al., 1996).

Rab11 was shown to control transferrin recycling via the perinuclear recycling endosome in a slower process than rab4 dependent recycling (Ullrich et al., 1996). The first direct comparison of their respective rates showed that rab4 dependent recycling had a half-life of 5-10 minutes compared to 15-30 minutes for rab11 dependent recycling (Hopkins & Trowbridge, 1983). A role for rab11 in exocytosis from the Golgi was shown by expression of dominant-negative rab11, which caused VSV-G protein to accumulate in the Golgi (Chen et al., 1998). Rab11 effectors identified to date include Rab11BP/rabphilin-11, (Mammoto et al., 1999; Zeng et al., 1999) and EF-hands-containing Rab11-interacting protein (Eferin) (Prekeris et al., 2001). A further study identified four effectors for rab11: Rab11-Family Interacting Protein 1 (Rab11-FIP1), Rab11-Family Interacting Protein 2 (Rab11-FIP2), Rab11-Family Interacting Protein 3 (Rab11-FIP3) and pp75/Rip11. Rab11 has known myosin binding activity through the effector FIP2 (Hales et al., 2001).

1.5.3 Rab9

Rab9 is found on the late endosome and Golgi, and regulates movement of cargoes including mannose 6-phosphate receptors (Lombardi et al., 1993). Mannose 6-phosphate receptors (MPRs) transport lysosomal hydrolases from the Golgi to prelysosomes and then return to collect more cargo. One requirement for this movement from late endosomes to Golgi is an effector protein known as tail-interacting protein of 47 kD (TIP47). Another is an effector present on the Golgi known as p40 (Diaz et al., 1997).
1.5.4 Rab7

When rab7 was first identified, it was observed to localise to the late endosome (Chavrier et al., 1990). It was later found to regulate trafficking from the early to late endosome with dominant-negative forms of rab7 (N125I and T22N) blocking movement of the VSV-G protein and causing it to accumulate in transferrin-positive early endosomes (Feng et al., 1995). These mutants also blocked degradation of $^{125}$-low density lipoproteins (LDL) but not their uptake (Vitelli et al., 1997). Rab7 is also required to maintain the integrity of the lysosome (Bucci et al., 2000).

Rab7 effectors have been identified, the first of which is rab-interacting lysosomal protein (RILP), a 45kDa protein that specifically binds to rab7-GTP at its C-terminus. Expression of a truncated form of RILP slows degradation of LDL and causes dispersal of lysosomes, similar to the effects seen when dominant-negative rab7 is expressed (Cantalupo et al., 2001). It has further been shown that RILP recruits dynein/dynactin and therefore connect the late endosome/lysosome to the microtubule network (Jordens et al., 2001).

Another recently identified rab7 effector is Rab7-interacting RING finger protein (Rabring7), which is speculated to act on the homotypic fusion and vacuole protein sorting (HOPS) complex. This complex regulates heterotypic fusion of late endosomes with lysosomes and homotypic fusion of lysosomes leading to lysosome biogenesis (Mizuno et al., 2003). Rabring7 also appears to have a role in lysosomal acidification, possibly by acting on the vacuolar ATPase, whereas RILP does not affect this process.
There is only one rab7 GEF identified so far, the Vps39 subunit of the class C VPS/HOPS complex (Wurmser et al., 2000) and one GAP, TBC domain family, member 15 (TBC1D15) (Zhang et al., 2005).

Rab7 has recently been shown to be required for the late maturation of autophagic vacuoles (Gutierrez et al., 2004; Jager et al., 2004). This will be further discussed in section 1.7 'Autophagy'.

1.5.5 Rab7 and viruses

A number of studies have been carried out investigating the effects of dominant-negative rab GTPases on virus infection. The first of these was a study involving adenovirus. Dominant-negative rab5 was shown to impair virus uptake, whereas overexpression of wild-type rab5 increased it (Rauma et al., 1999). A study of enveloped viruses showed a requirement for both rab5 and rab7 in influenza virus infection, but only for rab5 in Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV). This observation supports the observation that influenza requires a much lower pH for uncoating than the other viruses and was therefore thought to traffic to the late endosome (Sieczkarski & Whittaker, 2003). A screen of cellular genes required for Marburg virus infection showed rab9 as a candidate, and RNAi depletion of rab9 was shown to inhibit infection of a number of enveloped viruses including Ebola, Marburg, and measles viruses, but not the non-enveloped reovirus (Murray et al., 2005). Rab11 was also shown to be required for HIV infection, consistent with the view that HIV assembly initiates from the late endosome and then the Golgi prior to budding from the plasma membrane (Murray et al., 2005).
1.6 Autophagy

Rab7 is also involved in regulating the autophagic pathway. Autophagy is a cellular response to stress conditions such as amino acid starvation. The process of autophagy occurs in stages, with multiple factors regulating each stage. The mechanism of formation of the characteristic double-membraned vesicles is a current 'hot topic', as autophagy has been linked to many diseases (including cancer) as well as being implicated in the life cycles of bacteria and viruses.

1.6.1 The autophagic pathway

The process of autophagy allows the cell to survive during short term amino acid starvation (Mizushima et al., 2002). An area of cytoplasm and/or organelles is engulfed by an isolation membrane and the autophagosome matures through a series of steps before being directed to the lysosome where the contents are degraded and recycled (see figure 1.10). In yeast cells, autophagosomes form from the preautophagosomal structure (PAS), but in mammalian cells the origin of the membranes is unclear, some evidence suggesting that the membranes are derived from the endoplasmic reticulum (Dunn, 1994). The process of autophagy begins with the dephosphorylation and thus inactivation of ToR (target of rapamycin). This is an inhibitor of autophagy under normal conditions and dephosphorylation can be induced either by starvation or treatment with the drug rapamycin (Noda & Ohsumi, 1998). The formation of the isolation membrane requires three proteins: Atg5 conjugated to Atg12 and Atg8 (Mizushima et al., 1998). Atg (autophagy-related) genes were identified in yeast strains defective for autophagy. After the
Figure 1.11 Cellular autophagy

An isolation membrane forms around an area of cytoplasm and/or organelles. This becomes double membraned and LC3 can be detected at this point. LAMP associates with the mature autophagosome. Rab7 is required for late maturation, prior to fusion with the lysosome. This is discussed in section 1.7.
autophagosome is sealed, Atg5/12 can no longer be detected (Mizushima et al., 2001). The mammalian homologue of Atg8 is known as microtubule-associated protein-1 light-chain 3 (LC3). This is required for (and is a specific marker of) autophagy (Kabeya et al., 2000). Although LC3 in the outer membrane of the early autophagosome appears to be cleaved and possibly recycled, it remains bound to the inner membrane at all stages of autophagy. The outer membrane has no particular markers at early stages, but lysosomal proteins can be detected in the more mature autophagosomes. These include lysosomal membrane proteins LAMP1 and LAMP2 (Tanaka et al., 2000) and lysosomal enzymes Cathepsin D, B and L (Koike et al., 2000). It is thought that maturation of the autophagosome is a stepwise process beginning with fusion with early endosomes (Liou et al., 1997; Tooze et al., 1990) and then with multivesicular late endosomes (Berg et al., 1998), forming the amphisome. This is prior to fusion with lysosomes (Gordon et al., 1992). The markers found in late autophagic vacuoles could therefore have come from late endosomes or lysosomes. In early autophagosomes the pH has been measured at 6.4, dropping to 5.7 in the autolysosome (Tanaka et al., 2000). This may be due to the acquisition of proton pumps and lysosomal proteins through fusion events.

One factor present in both early and late autophagosomes is rab7 (see section 1.9 ‘Rab GTPases’), although its association is greater at late time points (Jager et al., 2004). In cells transfected with a dominant-negative rab7 or rab7-specific siRNA, early autophagosomes accumulate, indicating that rab7 is required for their final maturation (Gutierrez et al., 2004; Jager et al., 2004). A tracer taken up into early endosomes can be delivered to autophagosomes in rab7 deficient cells, but a lysosomal tracer cannot (Gutierrez et al., 2004). Rab24 is also implicated in
autophagy as it redistributes on induction of autophagy to LC3 positive compartments (Munafo & Colombo, 2002).

1.6.2 Bacteria replication vesicles

Bacteria have evolved a number of strategies in order to infiltrate host cells (Finlay & Falkow, 1997). Some enter the endocytic pathway and replicate in endosomes, while others subvert cellular autophagy. In both cases the bacteria require a vesicle where they can be hidden from the host cell response during replication. Many bacteria have mechanisms to impair the maturation of autophagosomes, thus preventing their fusion with lysosomes which would lead to degradation of the bacteria. For example, *Mycobacterium tuberculosis* replicates in vesicles lacking the V-ATPase, preventing acidification (Sturgill-Koszycki et al., 1994). *Salmonella typhimurium* traffics to a late endosome-like vacuole that cannot fuse with the lysosome (Meresse et al., 1999). The bacterium secretes a protein known as SifA that mimics the rab7 effector, RILP. SifA binds to rab7 in the place of RILP, preventing recruitment of microtubules and fusion with the lysosome (Harrison et al., 2004). Other bacteria able to exploit autophagy include *Coxiella burnetii* (Gutierrez et al., 2005), *Brucella abortus* (Dorn et al., 2002), *Legionella pneumophila* (Amer & Swanson, 2005) and *Porphyromonas gingivalis* (Dorn et al., 2001).

1.6.3 Autophagy and viruses

There is increasing evidence for the involvement of autophagy in viral infection. Two groups of viruses, the nidovirales and the picornaviridae, induce the formation of double membraned vesicles. The nidovirales (arteriviruses and coronaviruses)
replicate on vesicles smaller than autophagosomes, about 100-300nm as opposed to 500-1000nm (Gosert et al., 2002; Pedersen et al., 1999; Prentice et al., 2004). In poliovirus infected cells, double membraned vesicles of around 200-400nm can be seen (Dales et al., 1965; Suhy et al., 2000). These vesicles co-localise with LC3, and virus infection is reduced in LC3 or Atg12 knockout cells (Jackson et al., 2005). This implies an autophagic origin for the replication vesicles, in contrast to the previous suggestion that they are derived from COPII vesicles (Rust et al., 2001).

1.7 Aims

FMDV infection requires clathrin-dependent endocytosis and the low pH within endosomes. However, the precise endosomal compartments where capsid disassembly and vRNA membrane penetration take place are not known. The primary objective of the work described in this thesis is to characterise further the entry of FMDV into cells. The major approach has been the use of dominant-negative rab GTPases. Rab GTPases regulate many aspects of intracellular membrane transport and a number of rabs have been identified that regulate specific steps of endocytosis. Expression of dominant-negative rabs can therefore be used to investigate virus entry and trafficking pathways.
Chapter two: Methods

2.1 Cell culture

The porcine kidney cell line IBRS2 was cultured in Glasgow Eagles media (GMEM-IAH) supplemented with 10% adult bovine serum (ABS-Sigma), 20mM glutamine, 100 si units per ml penicillin and 100µg per ml streptomycin.

Baby hamster kidney cells (BHK) were grown in Dulbecco’s modified Eagles media (DMEM-Sigma) supplemented with 10% foetal calf serum (FCS-Sigma), 20mM glutamine, 100 si units per ml penicillin and 100µg per ml streptomycin.

2.1.1 Cell passage

Once confluent, cell monolayers were washed with 0.25% versine-trypsin and incubated at 37°C for approximately 5 minutes in the presence of 0.25% versine-trypsin. Cells were collected and pelleted using a Sorval Legend RT centrifuge for 3 minutes at 1000rpm. Cells were resuspended in culture medium and seeded at the required dilution into a fresh tissue culture flask.

2.2 Virus

All experiments use the FMDV strain O1Kcad2, a non-heparan sulphate binding virus. Working stocks were grown using primary bovine thyroid cells (BTY) and purified virus was prepared on sucrose gradients. All virus stocks were stored at -80°C.
2.2.1 Preparation of virus working stocks

Primary bovine thyroid cells (BTY) were grown in 175cm² tissue culture flasks until confluent. The cells were then washed with PBS and an aliquot of a previous virus stock was added to the cells. This was incubated in a 37°C incubator with 5% CO₂ for 15 minutes before adding 20ml pre-warmed viral growth medium (VGM) consisting of normal growth medium with 1% serum. The cells were returned to 37°C until cytopathic effect (CPE) was observed. The cells were freeze thawed to increase yield. Working stocks of virus were centrifuged before use to remove cell debris. The supernatant was aliquoted into 1.5ml screw capped eppendorf tubes and stored at -80°C.

2.2.2 Virus titration by plaque assay

60mm tissue culture dishes of BHK cells were cultured overnight in order to reach approximately 80% confluency. The cells were washed with PBS and 100μl virus diluted in DMEM added at tenfold dilutions. The dishes were incubated at 37°C for 15 minutes before being overlaid with 4ml Eagle’s overlay (see appendix I) at 45°C. The overlay was allowed to set at room temperature and the cells returned to 37°C for 48 hours. Plaques were visualised by fixing and staining with methylene blue and 4% paraformaldehyde in PBS for 24 hours.

2.3 Antibodies, fluorescent ligands and pharmacological inhibitors

Anti-FMDV antibodies used were monoclonal antibody D9 (mouse IgG2a), which recognises antigenic site 1 of type O FMDV, anti-3A monoclonal antibody 2C2 (mouse IgG2a) and a rabbit polyclonal serum which recognises the FMDV type O
capsid proteins. Integrin αVβ8 was detected using monoclonal antibody 14E5 (mouse IgG1). A variety of antibodies which recognise proteins localising to specific cellular compartments were used in this study. Early endosomes were labelled using anti-EEA1 clone 14 (mouse IgG1-BD Transduction Laboratories). The transferrin receptor was detected using a monoclonal antibody H68.4 (mouse IgG1-Zymed). The mannose 6-phosphate receptor was detected with a monoclonal antibody (mouse IgG2a-Affinity BioReagents). For the AP180C experiment, myc-tagged AP180C protein was detected using the anti-c-myc antibody 9E10 (mouse IgG-Developmental Studies Hybridoma Bank). All Alexa-fluor secondary antibodies were obtained from Molecular Probes (Invitrogen), as were the Alexa-fluor conjugated transferrin, DilLDL and lysotracker red. Pharmacological inhibitors used were nocodazole (Sigma) at 10mg/ml in DMSO and concanamycin A (Fluka) at 10mg/ml in DMSO. Antibodies used for western blotting were an anti-rab7 antibody (obtained from Suzanne Pfeffer), a rabbit polyclonal anti-rab5 antibody (Stressgen) and a mouse monoclonal anti-β-actin antibody (Sigma).

2.4 Virus infectivity assays

Infection was quantified using two methods; ELIspot assay or immunofluorescence.

2.4.1 ELIspot assay

For quantification of effects of pharmacological inhibitors on the number of FMDV infected cells, the ELIspot assay was used. Cells were cultured in 96 well plates overnight until approximately 70-80% confluent. The cells were washed and pre-treated with DMEM with or without drug for 30 minutes at 37°C. The cells were washed with DMEM before adding FMDV O1Kcad2 with or without drug for 1
hour. The cells were washed and incubated at 37°C for a further four hours in DMEM in the presence or absence of drug. As the stock drug solutions were prepared in DMSO, the equivalent concentration of DMSO was added to the DMEM in 'no drug' controls. This was to discount the possibility that effects on infection could be attributed to the DMSO. The cells were then fixed for 1 hour using 4% paraformaldehyde. For virus labelling, cells were washed 5 times with PBS then permeabilised using 0.1% Triton X-100 in PBS for 15 minutes. They were washed again and incubated in blocking buffer for 30 minutes (140mM NaCl, 1mM CaCl₂, 0.5mM MgCl₂, 10% normal goat serum and 1% fish gelatin) to reduce non-specific binding. Primary antibody 2C2 (mouse IgG2a) diluted 1/1000 in block buffer was then added for 1 hour at room temperature. Mab 2C2 recognises FMDV non-structural protein 3A, which is a marker for virus replication. The cells were washed and incubated for 1 hour with a biotinylated goat anti-mouse IgG secondary antibody at 1/500 in block buffer. This was followed by incubation with a streptavidin-conjugated alkaline phosphatase (Caltag laboratories) at 1/1000 in block buffer for 45 minutes. The alkaline phosphatase substrate (BIO-RAD) was then added for 6-7 minutes until a colour change was observed by light microscopy. Cells were then washed with distilled water and allowed to dry. Infected cells appear blue under a light microscope and were counted using an ELIspot plate reader (Zeiss).

2.4.2 Immunofluorescence assay

Cells were seeded on 13mm glass coverslips (BDH) to be approximately 80% confluent and transiently transfected (see section 2.6.2) using lipofectamine 2000 (Invitrogen) with mammalian expression plasmids expressing a number of enhanced green fluorescent protein (EGFP) fused rab GTPase proteins or myc-tagged...
AP180C. After incubation at 37°C for 6 hours, cells were washed and infected with FMDV O1Kcad2 for 3 hours at MOI=1. Where bovine enterovirus (BEV) was used, the infection time was extended to 6 hours. The cells were then fixed with 4% paraformaldehyde in PBS for 40 minutes and processed for confocal microscopy. Cells expressing EGFP fused rab GTPase proteins appear green, and AP180C expressing cells were labelled with an anti-c-myc antibody (9E10) and Alexa-488 conjugated goat anti-mouse IgG secondary antibody. Infected cells were labelled with rabbit polyclonal serum to the FMDV capsid and an Alexa-568 conjugated goat anti-rabbit IgG secondary antibody. The number of cells expressing the transfected protein (AP180C or rab GTPase) that were infected and the number of non-expressing cells infected were counted.

2.5 Immunofluorescence microscopy

2.5.1 Detection of intracellular antigen

Cells were seeded overnight on 13mm glass coverslips (BDH) to be approximately 80% confluent and fixed with 4% paraformaldehyde in PBS for 40 minutes. Cells were washed three times with PBS and permeabilised using 0.1% Triton X-100 in PBS for 30 minutes. The cells were washed again and incubated in blocking buffer (see appendix I) for 30 minutes to reduce non-specific binding. The cells were incubated with primary antibodies diluted in blocking buffer at the appropriate concentration for 1 hour at room temperature. This was followed by three five minute washes with PBS to remove unbound antibody. The Alexa fluor conjugated secondary antibodies (488nm-green or 568nm-red) were diluted in blocking buffer at a concentration of 1:200 and incubated with the cells for a further 1 hour at room temperature. The cells were then washed again in PBS for five minutes, three times.
Finally, the cells were washed with distilled water and mounted in Vectashield mounting medium containing DAPI (for DNA labelling) and sealed using clear nail varnish.

2.5.2 Detection of surface antigen

The procedure above was modified for detection of surface antigen. Cells were seeded on coverslips as before, and cooled on ice. Non-specific binding was blocked by incubation with cold blocking buffer on ice for 30 minutes. For detection of receptor αVβ8, the primary antibody 14E5 was diluted at 1:1 in cold GMEM and incubated with the cells on ice for 1 hour, followed by washing three times for five minutes each, with cold GMEM. A goat anti-mouse IgG1 Alexa conjugated secondary antibody was then added at 1:200 in cold GMEM and cells were incubated on ice for a further 1 hour. The cells were then fixed with 4% cold paraformaldehyde at the end and mounted as described in section 2.5.1.

2.5.3 Ligand internalisation

2.5.3.1 Virus binding and internalisation

Cells were incubated with purified FMDV at 5μg/ml diluted in cold GMEM for 45 minutes on ice. Unbound virus was removed by washing with cold GMEM three times, for five minutes each time. For internalisation studies, warm GMEM was then added and the cells were incubated at 37°C for the times indicated, then fixed with 4% cold paraformaldehyde and permeablised using 0.1% Triton X-100 in PBS for 30 minutes. Virus was detected using Mab D9 (5μg/ml) and a goat anti mouse IgG2a Alexa conjugated secondary antibody. For labelling of surface virus, the
warming step was removed, and labelling with D9 was carried out immediately using cold reagents, on ice. Fixation with 4% cold paraformaldehyde was at the end of the experiment. Coverslips were processed as described in section 2.5.1.

2.5.3.2 Transferrin binding and internalisation

Cells were washed with GMEM and incubated at 37°C for 45 minutes in GMEM to deplete the cells of iron. This process will enhance transferrin uptake. Alexa 568nm conjugated transferrin was diluted in GMEM at 25μg/ml and added to the cells at 4°C for 45 minutes to allow binding. The cells were washed with cold GMEM to remove unbound transferrin and internalisation initiated by washing with warm GMEM, followed by incubation at 37°C for the times indicated. For some experiments, labelled transferrin was chased out of the cells using holo-transferrin (unlabelled), also diluted in GMEM at a concentration of 25μg/ml. At the end of the experiment, cells were fixed with 4% cold paraformaldehyde and the coverslips processed as described in section 2.5.1.

2.5.3.3 LDL binding and internalisation

Cells were incubated with DilLDL at 10μg/ml diluted in cold GMEM for 45 minutes on ice. Unbound LDL was removed by washing with cold GMEM three times, for five minutes each time. Warm GMEM was then added and the cells were incubated at 37°C for the times indicated. At the end of the experiment, cells were fixed with 4% cold paraformaldehyde and the coverslips processed as described in section 2.5.1.
2.5.3.4 Labelling of acidic compartments with lysotracker red

Cells were incubated with lysotracker red at 100nM diluted in warm GMEM at 37°C for the times indicated, then fixed with 4% cold paraformaldehyde. The coverslips were processed as described in section 2.5.1.

2.5.4 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 cell(s) in question, with the exception of figures 5.8 and 5.9 which show an overlay of a number of sections through the cell. In order to reduce crosstalk between detection channels, data were collected sequentially. Images were processed using Adobe Photoshop software.

2.6 DNA techniques

2.6.1 Mammalian expression plasmids

Plasmids for expression of canine rab proteins fused to EGFP were obtained from Dr S.S. Ferguson (Robarts Research Institute, Canada). The plasmids were constructed using pEGFP-C1 or pEGFP-C2 (Clontech). These plasmids were transformed into competent *E.coli* JM109 and grown up overnight in 100ml LB with kanamycin (50μg/ml). Plasmid DNA was extracted using the Qiagen midi-prep system according to the manufacturer’s instructions.
2.6.2 Transient transfection of mammalian cells with plasmid DNA

Cells were seeded overnight on 13mm glass coverslips (BDH) in the absence of Penicillin/Streptomycin to be approximately 80% confluent. For each coverslip, 1μg plasmid DNA was diluted into 50μl Opti-MEM (Gibco) in one microfuge tube, with 3μl lipofectamine 2000 transfection reagent (Invitrogen) diluted into 50μl Opti-MEM (Gibco) in a separate microfuge tube. These were incubated at room temperature for 5 minutes. The contents of both tubes were then combined and incubated for a further 20 minutes. This mixture was added to the cells and media. The cells were then incubated at 37°C for six hours. At this point, cells were either fixed with 4% paraformaldehyde or infected with virus for 3 hours (FMDV) or 6 hours (BEV-see section 2.4.2). Coverslips were processed as described in section 2.5.1.

2.7 RNA techniques

2.7.1 Transfection of mammalian cells with siRNA

Cells were seeded overnight in 24-well or 96-well plates in the absence of antibiotics to be approximately 30-50% confluent. For each well of a 24-well plate, 20pmol siRNA was diluted into 50μl Opti-MEM (Gibco) in one microfuge tube, with 1μl siPORTamine transfection reagent (Ambion) diluted into 50μl Opti-MEM (Gibco) in a separate microfuge tube. For a 96 well plate, this was scaled down to 5pmol siRNA in 10μl Opti-MEM (Gibco) in one microfuge tube, and 0.25μl siPORTamine transfection reagent (Ambion) in 10μl Opti-MEM (Gibco) in the other. These were incubated at room temperature for 10 minutes. The contents of both tubes were then combined and incubated for a further 10 minutes. All reactions were carried out
using RNase-free tubes and tips. The complexes were then added to the cells and media. The cells were then incubated at 37°C for 72 hours. At this point, cells in 24-well plates were infected with FMDV for 3 hours and then acid washed to remove non-internalised virus. VGM was added and media samples taken at various time points to assay for virus production. These samples were titrated on BHK cells as described in section 2.2.2. The cells in 96-well plates were infected with FMDV and processed for the ELIspot infection assay as described in section 2.4.1.

2.7.2 Total RNA extraction

BHK cells were grown in a 175cm² flask until confluent. 1ml FMDV O1BFS (MOI=10) was then added and the flask was incubated at 37°C for 15 minutes. 10ml VGM was then added and infection allowed to proceed at 37°C for 2 hours. Total RNA was extracted from the cells using Trizol (Life Technologies) according to the manufacturer’s instructions. The final RNA pellet was resuspended in RNase-free water in RNase-free tubes and stored at -80°C.

2.7.3 Electroporation of mammalian cells with RNA

Cells were grown in 6mm dishes until 80% confluent. They were then transfected with mammalian expression plasmids expressing rab proteins fused to EGFP. After 6 hours, the cells were removed using trypsin and pelleted by centrifugation at 1000rpm for 3 minutes at 4°C. Cells were washed once with PBS and re-pelleted. Cells were counted using a haemocytometer and resuspended at 2 million cells per ml in cold siPORT siRNA Electroporation Buffer (Ambion). The cells were transferred into ice cold electroporation cuvettes (BioRad) with a 0.4cm gap length and 1µg RNA added. The cells and RNA were mixed gently and electroporated at
2000V for three pulses using a BTX T820 electro square porator. The cuvettes were allowed to stand at room temperature for 10 minutes and then the contents of each cuvette split between two coverslips in a 24-well plate. 500µl VGM was then added and the cells were incubated at 37°C for 6 hours, and then fixed with 4% paraformaldehyde. Coverslips were processed as described in section 2.5.1. Transfection and infection were assayed as in section 2.4.2.

2.8 Protein techniques

2.8.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used for protein analysis. Samples were prepared by lysing cells with lysis buffer C (see appendix I) and centrifugation to pellet cell debris. The supernatant was then mixed with 3X SDS sample buffer (New England Biolabs) and 40mM dithiothreitol (DTT) (NEB). Samples were then heated at 95°C for 5 minutes and 15µl run on a 10% gel (see appendix I). Gels were cast and run using a BioRad mini protean III system, in SDS running buffer (see appendix I) at 200volts/400mA for approximately 1 hour.

2.8.2 Western Blotting

Proteins were transferred from the SDS-PAGE gel onto a nitrocellulose membrane (Amersham) using transfer buffer (see appendix I) at 100volts/400mA for 90 minutes. The membranes were blocked to reduce non-specific binding for 1 hour using PBS containing 5% Marvel milk powder. Primary antibodies at 1/1000 (for rab5 and rab7) or 1/5000 (for actin) were added to 5ml blocking buffer and incubated at 4°C overnight on a Spiramax 5 (Denley). The membrane was washed with PBS and 0.1% Tween-20 four times, for 15 minutes each time, with shaking. The
appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were
then added to 5ml blocking buffer at 3/5000 and incubated for a further 1 hour at
4°C. This was followed by another washing step as before. The proteins were
visualised using electrochemiluminescence (ECL) reagents (Pierce) according to the
manufacturer’s protocol. The membranes were exposed to X-ray film (Kodak)
which was processed using a Compact X4 X-Ograph.
Chapter three: αvβ8-mediated infection of IBRS2 cells by FMDV

3.1 Introduction

One of the main objectives of this thesis is to identify the cellular compartment from which FMDV infection occurs. The cellular uptake pathway of FMDV has been studied using a human carcinoma cell line expressing αVβ6 (SW480β6). These studies showed that FMDV infection requires clathrin-dependent endocytosis and the low pH within endosomes. During uptake, virus was seen to co-localise with markers of early and recycling endosomes, but not with markers of late endosomes or lysosomes. This implicates one of the two former compartments as possible sites of FMDV infection. This was supported by the observation that cell treatment with the drug nocodazole, which disrupts microtubules and inhibits vesicular trafficking from early to late endosomes, had no effect on FMDV infection in SW480β6 cells (Berryman et al., 2005).

For the work carried out in this thesis, IBRS2 cells have been used. This is a kidney cell line derived from the natural porcine host of FMDV.

The main receptor for FMDV on IBRS2 cells is αVβ8. This has been demonstrated using an anti-αVβ8 specific antibody to block virus binding and infection (Burman et al., 2006).

The experiments carried out in this chapter will provide the first evidence of the FMDV cell entry pathway mediated by αVβ8.
3.2 αVβ8-mediated FMDV infection of IBRS2 cells is dependent on clathrin-dependent endocytosis

The method chosen to determine whether FMDV infection of IBRS2 cells is dependent on clathrin-dependent endocytosis was transient transfection with the AP180 C-terminal (AP180C). AP180 is an adaptor molecule, binding inositol polyphosphates at its N-terminus and clathrin and AP-2 at its C-terminus. This aids clathrin cage assembly and anchors clathrin to forming endocytic vesicles. Overexpression of the C-terminus of AP180 alone causes inhibition of clathrin-dependent endocytosis by sequestering clathrin away from the vesicles (Ford et al., 2001).

A plasmid encoding AP180C fused to a c-myc tag was transfected into IBRS2 cells on coverslips. At 6 hours post-transfection, the cells were infected with FMDV 01Kcad2 (MOI=1) for 3 hours. Cells were then fixed and processed for immunofluorescence confocal microscopy. AP180C expression was detected by labelling with the anti-myc antibody 9E10 and an Alexa-488 conjugated secondary antibody. Infected cells were detected by labelling with a rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa-568 conjugated secondary antibody.

Figure 3.1 (A) and (B) show representative cells at low and high magnification with AP180C shown in green and FMDV shown in red. This experiment showed that the majority of AP180C expressing cells are not infected with FMDV. This observation was quantified by counting 1) AP180C-expressing, FMDV-infected cells, and 2) non-expressing FMDV-infected cells on randomly selected fields of view on the
Chapter three

avß8-mediated infection of IBRS2 cells by FMDV

Figure 3.1 Expression of dominant-negative AP180C inhibits FMDV infection

IBRS2 cells were transfected with an expression plasmid for AP180C fused to a c-myc tag. At 6 hours post-transfection the cells were infected with FMDV 01Kcad2 for 3 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with an anti-myc antibody (9E10) and an Alexa 488nm conjugated secondary antibody (green). FMDV was labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10µm.

Panel A shows a representative image of AP180C transfected cells infected with FMDV. Panel B shows a higher power image. The majority of AP180C expressing cells are not infected by FMDV. Panel C shows quantification of these observations. A reduction in FMDV infection in AP180C expressing cells of approximately 87% was seen when compared to the non-expressing cells.
confocal microscope. Figure 3.1 (C) shows that cells expressing AP180C were only 4% infected (n=1417) as compared to 30% in non-expressing cells (n=3473). This corresponds to an inhibition of ~87%. This indicates that FMDV requires clathrin-dependent endocytosis for αVβ8-mediated infection of IBRS2 cells.

3.3 αVβ8-mediated FMDV infection of IBRS2 cells is dependent on endosomal acidification

FMDV infection of SW480β6 cells requires a low pH step (Berryman et al., 2005) and is inhibited by cell treatment with concanamycin A which raises endosomal pH by inhibiting the vacuolar H⁺-ATPase responsible for acidification of the endosome (Woo et al., 1992). IBRS2 cells in 96-well plates were treated with 1μM concanamycin A using three different treatments; 1) the drug was added to the cells for 30 minutes prior to incubation with FMDV 01Kcad2 and remained present throughout the 1 hour incubation with virus. The cells were then washed to remove excess virus and infection was allowed to continue for a further 4 hours at 37°C; 2) the cells were treated as above but the drug remained present throughout the assay; 3) the drug was added after the virus inoculum was removed and remained present throughout the rest of the assay. Infection was quantified by ELIspot infection assay (Berryman et al., 2005). An anti-3A antibody was used to label FMDV infected cells, followed by a biotinylated secondary antibody and streptavadin-conjugated alkaline phosphatase (see chapter two, section 2.4.1). On addition of enzyme substrate the infected cells are stained blue/black. On figures 3.2 and 3.3 the numbers on each panel (panel A) or below the bars (panel B) indicate the cell treatment with concanamycin A as described above.
<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>No Drug</th>
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Chapter three

avf8-mediated infection of IBRS2 cells by FMDV
Chapter three  

avB8-mediated infection of IBRS2 cells by FMDV

Figure 3.2 Concanamycin A inhibits FMDV infection

IBRS2 cells in a 96-well plate were incubated with FMDV 01Kcad2 for 1 hour at 37°C. The cells were washed to remove excess virus and infection was allowed to continue for a further 4 hours. Cells were treated with 1μM concanamycin A either:

1) The drug was added to the cells for 30 minutes prior to incubation with FMDV 01Kcad2 and remained present throughout the 1 hour incubation with virus.
2) The cells were treated as above but the drug remained present throughout the assay.
3) The drug was added after the virus inoculum was removed and remained present throughout the rest of the assay.

An anti-3A antibody was used to label infected cells (see chapter two, section 2.4.1) followed by a biotinylated secondary antibody and streptavidin-conjugated alkaline phosphatase. On addition of the enzyme substrate, infected cells are stained blue/black. Numbers of infected cells were quantified using an ELIspot plate reader (see chapter two section 2.4.1).

Panel A shows representative images of wells read by the ELIspot reader at 2.5X magnification. The number on each panel refers to the drug treatments described above. Each experiment was carried out in triplicate wells, and on three separate occasions. Panel B shows mean infected cells +/- one standard deviation. On the figure, the number of infected cells in the drug treated wells is expressed as a percentage of the number of infected cells in the mock-treated wells (i.e. no drug treatment). 'Background' refers to uninfected cells processed through the assay.

Concanamycin A inhibits FMDV infection by over 80% when cells were pre-treated with the drug, or in its presence throughout the experiment (treatment 1 and 2). The addition of concanamycin A after the virus inoculum had been removed has little effect on infection.
Figure 3.2 (A) shows an example of ELIspot well images at 2.5X magnification. Black dots representing infected cells were counted by the ELIspot reader. Each experiment was carried out in triplicate wells, and on three separate occasions. This is represented in figure 3.2 (B). On the figure, the number of infected cells in the drug treated wells is expressed as a percentage of the number of infected cells in the mock-treated wells (i.e. no drug treatment). These results show that concanamycin A inhibits FMDV infection by over 80% when the cells were pre-treated with the drug, or when it is present throughout the experiment (treatments 1 and 2, see above). In contrast, when the drug is added after the virus inoculum was removed (treatment 3), infection was not inhibited. These data indicate that a low pH step is required for an early event in infection, but not for intracellular virus replication.

3.4 αVβ8-mediated FMDV infection of IBRS2 cells is not dependent on an intact microtubule network

A requirement for the microtubule network during infection has been shown for a number of viruses including herpes simplex virus (HSV) (Sodeik et al., 1997), human immunodeficiency virus (HIV) (McDonald et al., 2002), adenovirus (Ad) (Suomalainen et al., 1999), simian virus 40 (SV40) (Pelkmans et al., 2001) and influenza virus (Sampaio et al., 2005). These viruses all require trafficking to the nucleus for replication, a process dependent on intact microtubules. However, vaccinia virus (VV) replicates in cytoplasmic viral factories and also requires microtubules (Carter et al., 2003). An intact microtubule network is required for vesicular trafficking from early to late endosomes (De Brabander et al., 1988). αVβ6-mediated infection of SW480 cells does not require virus transport to late endosomal compartments and therefore does not require intact microtubules and is
not inhibited by nocodazole. Nocodazole disrupts the microtubule network by depolymerising tubulin (Bomsel et al., 1990; Gruenberg et al., 1989).

IBRS2 cells in 96-well plates were pre-treated with 10µM nocodazole using the same cell-treatments as described above for concanamycin; 1) the drug was added to the cells for 30 minutes prior to incubation with FMDV 01Kcad2 and remained present throughout the 1 hour incubation with virus. The cells were then washed to remove excess virus and infection was allowed to continue for a further 4 hours at 37°C; 2) the cells were treated as above but the drug remained present throughout the assay; 3) the drug was added after the virus inoculum was removed and remained present throughout the rest of the assay. The number of infected cells was then quantified using the ELIspot assay as described above. Disruption of the microtubule network was verified by immunofluorescence labelling of tubulin (data not shown). Figure 3.3(A) shows an example of ELIspot well images at 2.5X magnification. Figure 3.3(B) shows the number of infected cells in the drug treated wells as a percentage of the number of infected cells in the mock-treated wells (i.e. no drug treatment). These experiments show that at whichever stage nocodazole was added, there is no significant effect on infection. This suggests that FMDV infection of IBRS2 cells does not require the microtubule network and implies that virus does not traffic to late endosomes during entry.

3.5 Discussion

The early events in entry of FMDV into SW480β6 cells (mediated by αVβ6) have been documented previously (Berryman et al., 2005). In chapter three, the first evidence for the cell-entry pathway of FMDV mediated by αVβ8 is described. In
Chapter three

avβ8-mediated infection of IBRS2 cells by FMDV
Figure 3.3 Nocodazole does not inhibit FMDV infection

IBRS2 cells in a 96-well plate were incubated with FMDV 01Kcad2 for 1 hour at 37°C. The cells were washed to remove excess virus and infection was allowed to continue for a further 4 hours. Cells were treated with 10 μM nocodazole either:
1) The drug was added to the cells for 30 minutes prior to incubation with FMDV 01Kcad2 and remained present throughout the 1 hour incubation with virus.
2) The cells were treated as above but the drug remained present throughout the assay
3) The drug was added after the virus inoculum was removed and remained present throughout the rest of the assay.

An anti-3A antibody was used to label infected cells (see chapter two section 2.4.1), followed by a biotinylated secondary antibody and streptavadin-conjugated alkaline phosphatase. On addition of the enzyme substrate, infected cells are stained blue/black. Numbers of infected cells were quantified using an ELISpot plate reader (see chapter two section 2.4.1).

Panel A shows representative images of wells read by the ELISpot reader at 2.5X magnification. The numbers refer to the drug treatments described above. Each experiment was carried out in triplicate wells, and on three separate occasions. Panel B shows mean infected cells +/- one standard deviation. On the figure, the number of infected cells in the drug treated wells is expressed as a percentage of the number of infected cells in the mock-treated wells (i.e. no drug treatment). 'Background' refers to uninfected cells processed through the assay.

Nocodazole appears to have little effect on infection when present at any point during the experiment. This means that FMDV infection is unlikely to be microtubule-dependent, and that the virus is unlikely to traffic from early- to late endosomes during cell entry.
SW480β6 cells, inhibition of clathrin-dependent endocytosis by over-expression of AP180C inhibited FMDV infection by ~90% (Berryman et al., 2005). In IBRS2 cells, the inhibition due to AP180C expression was ~87%, suggesting that FMDV also requires clathrin-dependent endocytosis to infect IBRS2 cells.

During infection of SW480β6 cells, FMDV enters early endosomes. From here, virus traffics to recycling endosomes but not to late endosomes or lysosomes. Hence, disruption of the microtubule network by nocodazole had no effect on infection of SW480β6. The experiments in chapter three show that infection of IBRS2 cells is also not inhibited by nocodazole, suggesting that microtubules are not required for FMDV infection of this cell line. This suggests that, as for SW480β6 cells, FMDV does not traffic to late endosomes or lysosomes during entry and that infection probably occurs from within early or recycling endosomes.

Cell treatment with concanamycin A (which raises endosomal pH) also inhibits infection of SW480β6 cells, demonstrating that FMDV infection requires a low pH step. 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, suggesting that the low pH within endosomes is required for capsid disassembly and delivery of vRNA across the endosomal membrane into the cytosol. The work described in chapter three shows that infection of IBRS2 cells is also inhibited by concanamycin A which suggests that, as for SW480β6, the prevailing low pH within endosomes is required for capsid disassembly and delivery of vRNA into the cytosol.
To conclude, the work described in this chapter is the first to document the FMDV cell entry pathway mediated by αVβ8. These studies show that the early events in αVβ8-mediated infection of IBRS2 cells by FMDV are similar to those observed in αVβ6-expressing SW480 cells as infection is dependent on clathrin-dependent endocytosis and the low pH within endosomes but does not require an intact microtubule network.
Chapter four: Effects of rab GTPases on vesicular trafficking

4.1 Introduction

The data presented in chapter three confirms that infection of IBRS2 cells by FMDV is dependent on clathrin-dependent endocytosis and the low pH within endosomes but does not require an intact microtubule network. This is in agreement with published observations concerning FMDV infection of SW480 cells expressing αVβ6 (Berryman et al., 2005). To further investigate the route taken by FMDV during infection of IBRS2 cells, dominant-negative rab GTPases will be used.

Rab GTPases are involved in vesicular trafficking between different endocytic compartments (Zerial & McBride, 2001). They are enriched in specific membrane vesicles and are involved in endosome formation, movement and docking with target vesicles. As with all GTPases, they cycle between active, GTP-bound and inactive, GDP-bound forms (see chapter one, section 1.5). Rab5 is involved in the formation of early endosomes. Rab4 and rab11 are required for fast and slow recycling of receptors to the cell surface, respectively. Rab9 controls vesicular trafficking from the late endosome to the Golgi. Rab7 is involved in vesicular trafficking from early endosomes to late endosomes and lysosomes (see chapter one, section 1.5).

Dominant-negative forms of each of these rab GTPases have been used to study vesicular trafficking through the endosomal pathway and their effects have been well characterised. There are two different mutations used to create dominant-negative rab proteins. The first is a substitution of asparagine for either serine or threonine in the GKT/S region (rabS/T-N), resulting in an increased affinity of the rab protein for GDP rather than GTP (Feig & Cooper, 1988). This causes the rab protein to be
predominantly GDP bound and therefore inactive. The second dominant-negative is created by a mutation of N-I in the NKXD nucleotide binding region (rabN-I) which renders the protein unable to bind nucleotides and again inactive (Pai et al., 1989). Another mutation creates a constitutively active rab protein, leucine to glutamine in the WDTAGQE region (rabQ-L). This inhibits the intrinsic GTPase activity of the rab protein and causes it to become locked in its active state (Der et al., 1986).

The wild-type rab proteins are enriched in the membranes of one or more cellular compartments and should co-localise with specific markers of these compartments. Expression of dominant-negative rab GTPases should inhibit vesicle trafficking at the specific stage at which the wild-type rab protein normally acts.

Rab5 is found on early endosomes and would be expected to co-localise with markers of this compartment, such as early endosomal antigen-1 (EEA-1) (Mu et al., 1995). The dominant-negative form of rab5 would be expected to block formation of, and ligand (e.g. transferrin and low-density lipoprotein) trafficking through, the early endosome (Bucci et al., 1992).

Rab4 and rab11 are both found on early- and recycling endosomes and would be expected to co-localise with both EEA1 and the transferrin receptor, a marker for recycling endosomes. Dominant-negative forms of these rabs would be expected to block recycling of transferrin to the cell surface (van der Sluijs et al., 1992)(Ullrich et al., 1996).

Rab7 is predominantly found on late endosomes and lysosomes, with some also being found on early endosomes (Meresse et al., 1995). Markers for late endosomes and lysosomes include the lysosome-associated membrane proteins (LAMPS) and
the mannose 6-phosphate receptor (CI-MPR). Dominant-negative rab7 would be expected to inhibit trafficking of ligands such as LDL from early endosomes to late endosomes and lysosomes (Vitelli et al., 1997).

Rab9 controls vesicular trafficking from late endosomes to the Golgi. It would be expected to co-localise with CI-MPR and Golgi markers such as GM130 and GM130 (Lombardi et al., 1993).

The effects of dominant-negative rab4, 5, 7, 9 and 11 expression on vesicular trafficking were determined by transient expression of wild-type and dominant-negative rab proteins fused to enhanced green fluorescent protein (EGFP) in IBRS2 cells. The cells were transfected with mammalian expression plasmids containing the rab-EGFP fusion and the cellular location of the rab protein, and its effect on vesicular trafficking, were determined by immunofluorescence confocal microscopy. Specific cellular compartments were identified by labelling with antibodies to proteins found predominantly on one particular vesicle.

4.2 Expression of EGFP-rab fusion proteins in IBRS2 cells

The mammalian expression plasmids used in this study encode rab proteins fused to EGFP. It was important to verify that the rab proteins were not cleaved from EGFP when the fusion protein is expressed in cells. IBRS2 cells were transfected with each expression plasmid and incubated at 37°C for 6 hours. The cell lysates were analysed by western blotting using an anti-EGFP antibody (see chapter two, section 2.8). Figure 4.1 shows a western blot of the rab mutants used in this study. The size of EGFP alone is 27kDa and when fused to rab proteins should be between 46kDa and 53 kDa, depending on the size of the rab protein.
Western blot analysis of lysates from 1BRS2 cells transfected with mammalian expression plasmids for rab proteins fused to EGFP. EGFP was detected by a polyclonal anti-EGFP antibody (Abcam). One band ~50 kDa shows that the EGFP signal detected in the transfected cells represents the intact EGFP-rab fusion proteins.
Figure 4.1 indicates that there is little cleavage of EGFP from the rab protein in IBRS2 cells as there is a strong band at around 50 kDa in each case but at around 30 kDa there is either a faint band or none at all. This data shows that when EGFP is detected in IBRS2 cells by confocal microscopy, it can be assumed that it is as a fusion protein with the rab GTPase of interest.

4.3 Characterisation of wild-type, dominant-negative and constitutively active rab5

Three rab5 constructs were used in this study: wild-type rab5 (wtrab5), dominant-negative rab5-S34N (dnrab5) and constitutively active rab5-Q79L (carab5). All were fused to EGFP. These plasmids were obtained from a research group who used them to study trafficking of G protein-coupled receptors (GPCRs) (Seachrist & Ferguson, 2003). Rab5 controls formation of early endosomes, and wtrab5 should localise to this compartment.

4.3.1 Rab5 and early endosomal antigen 1 (EEA1)

Early endosomal antigen 1 (EEA1) is a specific marker for early endosomes. IBRS2 cells were transfected with expression plasmids for wtrab5, dnrab5 or carab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.2 shows the expression pattern of wtrab5 and EEA1. Panel A shows that EEA1 labelling is similar in cells expressing wtrab5 as in non-expressing cells.
IBRS2 cells were transfected with an expression plasmid for wtrab5 fused to EGFP(green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1); a marker for early endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows labelling for EEA-1 (red),
Panel B shows wtrab5-EGFP (green)
Panel C is an overlay of panels A and B showing co-localisation of EEA1 and wtrab5 (yellow).
Panel C shows that as expected, wtrab5 co-localises with EEA1, indicating that rab5 is localised to early endosomes and the presence of the GFP tag does not affect this localisation.

Figure 4.3, panel B shows that dnrab5 is mainly cytosolic with a diffuse pattern. Panels A and D show that EEA1 labelling in dnrab5 expressing cells is markedly reduced (arrowheads) compared to non-expressing cells (arrows). This indicates that expression of dnrab5 inhibits the biogenesis of early endosomes.

Figure 4.4, panel A shows that expression of carab5 causes formation of characteristic enlarged endosomes. The non-expressing cell has a normal pattern of EEA1 distribution. Panel C shows that carab5 and EEA1 co-localise, providing further evidence that rab5 is localised to early endosomes.

### 4.3.2 Rab5 and transferrin receptor (TfR)

The transferrin receptor (TfR) is a marker for early- and recycling endosomes. IBRS2 cells were transfected with expression plasmids for wtrab5, dnrab5 or carab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.5 shows the expression pattern of wtrab5 and TfR. Panel C shows that wtrab5 co-localises with TfR, presumably due to the presence of some TfR on early endosomes. Unlike EEA-1, not all the TfR is localised with wtrab5, the remainder likely to be located on recycling endosomes.
IBRS2 cells were transfected with an expression plasmid for dnrab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1); a marker for early endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for EEA-1 (red), Panels B and E show dnrab5-EGFP (green) Panel C is an overlay of panels A and B, Panel F is an overlay of panels D and E. Both show that dnrab5 expressing cells are unable to form early endosomes.
Figure 4.4. Immunofluorescence confocal microscopy showing co-localisation of constitutively active rab5 (carab5-EGFP) with early endosomes

IBRS2 cells were transfected with an expression plasmid for carab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1), a marker for early endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10µm.

Panel A shows labelling for EEA-1 (red), Panel B shows carab5-EGFP (green) Panel C is an overlay of panels A and B showing that cells expressing carab5 have enlarged endosomes and carab5 co-localises with EEA1 (yellow).
Figure 4.5. Immunofluorescence confocal microscopy showing co-localisation of wild-type rab5 (wtrab5-EGFP) with transferrin receptor positive compartments

IBRS2 cells were transfected with an expression plasmid for wtrab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR); a marker for early endosomes and recycling endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows labelling for TfR (red),
Panel B shows wtrab5-EGFP (green)
Panel C is an overlay of panels A and B showing partial co-localisation of wtrab5 and TfR (yellow).
Figure 4.6, panels A and D show that the extent of TfR labelling in dnrab5 expressing cells appears reduced when compared to non-expressing cells. This is probably due to the reduction in early endosome biogenesis in dnrab5 expressing cells.

Figure 4.7, panel C shows co-localisation of carab5 and TfR. Again, in cells expressing carab5 a proportion of the TfR co-localises with rab5 on early endosomes, with the remainder likely to be on recycling endosomes.

4.3.3 Rab5 and mannose 6-phosphate receptor (CI-MPR)

The markers normally used to study late endosomes and lysosomes are lysosomal-associated membrane proteins (LAMPs). However, cross-reactive LAMP-1/LAMP-2 antibodies for porcine (IBRS2) cells were not identified. Instead the Mannose 6-phosphate receptor (CI-MPR), also a marker for late endosomes and lysosomes was used. IBRS2 cells were transfected with expression plasmids for wtrab5, dnrab5 or carab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose 6-phosphate receptor (CI-MPR), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.8 shows the expression pattern of wtrab5 in panel B and dnrab5 in panel E. Panels A and D show the pattern of CI-MPR labelling. The overlays in panels C and F show that, as expected, little or no co-localisation was observed between wtrab5 or dnrab5 with CI-MPR, and expression of neither form of rab5 has affected the pattern of CI-MPR labelling. This suggests that late endosomes and lysosomes remain intact in cells expressing dnrab5.
Figure 4.6. Immunofluorescence confocal microscopy showing inhibition of early endosome formation in dominant-negative rab5 (dnrab5-EGFP) expressing cells.

IBRS2 cells were transfected with an expression plasmid for dnrab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR); a marker for early endosomes and recycling endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for TfR (red), Panels B and E show dnrab5-EGFP (green)
Panel C is an overlay of panels A and B. Panel F is an overlay of panels D and E. Both show that dnrab5 expressing cells appear to contain fewer TfR positive compartments.
IBRS2 cells were transfected with an expression plasmid for carab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR); a marker for early endosomes and recycling endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows labelling for TfR (red),
Panel B shows carab5-EGFP (green)
Panel C is an overlay of panels A and B showing partial co-localisation of carab5 with TfR (yellow).
Figure 4.8. Immunofluorescence confocal microscopy showing wild-type rab5 (wtrab5-EGFP) does not co-localise with late endosomes and expression of dominant-negative rab5 (dnrab5-EGFP) does not inhibit formation of late endosomes

IBRS2 cells were transfected with expression plasmids for wtrab5 and drab5 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose-6-phosphate receptor (CI-MPR); a marker for late endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for CI-MPR (red), Panel B shows wtrab5-EGFP (green) and panel E shows drab5-EGFP (green). Panel C is an overlay of panels A and B, showing that wtrab5 does not co-localise with late endosomes. Panel F is an overlay of panels D and E, showing that expression of drab5 does not inhibit the biogenesis of late endosomes.
4.3.4 Rab5 and transferrin uptake

The effect of dnrab5 expression on uptake of labelled transferrin was investigated. Transferrin is a ligand which is taken up by clathrin-dependent endocytosis into early endosomes and recycled back to the plasma membrane via recycling endosomes. IBRS2 cells were transfected with expression plasmids for wtrab5 or dnrab5 fused to EGFP (green). At 6 hours post-transfection, Alexa 568nm labelled transferrin (red) was taken up into IBRS2 cells for 5 minutes. At this time point the transferrin should be in early compartments (early- or recycling endosomes).

Figure 4.9, panel B shows the wtrab5 expressing cell, and panel E shows the dnrab5 expressing cell. Panel A shows that the cells expressing wtrab5 have a normal pattern of transferrin uptake, with some clearly co-localising with rab5 (panel C) therefore in early endosomes. Panel D shows that cells expressing dnrab5 have taken up less labelled transferrin when compared to non-expressing cells, indicating that trafficking through early endosomes is inhibited. This data is consistent with that in figure 4.3, which shows that cells expressing dnrab5 have fewer early endosomes than those expressing wtrab5.

4.4 Characterisation of wild-type and dominant-negative rab4

The mammalian expression plasmids for rab4 used in this study are wild-type rab4 (wtrab4) and dominant-negative rab4-N121I (dnrab4). Both are fused to EGFP. Rab4 controls rapid recycling directly from early endosomes to the plasma membrane. Wt rab4 should localise to early endosomes and possibly recycling endosomes.
Figure 4.9. Immunofluorescence confocal microscopy showing differences in uptake of labelled transferrin in wild-type rab5 (wtrab5-EGFP) or dominant-negative rab5 (dnrab5-EGFP) expressing cells.

IBRS2 cells were transfected with expression plasmids for wtrab5 or drnab5 fused to EGFP (green). At 6 hours post-transfection Alexa 568nm labelled transferrin (red) was added to the media at 50μg/ml and allowed to internalise for 5 minutes. The cells were then fixed with paraformaldehyde. At this time point, the red transferrin should still be in early compartments. Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for transferrin (red), Panel B shows wtrab5-EGFP (green) and panel E shows drnab5-EGFP (green) Panel C is an overlay of panels A and B, showing that uptake of labelled transferrin is not inhibited in wtrab5 expressing cells. Panel F is an overlay of panels D and E, showing that expression of drnab5 inhibits uptake of labelled transferrin.
4.4.1 Rab4 and early endosomal antigen 1 (EEA1)

Early endosomal antigen 1 (EEA1) is a specific marker for early endosomes. IBRS2 cells were transfected with expression plasmids for wtrab4 or dnrab4 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.10 shows the pattern of expression of wtrab4 (panel B) and dnrab4 (panel E). As expected, panel C shows that wtrab4 (green) partially co-localises with EEA1 (red, panel A) indicating that some wtrab4 is located on early endosomes. The wtrab4 that is not co-localised with EEA1 is presumably located on recycling endosomes. Panel D shows that as expected, expression of dnrab4 does not affect EEA1 labelling when compared to non-expressing cells, indicating that the integrity of early endosomes are not affected by dnrab4 expression.

4.4.2 Rab4 and transferrin receptor (TfR)

The transferrin receptor (TfR) is a specific marker for early and recycling endosomes. IBRS2 cells were transfected with expression plasmids for wtrab4 or dnrab4 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.11 shows the expression pattern of wtrab4 (green) in panels B and E and the
Figure 4.10. Immunofluorescence confocal microscopy showing partial co-localisation of wild-type rab4 (wtrab4-EGFP) with early endosomes and that expression of dominant-negative rab4 (dnrab4-EGFP) does not affect the integrity of early endosomes

IBRS2 cells were transfected with expression plasmids for wtrab4 and drab4 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1); a marker for early endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for EEA1 (red), Panel B shows wtrab4-EGFP (green) and panel E shows dnrab4-EGFP (green) Panel C is an overlay of panels A and B, showing partial co-localisation of wtrab4 with early endosomes. Panel F is an overlay of panels D and E, showing that expression of dnrab4 does not affect the integrity of early endosomes.
IBRS2 cells were transfected with an expression plasmid for wtrab4 fused to EGFP(green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR); a marker for early endosomes and recycling endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for TfR (red), Panels B and E show wtrab4-EGFP(green)
Panel C is an overlay of panels A and B. Panel F is an overlay of panels D and E. Both show that wtrab4 is in the same region of the cell as TfR positive compartments.
labelling pattern of TfR (red) in panels A and D. The overlays (panels C and F) show that the majority of wtrab4 is in the same region of the cell as TfR, and some is co-localised (yellow, panels C and F).

Figure 4.12 shows the expression pattern of dnrab4 in Panel B and TfR in panel A. Expression of dnrab4 does not appear to have a significant effect on the distribution of TfR when compared to non-expressing cells. This indicates that the integrity of recycling endosomes is not dependent on the presence of an active rab4.

4.4.3 Rab4 and mannose 6-phosphate receptor (CI-MPR)

Mannose 6-phosphate receptor (CI-MPR) is a marker for late endosomes and lysosomes. IBRS2 cells were transfected with expression plasmids for wtrab4 or dnrab4 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose 6-phosphate receptor (CI-MPR), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.13 shows the expression pattern of wtrab4 (green) in panel B and CI-MPR (red) in panel A. As expected, there does not appear to be any co-localisation between wtrab4 and CI-MPR (panel C).

Figure 4.14 shows the expression pattern of dnrab4 (green) in panels B and E and CI-MPR (red) in panels A and D. As expected, there does not appear to be any co-localisation between dnrab4 and CI-MPR (panels C and F). Expression of dnrab4 does not affect the distribution of CI-MPR when compared to non-expressing cells. This indicates that dnrab4 expression is unlikely to affect the integrity of late
Figure 4.12. Immunofluorescence confocal microscopy showing that expression of dominant-negative rab4 (dnrab4-EGFP) does not affect the pattern of labelling of transferrin receptor-positive compartments.

IBRS2 cells were transfected with an expression plasmid for dnrab4 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR); a marker for early endosomes and recycling endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows labelling for TfR (red),
Panel B shows dnrab4-EGFP (green)
Panel C is an overlay of panels A and B, showing that expression of dnrab4 does not affect the pattern of TfR positive compartment labelling.
IBRS2 cells were transfected with an expression plasmid for wtrab4 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose-6-phosphate receptor (CI-MPR); a marker for late endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10µm.

Panel A shows labelling for CI-MPR (red).
Panel B shows wtrab4-EGFP (green).
Panel C is an overlay of panels A and B, showing that wtrab4 does not co-localise with late endosomes.
Figure 4.14. Immunofluorescence confocal microscopy showing expression of dominant-negative rab4 (dnrab4-EGFP) does not affect the integrity of late endosomes.

IBRS2 cells were transfected with an expression plasmid for dnrab4 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose-6-phosphate receptor (CI-MPR); a marker for late endosomes and an Alexa 568 nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10 μm.

Panels A and D show labelling for CI-MPR (red), Panels B and E show dnrab4-EGFP (green) Panel C is an overlay of panels A and B, and panel F is an overlay of panels D and E. Both show that expression of dnrab4 does not affect the integrity of late endosomes.
Chapter four

Effects of rab GTPases on vesicular trafficking

endosomes or lysosomes.

4.5 Characterisation of wild-type and dominant-negative rab11

The mammalian expression plasmids for rab11 used in this study are wild-type rab11 (wtrab11) and dominant-negative rab11-S25N (dnrab11). Both are fused to EGFP. Rab11 controls a slow recycling pathway from early endosomes to the plasma membrane via perinuclear recycling endosomes. Wrab11 should localise to early and recycling endosomes.

4.5.1 Rab11 and early endosomal antigen 1 (EEA1)

Early endosomal antigen 1 (EEA1) is a specific marker for early endosomes. IBRS2 cells were transfected with expression plasmids for wtrab11 or drab11 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.15 shows the expression pattern of wtrab11 (green) in panel B and drab11 (green) in panel E. EEA1 (red) is shown in panels A and D. Panel C shows a small amount of wtrab11 co-localised with EEA1, with the remainder presumably located on recycling endosomes. Panel D shows that expression of drab11 does not affect the integrity of early endosomes, its action being downstream of this compartment.

4.5.2 Rab11 and transferrin receptor (TfR)

The transferrin receptor (TfR) is a specific marker for early and recycling
Figure 4.15. Immunofluorescence confocal microscopy showing that expression of wild-type rab11 (wtrabl1-EGFP) or dominant-negative rab11 (dnrab11-EGFP) do not affect the integrity of early endosomes.

IBRS2 cells were transfected with expression plasmids for wtrabl1 or drnabl1 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1); a marker for early endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for EEA1 (red), Panel B shows wtrabl1-EGFP (green) and panel E shows dnrabl1-EGFP (green) Panel C is an overlay of panels A and B and panel F is an overlay of panels D and E. Both show that expression of wtrabl1 or dnrabl1 do not affect the integrity of early endosomes.
endosomes. IBRS2 cells were transfected with expression plasmids for wtrab11 or dnrab11 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.16 shows the expression pattern of wtrab11 (green) in panel B and dnrab11 (green) in panel E. TfR (red) is shown in panels A and D. As expected, there is some co-localisation of wtrab11 with TfR, and its distribution is the same in expressing and non-expressing cells. Expression of dnrab11 appears to have affected the pattern of TfR distribution when compared to non-expressing cells. The TfR is in a pattern more indicative of early endosomes, consistent with the idea that dominant-negative rab11 inhibits the formation of perinuclear recycling endosomes.

4.5.3 Rab11 and mannose 6-phosphate receptor (CI-MPR)

Mannose 6-phosphate receptor (CI-MPR) is a marker for late endosomes and lysosomes. IBRS2 cells were transfected with expression plasmids for wtrab11 or dnrab11 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose 6-phosphate receptor (CI-MPR), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.17 shows the expression pattern of wtrab11 (green) in panel B and dnrab11 (green) in panel E. CI-MPR (red) is shown in panels A and D. As expected, there is no co-localisation of wtrab11 with CI-MPR (panels C and F). Expression of dnrab11 does not affect the distribution of CI-MPR when compared to non-
Figure 4.16. Immunofluorescence confocal microscopy showing expression of dominant-negative rab11 (dnrab11-EGFP), but not wild-type rab11 (wtrab11-EGFP) leads to reduced labelling of transferrin receptor-positive compartments.

IBRS2 cells were transfected with expression plasmids for wtrab11 and dnrab11 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR); a marker for early endosomes and recycling endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for TfR (red), Panel B shows wtrab11-EGFP (green) and panel E shows dnrab11-EGFP (green) Panel C is an overlay of panels A and B, showing that expression of wtrab11 does not affect the pattern of labelling of transferrin receptor-positive compartments. Panel F is an overlay of panels D and E, showing that expression of dnrab11 leads to reduced labelling of transferrin receptor-positive compartments.
Figure 4.17. Immunofluorescence confocal microscopy showing wild-type rab11 (wtrab11-EGFP) does not co-localise with late endosomes and expression of dominant-negative rab11 (dnrab11-EGFP) does not inhibit formation of late endosomes

IBRS2 cells were transfected with expression plasmids for wtrab11 or drab11 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose-6-phosphate receptor (CI-MPR); a marker for late endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for CI-MPR (red), Panel B shows wtrab11-EGFP (green) and panel E shows drab11-EGFP (green) Panel C is an overlay of panels A and B, showing that wtrab11 does not co-localise with late endosomes. Panel F is an overlay of panels D and E, showing that expression of drab11 does not inhibit the biogenesis of late endosomes.
expressing cells and therefore is unlikely to affect the integrity of late endosomes or lysosomes.

4.5.4 Rab11 and transferrin uptake

The effect of dnrab11 expression on uptake of labelled transferrin was investigated. Transferrin is a ligand which is taken up by clathrin-dependent endocytosis into early endosomes and recycled back to the cell surface via recycling endosomes. IBRS2 cells were transfected with an expression plasmid for dnrab11 fused to EGFP (green). At 6 hours post-transfection, Alexa 568nm labelled transferrin was taken up into IBRS2 cells for 5 minutes and then chased with unlabelled transferrin for 25 minutes. At this time point the red transferrin should have been chased out of early compartments (early- and recycling endosomes) and recycled out of the cell.

Figure 4.18, panel A shows dnrab11 expressing cells. In panel B the non-expressing cells have reduced levels of red transferrin as it has been chased out by the unlabelled transferrin. However, in the dnrab11 expressing cells, the red transferrin has been trapped in early compartments, indicating that dnrab11 blocks recycling of ligands to the cell surface.

4.6 Characterisation of wild-type and dominant-negative rab7

The mammalian expression plasmids for rab7 used in this study are wild-type rab7 (wtrab7) and dominant-negative rab7-N125I (dnrab7). Both are fused to EGFP. Rab7 controls trafficking from early to late endosomes and lysosomes, and wtrab7 should localise to early endosomes, late endosomes and lysosomes. Another form of dominant-negative rab7 (T22N) will be used later in this study (see chapter six).
IBRS2 cells were transfected with an expression plasmid for dnrab11 fused to EGFP (green). At 6 hours post-transfection Alexa 568nm labelled transferrin (red) was added to the media at 50μg/ml and then chased with unlabelled transferrin for 25 minutes. The cells were then fixed with paraformaldehyde. At this time point the red transferrin should have been chased out of early compartments (early- or recycling endosomes) and recycled. Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows dnrab11-EGFP (green)
Panel B shows transferrin (red)
Panel C is an overlay of panels A and B, showing that expression of dnrab11 inhibits recycling of labelled transferrin from early compartments.
4.6.1 Rab7 and early endosomal antigen 1 (EEA1)

Early endosomal antigen 1 (EEA1) is a specific marker for early endosomes. IBRS2 cells were transfected with expression plasmids for wtrab7 or dnrab7 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.19 shows the expression pattern of wtrab7 (green) in panel B and dnrab7 (green) in panel E. EEA1 (red) is shown in panels A and D. Panel C shows a small amount of wtrab7 co-localised with EEA1, indicating that it can be found on early endosomes. The majority of wtrab7 is presumably on late endosomes and lysosomes. Expression of dnrab7 does not affect the integrity of the early endosome (panel D), its action being downstream of this compartment.

4.6.2 Rab7 and transferrin receptor (TfR)

The transferrin receptor (TfR) is a specific marker for early and recycling endosomes. IBRS2 cells were transfected with expression plasmids for wtrab7 or dnrab7 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.20 shows the expression pattern of wtrab7 (green) in panel B and dnrab7 (green) in panel E. TfR (red) is shown in panels A and D. Panel C shows no co-
Figure 4.19. Immunofluorescence confocal microscopy showing expression of wild-type rab7 (wtrab7-EGFP) or dominant-negative rab7 (dnrab7-N125I-EGFP) do not affect the integrity of early endosomes.

IBRS2 cells were transfected with expression plasmids for wtrab7 or dnrab7-N125I fused to EGFP(green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to early endosomal antigen 1 (EEA-1); a marker for early endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for EEA1(red), Panel B shows wtrab7-EGFP(green) and panel E shows dnrab7-N125I-EGFP(green) Panel C is an overlay of panels A and B and panel F is an overlay of panels D and E. Both show that expression of wtrab11 or dnrab11 does not affect the integrity of early endosomes.
Figure 4.20. Immunofluorescence confocal microscopy showing expression of wild-type rab7 (wtrab7-EGFP) or dominant-negative rab7 (dnrab7-EGFP) do not affect labelling of transferrin receptor-positive compartments.

IBRS2 cells were transfected with expression plasmids for wtrab7 or dnrab7 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to transferrin receptor (TfR); a marker for early endosomes and recycling endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for TfR (red), Panel B shows wtrab7-EGFP (green) and panel E shows dnrab7-EGFP (green) Panel C is an overlay of panels A and B and panel F is an overlay of panels D and E. Both show that expression of wtrab7 or dnrab7 do not affect labelling of transferrin receptor-positive compartments.
localisation of wtrab7 with TfR, and expression of dnrab7 has not affected the pattern of TfR distribution when compared to non-expressing cells (panel F). This shows that early- and recycling endosomes remain intact in dnrab7 expressing cells.

4.6.3 Rab7 and mannose 6-phosphate receptor (CI-MPR)

Mannose 6-phosphate receptor (CI-MPR) is a marker for late endosomes and lysosomes. IBRS2 cells were transfected with expression plasmids for wtrab7 or dnrab7 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose 6-phosphate receptor (CI-MPR), conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.21 shows the expression pattern of wtrab7 (green) in panel B and dnrab7 (green) in panel E. CI-MPR (red) is shown in panels A and D. There no co-localisation of wtrab7 with CI-MPR and expression of dnrab7 does not affect its distribution. This is surprising, as CI-MPR is a marker for the late compartments whose biogenesis rab7 is supposed to control. However, there is evidence in the literature that CI-MPR does not always co-localise with rab7 (Meresse et al., 1995).

4.6.4 Rab7 and uptake of labelled low density lipoprotein (Dil-LDL)

The effect of dnrab7 expression on labelled low-density lipoprotein, Dil-LDL (red) uptake was investigated. Dil-LDL is a ligand which is taken up by clathrin-dependent endocytosis into early endosomes and traffics to late endosomes. IBRS2 cells were transfected with expression plasmids for wtrab7 or dnrab7 fused to EGFP (green).
Figure 4.21. Immunofluorescence confocal microscopy showing wild-type rab7 (wtrab7-EGFP) does not co-localise with late endosomes and expression of dominant-negative rab7 (dnrab7-EGFP) does not inhibit formation of late endosomes.

IBRS2 cells were transfected with expression plasmids for wtrab7 or drabar7 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a monoclonal antibody to mannose-6-phosphate receptor (CI-MPR); a marker for late endosomes and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and D show labelling for CI-MPR (red), Panel B shows wtrab7-EGFP (green) and panel E shows drabar7-EGFP (green) Panel C is an overlay of panels A and B, showing that wtrab7 does not co-localise with late endosomes. Panel F is an overlay of panels D and E, showing that expression of drabar7 does not inhibit the biogenesis of late endosomes.
At 6 hours post-transfection, Dil-LDL (red) was taken up into IBRS2 cells for 30 minutes. At this time point the Dil-LDL should have reached late endosomes.

Figure 4.22, panel A shows the wtrab7 expressing cell, and panel B shows the dnrab7 expressing cell. In panel C all cells have high levels of Dil-LDL in large vesicles close to the nucleus, in a distribution indicative of late endosomes. This shows that uptake of Dil-LDL is not inhibited by expression of wtrab7. However, in panel D, the dnrab7 expressing cell has an altered distribution of Dil-LDL when compared to non-expressing or wtrab7 expressing cells. The Dil-LDL is in smaller vesicles near to the periphery of the cell, suggesting that it has become trapped in early compartments. This indicates that dnrab7 expression inhibits trafficking of ligands to late endosomes.

4.6.5 Rab7 and labelling of acidic compartments with lysotracker red

The effect of dnrab7 expression on formation of acidic compartments was also investigated. Lysotracker red is a dye which labels highly acidic compartments such as late endosomes and lysosomes. IBRS2 cells were transfected with expression plasmids for wtrab7 or dnrab7 fused to EGFP (green). At 6 hours post-transfection, Lysotracker red was taken up into IBRS2 cells for 30 minutes. At this time point, lysotracker permeates acidic compartments, labelling them red.

Figure 4.23, panel A shows the wtrab7 expressing cell, and panel B shows the dnrab7 expressing cell. In panel C, all cells have high levels of red lysotracker in large vesicles close to the nucleus, in a distribution indicative of late endosomes and lysosomes. This shows that the uptake of lysotracker into late endosomes and lysosomes is not inhibited by expression of wtrab7. However, in the panel D, the
Figure 4.22 Immunofluorescence confocal microscopy showing expression of dominant-negative rab7 (dnrab7-EGFP), but not wild-type rab7 (wtrab7-EGFP) inhibits early- to late endosome trafficking of labelled low density lipoprotein (Dil-LDL).

IBRS2 cells were transfected with expression plasmids for wtrab7 and dnrab7 fused to EGFP(green). At 6 hours post-transfection Dil-LDL (red) was added to the media and allowed to internalise for 30 minutes. The cells were then fixed with paraformaldehyde. At this time point, the Dil-LDL should have reached late endosomes. Cell nuclei were stained with DAPI and are shown in blue. Bars=10µm.

Panel A shows the wtrab7 expressing cell (green), and panel C shows Dil-LDL (red). The wtrab7 expressing cell has an equal level of Dil-LDL when compared to non-expressing cells. Panel B shows the dnrab7 expressing cell (green), and panel D shows Dil-LDL (red). The dnrab7 expressing cell has a reduced level of Dil-LDL when compared to non-expressing cells, suggesting an inhibition in trafficking from early endosomes to late endosomes when dnrab7 is expressed.
Figure 4.23 Immunofluorescence confocal microscopy showing expression of dominant-negative rab7 (dnrab7-EGFP), but not wild-type rab7 (wtrab7-EGFP) inhibits biogenesis of late endosomes and lysosomes.

IBRS2 cells were transfected with expression plasmids for wtrab7 and dnrab7 fused to EGFP(green). At 6 hours post-transfection lysotracker red was added to the media and allowed to label acidic compartments for 30 minutes. The cells were then fixed with paraformaldehyde. At this time point, lysotracker red should label highly acidic compartments such as late endosomes and lysosomes. Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows the wtrab7 expressing cell (green), and panel C shows labelling for lysotracker red. The wtrab7 expressing cell has an equal level of lysotracker red labelling when compared to non-expressing cells. Panel B shows the dnrab7 expressing cell (green), and panel D shows labelling for lysotracker red. The dnrab7 expressing cell has a reduced level of lysotracker red labelling when compared to non-expressing cells, suggesting an inhibition in biogenesis of late endosomes and lysosomes when dnrab7 is expressed.
dnrab7 expressing cell has an altered distribution of red lysotracker when compared to non-expressing or wtrab7 expressing cells. The lysotracker is labelling smaller vesicles near to the periphery of the cell, suggesting labelling of early compartments only. This shows that dnrab7 expression inhibits biogenesis of late endosomes and lysosomes.

4.7 Characterisation of dominant-negative rab9

The only construct used for rab9 in this study is dominant-negative rab9-S21N (dnrab9) fused to EGFP. The reason for this is that overexpression of wild-type rab9 also acts as a dominant-negative inhibitor of rab9 function. Rab9 controls trafficking from late endosomes to the Golgi and wtrab9 localises to both of these compartments.

4.7.1 Rab9, early endosomal antigen 1 (EEA1) and transferrin receptor (TfR)

Early endosomal antigen 1 (EEA1) is a specific marker for early endosomes, and the transferrin receptor (TfR) is a specific marker for early and recycling endosomes. IBRS2 cells were transfected with an expression plasmid for dnrab9 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeablised with Triton X-100 and labelled with monoclonal antibodies to early endosomal antigen 1 (EEA-1) or transferrin receptor (TfR), both conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.24, panels B and E show the expression pattern of dnrab9 (green). Panel A shows EEA1 labelling and panel D, TfR (red). As expected, expression of dnrab9 does not appear to affect the integrity of early- or recycling endosomes.
Figure 4.24. Immunofluorescence confocal microscopy showing that expression of dominant-negative rab9 (dnrab9-EGFP) does not affect the integrity of early endosomes or transferrin receptor-positive compartments.

IBRS2 cells were transfected with an expression plasmid for dnrab9 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with monoclonal antibodies to early endosomal antigen 1 (EEA-1), or transferrin receptor (TfR); both with Alexa 568nm conjugated secondary antibodies (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows labelling for EEA-1 (red), and panel D shows labelling for TfR (red).
Panel B and E show dnrab9-EGFP (green).
Panel C is an overlay of panels A and B, showing that expression of dnrab9 does not affect the integrity of early endosomes. Panel F is an overlay of panels D and E, showing that expression of dnrab9 does not affect the pattern of labelling of transferrin receptor-positive compartments.
4.7.2 Rab9, mannose 6-phosphate receptor (CI-MPR) and Golgi (GM130)

Mannose 6-phosphate receptor (CI-MPR) is a marker for late endosomes and lysosomes. GM130 is an antibody to β-COP, a subunit of the Golgi coatomer complex. IBRS2 cells were transfected with an expression plasmid for dnrab9 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeablised with Triton X-100 and labelled with monoclonal antibodies to mannose 6-phosphate receptor (CI-MPR) or GM130, both conjugated with an Alexa 568nm secondary antibody (red).

Figure 4.25, panels B and E show the expression pattern of dnrab9 (green). Panel A shows CI-MPR labelling and panel D, GM130 (red). Expression of dnrab9 does not affect the distribution of CI-MPR, and they do not co-localise (panel C). However, dnrab9 does co-localise with GM130 (panel F).

4.8 Discussion

The effects of expression of both wild-type and dominant-negative rab GTPases on vesicular trafficking have been characterised in many previous studies. Rab5 controls formation of, and trafficking through, early endosomes. Previous studies have shown that rab5 co-localises with EEA1 on early endosomes (Stenmark et al., 1996). In this study, wtrab5 can be seen to co-localise with EEA1 in figure 4.2 and expression of wtrab5 does not affect the labelling pattern of markers of recycling endosome or late endosomes (figures 4.5 and 4.8). Expression of dnrab5 inhibits formation of early endosomes, with an accumulation of smaller vesicles being seen (Stenmark et al., 1994). In this chapter, inhibition of early endosome biogenesis by expression of dnrab5 is shown in figures 4.3 and 4.6 by reduced labelling of EEA1
IBRS2 cells were transfected with an expression plasmid for dnrab9 fused to EGFP (green). At 6 hours post-transfection the cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with monoclonal antibodies to mannose-6-phosphate receptor (CI-MPR), or the Golgi marker GM130; both with Alexa 568nm conjugated secondary antibodies (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10µm.

Panel A shows labelling for CI-MPR (red), and D shows labelling for GM130 (red) Panels B and E show dnrab9-EGFP (green)
Panel C is an overlay of panels A and B, showing that expression of dnrab9 does not affect the integrity of late endosomes. Panel F is an overlay of panels D and E, showing that expression of dnrab9 does not affect the Golgi network. There is some co-localisation between dnrab9 and GM130.
and TfR. Previous studies have observed an inhibition in endocytosis of transferrin when dnrab5 was expressed (Bucci et al., 1992; Papini et al., 1997; Stenmark et al., 1994), and these results were replicated in figure 4.9 by showing reduced uptake of labelled transferrin in dnrab5 expressing cells. Neither wtrab5 nor dnrab5 co-localise with, or affect the distribution of, a marker for late endosomes (figure 4.8).

Rab4 controls rapid recycling from the early endosome back to the plasma membrane (Sheff et al., 1999; van der Sluijs et al., 1992). It has been shown that wtrab4 is localised mainly to the early endosome and is thought to exert its effects on this compartment, but in some studies it also localises to recycling endosomes (Sonnichsen et al., 2000) The co-localisation of wtrab4 and EEA1 can be seen in figure 4.10 and with TfR (though to a lesser extent) in figure 4.11. Also in figure 4.10, it can be seen that expression of dnrab4 does not affect the integrity of the early endosome. In figure 4.12 it appears that expression of dnrab4 does not affect the distribution of TfR, in contrast to a study showing a moderate redistribution of TfR to the perinuclear area (McCaffrey et al., 2001). This may be due to cell type differences. Neither form of rab4 co-localises with, or affects the distribution of, a marker for late endosomes (figures 4.13 and 4.14).

Rab11 controls slower recycling via the perinuclear recycling endosome (Ullrich et al., 1996). There is a small amount of co-localisation between wtrab11 and EEA1 (figure 4.15), consistent with rab11 being found on early endosomes as well as recycling endosomes. The same figure shows that expression of dnrab11 does not affect the integrity of the early endosome. As expected, the distribution of wtrab11 overlaps with that of TfR and the pattern of TfR labelling changes on expression of dnrab11 (figure 4.16). The pattern of TfR labelling in dnrab11 expressing cells is
more indicative of early endosome labelling, suggesting that the TfR can enter the early endosome, but its trafficking from here to the recycling endosome is impaired. This is consistent with observations made in previous studies (Ullrich et al., 1996). Expression of dnrab11 also inhibits recycling of transferrin (Ren et al., 1998; Ullrich et al., 1996), in common with the result seen in figure 4.18. Neither form of rab11 co-localises with, or affects the distribution of, a marker for late endosomes (figure 4.17).

Rab7 controls vesicular trafficking from early- to late endosomes (Feng et al., 1995) and from late endosomes to lysosomes (Meresse et al., 1995). It has been shown recently that early endosomes may also contain rab7-positive domains, from which vesicles detach during transport to late endosomes (Vonderheit & Helenius, 2005). This explains the partial co-localisation seen between wtrab7 and EEA1 in figure 4.19. It is important to note also that in the same figure, expression of dnrab7 does not affect the integrity of the early endosome. Neither form of rab7 co-localises with, or affects the distribution of, TfR (figure 4.20). The ideal marker for late endosomes and lysosomes would have been lysosomal-associated membrane proteins (LAMPs), but unfortunately none of the antibodies available labelled in IBRS2 cells. The lack of a specific marker for late endosomes and lysosomes makes it difficult to show that wtrab7 is localised to the correct compartment and that expression of dnrab7 blocks trafficking to late endosomes. CI-MPR does not usually co-localise with wtrab7 (Meresse et al., 1995), in agreement with figure 4.21. One study shows a redistribution of CI-MPR from perinuclear areas to more peripheral vesicles in dnrab7 expressing cells (Press et al., 1998), however, this was not seen in figure 4.21. This may be due to differences in cell type. More convincingly,
expression of dnrab7 blocks trafficking of labelled LDL from early endosomes to late endosomes in figure 4.22. It also appears to inhibit biogenesis of highly acidic compartments such as late endosomes and lysosomes, as seen by labelling with the dye lysotracker red in figure 4.23. These reagents have been used to similar effect in previous studies (Bucci et al., 2000).

Rab9 controls vesicular trafficking from late endosomes to the Golgi (Lombardi et al., 1993). Figure 4.24 shows that as expected, expression of dnrab9 has no effect on early compartments. Similarly, figure 4.25 shows that expression of dnrab9 has no significant effect on the distribution of CI-MPR. This is may seem surprising, as movement of CI-MPR cargo from the late endosome to the Golgi is impaired in cells deficient in rab9 function (Riederer et al., 1994). However, it has been reported in the same study that steady-state distribution of CI-MPR itself is not affected in cells expressing dnrab9. The localisation of dnrab9 in the same study is in the perinuclear region (presumably late endosomes) whereas in figure 4.24, dnrab9 predominantly co-localises with the Golgi marker GM130. This may be due to differences in cell type.

To conclude, the majority of EGFP-tagged rab proteins appear to localise to the correct compartments, and all have the expected effects on vesicle trafficking. FMDV proteins co-localise with markers for early and recycling endosomes at early time points during infection. FMDV is thought to require early endosomes for pH dependent capsid disassembly and release of the viral RNA genome (Berryman et al., 2005). It is therefore likely that expression of dominant-negative rab5 will inhibit FMDV infection, as the results in this chapter show that the compartment is not formed correctly in cells expressing dnrab5. Expression of dnrab4, 7, 9, and 11
do not affect the integrity of the early endosome and for this reason may not be expected to inhibit FMDV infection. If there is a requirement for recycling endosomes, as suggested by the co-localisation of FMDV with markers of this compartment at early time points (Berryman et al., 2005), it may be predicted that dnrab11 could also inhibit infection. The effects of dnrab protein expression on FMDV infection will be investigated in chapter five.
Chapter five: Effects of rab GTPases on FMDV uptake and infection

5.1 Introduction

The results presented in chapter four verified that the wild-type and dominant-negative rab proteins were having the expected effects on vesicular trafficking in IBRS2 cells. Each rab GTPase specifically controls one or more stages of endocytosis and expression of dominant-negative rab proteins in IBRS2 cells was shown to inhibit biogenesis of cellular compartments and normal trafficking of ligands through these compartments (see chapter four). Therefore, it should be possible to investigate the route of FMDV infection by expressing dominant-negative rab proteins and determining their effects on FMDV uptake and infection.

5.2 Effect of dominant-negative rab GTPase expression on infection by FMDV

The effects of dominant-negative rab protein expression on FMDV infection of IBRS2 cells were determined by transient expression of wild-type and dominant-negative rab proteins fused to enhanced green fluorescent protein (EGFP) in IBRS2 cells. The cells, on coverslips, were transfected with mammalian expression plasmids containing rab-EGFP fusion proteins and their effects on FMDV infection were determined by immunofluorescence confocal microscopy. The rab expressing cells emitted green fluorescence. FMDV was labelled with rabbit polyclonal serum recognising the FMDV capsid and a goat anti-rabbit secondary antibody conjugated to Alexa 568nm which emits red fluorescence. To verify that the virus detected was new progeny virus rather than input virus, guanidine hydrochloride was added to control cells on coverslips in order to inhibit virus replication (Black & Brown, 1969). The levels of red fluorescence detected in infected, guanidine hydrochloride
treated cells was low, and set as the background level for the infection assay (data not shown). To quantify the effects of rab-EGFP expression on infection, fields of vision viewed down the confocal microscope were selected at random and images collected. The cells were then counted to determine 1) the proportion of non-expressing cells that were infected, and 2) the proportion of rab-expressing cells that were infected.

5.2.1 Effect of dominant-negative rab5 on FMDV infection of IBRS2 cells

The first rab protein investigated was rab5. This rab protein controls biogenesis of early endosomes (Bucci et al., 1992; Gorvel et al., 1991) and in IBRS2 cells expression of dominant-negative rab5 blocked formation of (figure 4.3), and ligand trafficking through (figure 4.9), these compartments. FMDV is transported to early endosomes in SW480 cells expressing αVβ6 via clathrin-dependent endocytosis (Berryman et al., 2005). The results in chapter three show that FMDV also uses this pathway to gain entry into IBRS2 cells. Expression of dominant-negative rab5 in IBRS2 cells would therefore be expected to reduce the number of early endosomes and inhibit FMDV infection.

IBRS2 cells were transfected with expression plasmids for wild-type (wtrab5), dominant-negative (dnrab5) or constitutively active rab5 (carab5) fused to EGFP. At 6 hours post-transfection, the cells were infected with FMDV 01Kcad2 for 3 hours. The cells were then fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Figure 5.1 shows that although cells expressing wtrab5 (green, panel A) or carab5 (green, panel C) can be
<table>
<thead>
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<th></th>
<th>rab5(wt)</th>
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<td>4.2%</td>
<td>19.5%</td>
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<tr>
<td>Cells Infected</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>% Expressing/non-expressing</td>
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<td>16%</td>
<td>71%</td>
</tr>
<tr>
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<td>106%</td>
</tr>
<tr>
<td>% Knockdown</td>
<td>77%</td>
<td>-6%</td>
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**Figure 5.1 Effect of dominant-negative rab5 on FMDV infection of IBRS2 cells**

IBRS2 cells were transfected with expression plasmids for wtrab5, dnrab5 or carab5 fused to EGFP. At 6 hours post-transfection, cells were infected with FMDV 01Kcad2 for 3 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=20µm.

Panel A shows wtrab5 and FMDV. Panel B shows dnrab5 and FMDV. Panel C shows carab5 and FMDV. In panels A and C rab5 expressing, infected cells are arrowed. Panel D shows percentage knockdown due to expression of dnrab5 and carab5.
infected by FMDV (red), the cells expressing dnrab5 (green, panel B) are rarely infected.

Figure 5.1, panel D quantifies the effect of expression of each form of rab5 on FMDV infection. This was achieved by counting 1) wtrab5, dnrab5 or carab5 expressing, FMDV-infected cells, and 2) non-expressing FMDV-infected cells on randomly selected fields of view on the confocal microscope (see above). Expression of wtrab5 results in a ~29% reduction in infection when compared to non-expressing cells on the same coverslip. This suggests that overexpression of wtrab5 has a slight inhibitory effect on FMDV infection. However, the inhibition due to expression of dnrab5 is greater, ~84%. When normalised to wtrab5 (i.e. the number of infected cells expressing wtrab5), the inhibition due to expression of dnrab5 is ~77%. This suggests that FMDV infection depends on the integrity of early endosomes. Expression of carab5 has little effect on FMDV infection when compared to wtrab5, despite the characteristic enlarged endosomes being clearly seen in carab5 expressing cells.

5.2.2 Effect of dominant-negative rab4 on FMDV infection of IBRS2 cells

Rab4 localises to early endosomes and is involved in receptor recycling back to the plasma membrane. A number of studies in different cell types have shown that rab4 is required for the rapid recycling of receptors directly from early endosomes to the cell surface (McCaffrey et al., 2001; Sheff et al., 1999; van der Sluijs et al., 1992; Ward et al., 2005). Some recent studies have also suggested a role for rab4 in the formation of recycling endosomes from early endosomes (Pagano et al., 2004; Ward et al., 2005). FMDV capsid proteins are localised to early- and recycling endosomes
at early times after infection (Berryman et al., 2005; O'Donnell et al., 2005). Therefore it is possible that the virus may need to enter recycling endosomes for infection.

IBRS2 cells were transfected with expression plasmids for wtrab4 or dnrab4 fused to EGFP. At 6 hours post-transfection, the cells were infected with FMDV 01Kcad2 for 3 hours. The cells were then fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Figure 5.2 shows that cells expressing wtrab4 (panel A) or dnrab4 (panel B) can be infected to a similar extent.

These observations were quantified (figure 5.2, panel C), by counting 1) wtrab4 or dnrab4 expressing, FMDV-infected cells, and 2) non-expressing FMDV-infected cells on randomly selected fields of view on the confocal microscope. Expression of wtrab4 results in a 19% reduction when compared to non-expressing cells on the same coverslip. This suggests that overexpression of wtrab4 has a slight inhibitory effect on FMDV infection. The inhibition when dnrab4 is expressed is very similar, 13%. If the proportion of wtrab4 expressing cells infected is set at 100%, there is very little difference when dnrab4 is expressed. These results show that rab4 is not required for FMDV entry and infection, suggesting that successful infection does not require delivery to recycling endosomes.

5.2.3 Effect of dominant-negative rab11 on FMDV infection of IBRS2 cells

Similar to rab4, rab11 is involved in receptor recycling to the plasma membrane. A number of studies have implicated rab11 in recycling of receptors from early- and
Figure 5.2 Effect of dominant-negative rab4 on FMDV infection of IBRS2 cells

IBRS2 cells were transfected with expression plasmids for wtrab4 and dnrab4 fused to EGFP. At 6 hours post-transfection, cells were infected with FMDV 01Kcad2 for 3 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=20μm.

Panel A shows wtrab4 and FMDV. Panel B shows dnrab4 and FMDV. In panels A and B, rab4 expressing, infected cells are arrowed. Panel C shows percentage knockdown due to expression of dnrab4.
recycling endosomes to the plasma membrane (Casanova et al., 1999; Green et al., 1997; Sheff et al., 1999; Ullrich et al., 1996). Recent studies have also implicated rab11 in the formation of recycling endosomes from early endosomes (Pagano et al., 2004; Ward et al., 2005). As mentioned above, FMDV capsid proteins are localised to early- and recycling endosomes at early times after infection (Berryman et al., 2005; O'Donnell et al., 2005). Therefore it is possible that the virus may enter recycling endosomes for infection.

IBRS2 cells were transfected with expression plasmids for wtrab11 or dnrab11 fused to EGFP. At 6 hours post-transfection, the cells were infected with FMDV 01Kcad2 for 3 hours. The cells were then fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Figure 5.3 shows that cells expressing both wtrab11 (panel A) or dnrab11 (panel B) can be infected to a similar extent.

These observations were quantified (figure 5.3, panel C), by counting 1) wtrab11 or dnrab11 expressing, FMDV-infected cells, and 2) non-expressing FMDV-infected cells on randomly selected fields of view on the confocal microscope. Expression of wtrab11 results in a 19% reduction when compared to non-expressing cells on the same coverslip. This suggests that overexpression of wtrab11 has a slight inhibitory effect on FMDV infection. However, the inhibition when dnrab11 is expressed is higher, 49%. If the proportion of wtrab11 expressing cells infected is set at 100%, there is an inhibition of 35% when dnrab11 is expressed. This suggests that FMDV may have a requirement for recycling endosomes for infection.
Figure 5.3 Effect of dominant-negative rab11 on FMDV infection of IBRS2 cells

IBRS2 cells were transfected with expression plasmids for wtrab11 and dnrab11 fused to EGFP. At 6 hours post-transfection, cells were infected with FMDV 01Kcad2 for 3 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=20μm.

Panel A shows wtrab11 and FMDV. Panel B shows dnrab11 and FMDV. In panels A and B, rab11 expressing, infected cells are arrowed. Panel C shows percentage knockdown due to expression of dnrab11.
5.2.4 Effect of dominant-negative rab7 on FMDV infection of IBRS2 cells

Rab7 regulates the degradative endocytic pathway from early- to late endosomes and from there to lysosomes (Feng et al., 1995) (Vitelli et al., 1997). FMDV does not co-localise with markers of these compartments during entry of SW480-β6 cells (Berryman et al., 2005). Movement of cargo from early- to late endosomes is dependent on the microtubule network (De Brabander et al., 1988), and disruption of this network with nocodazole has no effect on FMDV infection (see chapter three). These observations strongly suggest that FMDV does not need to enter late endosomes or lysosomes for infection.

IBRS2 cells were transfected with expression plasmids for wtrab7 or dnrab7 fused to EGFP. At 6 hours post-transfection, the cells were infected with FMDV 01Kcad2 for 3 hours. The cells were then fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Figure 5.4 (panel A) shows that, as expected, expression of wtrab7 does not affect infection by FMDV. However, surprisingly, it appears that expression of dnrab7 inhibits infection (panel B) as the majority of rab7 expressing cells are not infected by FMDV.

These observations were quantified (figure 5.4, panel C), by counting 1) wtrab7 or dnrab7 expressing, FMDV-infected cells, and 2) non-expressing FMDV-infected cells on randomly selected fields of view on the confocal microscope. Expression of wtrab7 results in a 32% reduction when compared to non-expressing cells on the same coverslip. This suggests that overexpression of wtrab7 has a slight inhibitory
**Figure 5.4** Effect of dominant-negative rab7 on FMDV infection of IBRS2 cells

IBRS2 cells were transfected with expression plasmids for wtrab7 and dnrab7 fused to EGFP. At 6 hours post-transfection, cells were infected with FMDV 01Kcad2 for 3 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=20µm.

Panel A shows wtrab7 and FMDV. Panel B shows dnrab7 and FMDV. In panel A, rab7 expressing, infected cells are arrowed. Panel C shows percentage knockdown due to expression of dnrab7.

<table>
<thead>
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<th>rab7(wt)</th>
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</tr>
</thead>
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<td>3593</td>
</tr>
<tr>
<td><strong>% Non-expressing Cells Infected</strong></td>
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</tr>
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<td>22%</td>
</tr>
<tr>
<td><strong>% Knockdown</strong></td>
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<td>78%</td>
</tr>
</tbody>
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effect on FMDV infection. However, the inhibition when dnrab7 is expressed is much higher, 85%. If the proportion of wtrab7 expressing cells infected is set at 100%, there is an inhibition of 78% when dnrab7 is expressed. This is unexpected, given that FMDV is thought not to require entry into late endosomes or lysosomes for infection. However, it appears that rab7 has a role in FMDV infection.

5.2.5 Effect of dominant-negative rab9 on FMDV infection of IBRS2 cells

Rab9 controls movement of cargo from late endosomes to the Golgi (Lombardi et al., 1993). It is not thought that FMDV enters late endosomes or the Golgi after internalisation, however, the Golgi may be a source of membranes for FMDV replication (see chapter one, section 1.4.5).

IBRS2 cells were transfected with an expression plasmid for dnrab9 fused to EGFP. At 6 hours post-transfection, the cells were infected with FMDV 01Kcad2 for 3 hours. The cells were then fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Figure 5.5 (panel A) shows that cells expressing dnrab9 can be infected by FMDV to a similar extent as non-expressing cells.

These observations were quantified (figure 5.5, panel C), by counting 1) dnrab9 expressing, FMDV-infected cells, and 2) non-expressing FMDV-infected cells on randomly selected fields of view on the confocal microscope. The proportion of non-expressing cells infected is set at 100%, in the absence of a wild-type rab9 control. Expression of dnrab9 inhibits FMDV infection by only 10%. This suggests that rab9 does not have a major role in FMDV infection and the virus does not
Figure 5.5 Effect of dominant-negative rab9 on FMDV infection of IBRS2 cells

IBRS2 cells were transfected with an expression plasmid for drab9 fused to EGFP. At 6 hours post-transfection, cells were infected with FMDV 01Kcad2 for 3 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=20μm.

Panel A shows drab9 and FMDV. Rab9 expressing, infected cells are arrowed. Panel B shows percentage knockdown due to expression of drab9.
require trafficking from late endosomes to the Golgi.

5.3 Effect of dominant-negative rab GTPase expression on αVβ8 expression

As rab proteins regulate endocytosis and receptor recycling, it is important to verify that the effects of dominant-negative rab protein expression on FMDV infection are not due to a reduction in receptor abundance at the cell surface. Integrins, the receptors for FMDV, are normally internalised via endocytosis and recycled back to the plasma membrane (Roberts et al., 2001). Inhibition of receptor recycling by expression of dominant-negative rab proteins could therefore reduce the abundance of receptors on the cell surface, inhibiting virus binding and therefore infection. Both wtrab5 and dnrab5, along with dnrab4 and dnrab11 (the rab proteins involved in recycling) were transiently expressed in IBRS2 cells as described previously. This was followed by labelling with an antibody to the primary receptor on these cells, αVβ8, and an Alexa 568nm conjugated secondary antibody (red). All labelling was carried out at 4°C to prevent antibody internalisation. The cells were fixed at the end of the experiment and then processed for immunofluorescence confocal microscopy.

Figure 5.6 shows expression of wtrab5 (panel A) or dnrab5 (panel C). Labelling of αVβ8 on the cell surface can be seen more clearly in panels B and D. The levels of receptor expression are similar on rab-expressing and non-expressing cells. Therefore, the inhibition of FMDV infection in dnrab5 expressing cells is unlikely to be due to reduced receptor availability.
Figure 5.6 Integrin αVβ8 expression is not altered in wtrab5 or dnrab5 expressing cells

IBRS2 cells were transfected with expression plasmids for wtrab5 or dnrab5 fused to EGFP. At 6 hours post-transfection, cells were labelled with an antibody against αVβ8 (14E5), and an Alexa 568nm conjugated secondary antibody (red). All labelling was carried out at 4°C, the cells fixed with 4% paraformaldehyde at the end of the experiment. Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A (wtrab5) and panel C (dnrab5) show αVβ8 labelling (red) on rab expressing (green) and non-expressing cells. For clarity, panels B and D show the same images as panels A and C with αVβ8 labelling only.

For both wtrab5 and dnrab5 expressing cells the level of integrin labelling appears similar to the non-expressing cells, indicating that dnrab5 does not inhibit cell surface expression of αVβ8.
Figure 5.7 shows a similar experiment for dnrab4 and dnrab11. Expression of dnrab4 (panel A) and dnrab11 (panel C) have little or no effect on surface αVβ8 expression when compared to non-expressing cells. For clarity, panels B and D show the same cells as in panels A and C, but with integrin expression only shown in red.

5.4 Effect of dominant-negative rab GTPase expression on FMDV binding

As there is no discernable effect of dominant-negative rab GTPase expression on cell surface receptor abundance, it is unlikely that virus binding will be impaired in cells expressing these rabs. This was verified by expression of dnrab5 and dnrab11 in IBRS2 cells, followed by labelling with an FMDV anti-VP1 antibody (Mab D9) and an Alexa 568nm conjugated secondary antibody (red). These rab proteins were chosen as both had inhibitory effects on FMDV infection. All labelling was carried out at 4°C, the cells being fixed at the end and processed for immunofluorescence confocal microscopy. Figure 5.8 shows expression of wtrab5 (panel A) or dnrab5 (panel C) and FMDV binding at the cell surface (red). Panels B and D show the same cells as panels A and C with FMDV binding shown as a series of stacked layers of the upper region of the cells. The cells expressing wtrab5 and dnrab5 appear to have similar levels of FMDV bound to the cell surface when compared to the adjacent non-expressing cells. Figure 5.9 shows a similar experiment for dnrab11. Similarly, expression of dnrab11 did not appear to reduce the level of virus binding when compared to the adjacent non-expressing cells.
Figure 5.7 Integrin αVβ8 expression is not altered in dnrab4 or dnrab11 expressing cells

IBRS2 cells were transfected with expression plasmids for dnrab4 or dnrab11 fused to EGFP. At 6 hours post-transfection, cells were labelled with an antibody against αVβ8 (14E5), and an Alexa 568nm conjugated secondary antibody (red). All labelling was carried out at 4°C, the cells fixed with 4% paraformaldehyde at the end of the experiment. Cell nuclei were stained with DAPI and are shown in blue. Bars=10µm.

Panel A (dnrab4) and panel C (dnrab11) show αVβ8 labelling (red) on rab expressing (green) and non-expressing cells. For clarity, panels B and D show the same images as panels A and C with αVβ8 labelling only.

For both dnrab4 and dnrab11 expressing cells the level of integrin labelling appears similar to the non-expressing cells, indicating that dnrab4/11 does not inhibit cell surface expression of αVβ8.
Figure 5.8 FMDV binding is not altered in wtrab5 or dnrab5 expressing cells

IBRS2 cells were transfected with expression plasmids for wtrab5 or dnrab5 fused to EGFP. At 6 hours post-transfection, purified FMDV was bound to the cell surface at 4 °C for 45 minutes. This was followed by labelling with an antibody against FMDV VP1 (D9), and an Alexa 568nm conjugated secondary antibody (red). All labelling was carried out at 4°C, the cells being fixed with 4% paraformaldehyde at the end of the experiment. Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A (wtrab5) and panel C (dnrab5) show FMDV binding (red) on rab expressing (green) and non-expressing cells. Panels B and D show the same images as panels A and C with FMDV binding shown as a series of layers through the upper region of the cells. The amount of virus detected on rab expressing and non-expressing cells is similar, indicating that expression of wtrab5 or dnrab5 do not inhibit virus binding.
Figure 5.9 FMDV binding is not altered in dnrab11 expressing cells

IBRS2 cells were transfected with an expression plasmid for dnrab11 fused to EGFP. At 6 hours post-transfection, purified FMDV was bound to the cell surface at 4 °C for 45 minutes. This was followed by labelling with an antibody against FMDV VP1 (D9), and an Alexa 568nm conjugated secondary antibody (red). All labelling was carried out at 4°C, the cells being fixed with 4% paraformaldehyde at the end of the experiment. Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A (dnrab11) shows FMDV binding (red) on rab expressing (green) and non-expressing cells. Panel B shows the same image as panel A with FMDV binding shown as a series of layers through the upper region of the cells. The amount of virus detected on dnrab11 expressing and non-expressing cells is similar, indicating that expression of dnrab11 does not inhibit virus binding.
5.5 Effect of dominant-negative rab GTPase expression on FMDV uptake

As expression of dnrab5 and dnrab7 both inhibited FMDV infection, it was important to verify whether the block in infection was at the stage of virus uptake. With dnrab5, it is likely that the inhibition is at this stage, but it is less likely for dnrab7. To investigate virus entry in dnrab5 and dnrab7 expressing cells, dnrab5 and dnrab7 were expressed in IBRS2 cells, followed by uptake of purified FMDV for 20 minutes at 37°C. The cells were then fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with an FMDV anti-VP1 antibody (Mab D9) and an Alexa 568nm conjugated secondary antibody (red). Figure 5.10 shows expression of dnrab5 (panel A) and dnrab7 (panel C) and FMDV uptake (panels B and D). Cells expressing dnrab5 can be seen to take up a lesser amount of virus, consistent with the cells having fewer early endosomes (see figure 4.3). In contrast, expression of dnrab7 did not appear to inhibit virus internalisation.

5.6 Discussion

The use of dominant-negative rab GTPases to block specific stages of endocytosis has been well documented (see chapter one, section 1.5). The results presented here have given some insights into which endocytic compartments are required for FMDV infection. Expression of dnrab5 inhibits FMDV infection by ~76% when compared to wtarb5, suggesting that the virus requires intact early endosomes for infection. This supports previous data showing co-localisation between FMDV and EEA1 (a marker for early endosomes) in SW480-β6 cells at early time points post-entry (Berryman et al., 2005). The inhibition of FMDV infection by dnrab5 expression seen in this study is therefore consistent with studies using other viruses.
Figure 5.10 FMDV uptake is inhibited in dnrab5, but not dnrab7 expressing cells

IBRS2 cells were transfected with expression plasmids for wtrab5 or dnrab5 fused to EGFP. At 6 hours post-transfection, purified FMDV was taken up into cells at 37°C for 20 minutes. Cells were fixed with 4% paraformaldehyde and permeablised with Triton X-100. This was followed by labelling with an antibody against FMDV VP1 (D9), and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel B shows FMDV labelling, with panel A showing which is the dnrab5 expressing cell. These pictures show that dnrab5 expressing cells do not internalise purified FMDV to the same extent as non-expressing cells. Panel D shows FMDV labelling, with panel C showing which is the dnrab7 expressing cell. These pictures show that dnrab7 expressing cells internalise purified FMDV to the same extent as non-expressing cells.
The first study showing an inhibition of virus infection by dnrab5 was carried out using adenovirus. This study documented a ~50% inhibition of infection in dnrab5 expressing cells (Rauma et al., 1999). In a similar study, Influenza virus infection was found to be inhibited by ~61% in dnrab5 expressing cells (Sieczkarski & Whittaker, 2003). The same study showed an inhibition in infection of other enveloped viruses, semliki forest virus (SFV) and vesicular stomatitis virus (VSV) when dnrab5 was expressed, although these effects were not quantified. A recent study investigating calicivirus infection showed an inhibition of infection of ~80% when dnrab5 was expressed (Stuart & Brown, 2006).

Lack of infection in dnrab5 expressing cells is consistent with virus uptake by clathrin-dependent endocytosis, as formation of early endosomes and uptake of labelled transferrin into early and recycling endosomes was inhibited in dnrab5 expressing cells (see chapter four). In the case of FMDV, it seems likely that inhibition of infection by dnrab5 is at the stage of virus uptake. In dnrab5 expressing cells, FMDV appeared to be taken up into small peripheral vesicles, most likely ‘nude’ clathrin-coated vesicles. As there were no early endosomes for these vesicles to fuse with, the virus would be unable to gain access to these acidic compartments for capsid uncoating. In wtrab5 expressing cells, FMDV uptake was normal as compared to non-expressing cells.

It was important to verify that the effects of dominant-negative rab protein expression on FMDV infection were not due to inhibition of receptor recycling, reducing the amount of available receptors on the cell surface. Expression of wtrab5 or dnrab5 did not affect the cell surface expression levels of αVβ8. Virus binding at the cell surface was also investigated in dnrab5 expressing cells. There was no
detectable reduction in virus binding in cells expressing dnrb5, when compared to non-expressing cells. This suggests that inhibition of FMDV infection in dnrb5 expressing cells is unlikely to be due to reduced receptor availability, or an inhibition in virus binding.

Expression of constitutively active rab5 (carab5) had little effect on FMDV infection when compared to wild-type. Although there is evidence that expression of carab5 enhances the rate of early endosome fusion, forming enlarged endosomes (Stenmark et al., 1994), there is no evidence that it increases rates of ligand uptake or recycling (Ceresa et al., 2001). This may explain why there is no significant increase in FMDV infection in cells expressing this mutant rab5 protein.

Expression of dnrb4 did not inhibit FMDV infection. This suggests that the rapid recycling pathway directly from early endosomes to the plasma membrane is not required for FMDV infection. Expression of dnrb11 has a slight inhibitory effect on FMDV infection and reduced recycling of labelled transferrin out of the cell. It seems therefore that the rab11-dependent recycling pathway may be more important than the rapid rab4-dependent pathway for FMDV infection. This may be due to the observation that the majority of integrins studied so far appear to use the rab11 dependent pathway for recycling (Jones et al., 2006) and incoming FMDV particles may follow the same trafficking route as their receptor. As recycling endosomes are less acidic than early endosomes (Gagescu et al., 2000), it is difficult to see any advantage to the virus entering the recycling pathway. It is possible that some virus particles do not uncoat in early endosomes and are carried rapidly into the recycling pathway along with the receptor. The virus may then experience a prolonged exposure to the acidic pH in recycling endosomes. However, the data in chapter five
show that FMDV has a greater requirement for early endosomes than recycling endosomes for infection. Expression of dnrab4 or dnrab11 did not appear to affect the cell surface expression levels of αVβ8. Similarly, there was also no detectable reduction in virus binding in cells expressing dnrab11 when compared to non-expressing cells. This suggests that the partial inhibition of FMDV infection in dnrab11 expressing cells is unlikely to be due to reduced receptor availability, or an inhibition in virus binding. It is important to note that in cells expressing dnrab4 and dnrab11, the early endosomes are intact, and therefore the virus has access to this acidic compartment for capsid uncoating.

Unexpectedly, expression of dnrab7 inhibited infection by FMDV. The inhibition is comparable to that seen with dnrab5. All evidence so far argues against a requirement for rab7-dependent compartments such as late endosomes and lysosomes in virus entry. In SW480-β6 cells, FMDV protein does not co-localise with LAMP-2, a marker for lysosomes (Berryman et al., 2005). Trafficking of ligands from early- to late endosomes needs microtubules; however FMDV has no requirement for these structures during infection as infection is not inhibited in cells treated with the microtubule-disrupting agent nocodazole (see chapter three). Other viruses shown to require rab7 for infection are known to enter late endosomes and require the lower pH in these compartments for genome release (for example, human rhinovirus (Prchla et al., 1994)). The study on influenza virus mentioned previously found that dnrab7 expression inhibited infection by ~50% (Sieczkarski & Whittaker, 2003). The reason given for this is that influenza virus requires a pH of 5.5 for infection. The pH in early endosomes is around 6.0 and in late endosomes it is 5.0 to 5.5. Therefore influenza virus must traffic to the late endosome in a rab7-dependent
manner for infection. In this same study, infection by Semliki Forest Virus (SFV) and Vesicular Stomatitis Virus (VSV) did not require rab7. This can be explained as the pH required for infection by these viruses are 6.2 and 6.3, respectively (Sieczkarski & Whittaker, 2003), which would permit infection to take place from within early endosomes. FMDV requires a pH just below neutral for uncoating and the environment in early endosomes is certainly sufficiently acidic for uncoating to occur (Curry et al., 1995). The data in chapter four showed that expression of dnrab7 inhibited trafficking of Dil-LDL and lysotracker to late compartments, and the data in this chapter showed that uptake of FMDV was not inhibited in dnrab7 expressing cells. These data suggest that FMDV does not require transport to late endosomes and rab7 is not required for virus uptake. It is therefore possible that rab7 may play some other role in FMDV infection. This will be further investigated in chapter six.

Expression of dnrab9 did not appear to inhibit FMDV infection. Rab9 controls trafficking of ligands from late endosomes to the Golgi. FMDV is not thought to enter either compartment, although the Golgi is known to disperse during infection (O'Donnell et al., 2001). FMDV does not co-localise with the Golgi at early time points post infection (O'Donnell et al., 2005) although the non-structural protein 2C does co-localise to some extent with markers of both the ER and Golgi during replication (Moffat et al., 2005). There is a possibility that the Golgi is a source of membranes for formation of the vesicles required for replication (see chapter one, section 1.4.5. An inhibition of trafficking from late endosomes to the Golgi is unlikely to affect the integrity of the Golgi itself. Expression of dnrab9 in CHO cells does not affect the morphology of the Golgi as seen by immunofluorescence.
It is interesting to note that dnrab9 in uninfected IBRS2 cells is distributed in a Golgi-like pattern, and in chapter four was seen to co-localise strongly with markers of the Golgi apparatus. In FMDV infected IBRS2 cells, dnrab9 was dispersed, consistent with the fragmentation of the Golgi during FMDV infection seen in the studies mentioned above (data not shown).

In summary, the results of chapter five show that early endosomes, and to a lesser extent recycling endosomes are required for infection by FMDV. Although expression of dnrab7 inhibited trafficking of Dil-LDL and lysotracker, it also inhibited FMDV infection, suggesting that rab7 may have a role in the virus life cycle.
Chapter six: The role of rab7 in FMDV infection

6.1 Introduction

Although the inhibitory effect of dominant-negative rab5 expression on FMDV infection was as expected, the inhibitory effect of dominant-negative rab7 expression was surprising (see chapter five). It is likely that inhibition by dnrab7 is not at the stage of virus uptake, as FMDV is internalised by IBRS2 cells to a similar extent in dnrab7 expressing cells when compared to non-expressing cells (figure 5.10). One possibility is that a later stage of infection, such as intracellular virus replication, is being inhibited. It is known that extensive membrane rearrangements take place within cells infected with positive-strand RNA viruses (see chapter one, section 1.4.5). Recent papers have suggested a link between the vesicles formed during positive-strand RNA virus infection and autophagosomes (Jackson et al., 2005; Prentice et al., 2004). It has also been shown that rab7 is involved in a late stage of autophagy (Gutierrez et al., 2004; Jager et al., 2004). This suggests a possible role for rab7 in FMDV infection. To further investigate the role of rab7 in FMDV infection, a second dominant-negative rab7 was obtained (from Maria Columbo, Argentina) with the rabS/T-N mutation, (T22N) rather than the rabN-I, (N125I) used in chapter five. The N125I mutant is unable to bind GTP or GDP and consequently remains primarily cytosolic. The T22N mutant binds preferentially to GDP and is inactive and therefore unable to recruit effector proteins. However GDP-bound rab proteins interact transiently with the cellular membranes they target (Seabra & Wasmeier, 2004). In HeLa cells, rab7-T22N has increased membrane binding ability when autophagy is activated (Gutierrez et al., 2004).
6.2 Expression of rab7-T22N inhibits trafficking of labelled low density lipoprotein (Dil-LDL)

The effect of dominant-negative rab7-N125I expression on trafficking of labelled low density lipoprotein, Dil-LDL (red) was investigated in chapter four. Dil-LDL is a ligand which is taken up by clathrin-dependent endocytosis into early endosomes from where it traffics to late endosomes and lysosomes. This experiment was repeated with the new dominant-negative rab7-T22N, to verify that this mutant also inhibits trafficking from early endosomes to late endosomes. Following transfection with an expression plasmid containing wtrab7-EGFP or rab7-T22N-EGFP, Dil-LDL (red) was taken up into IBRS2 cells for 30 minutes. At this time point the Dil-LDL should have reached late endosomes. Figure 6.1, panel A shows the wtrab7 expressing cell, and panel B shows the rab7-T22N expressing cell. In panel C, all cells have a similar level of Dil-LDL in large vesicles close to the nucleus, in a distribution indicative of late endosomes. This shows that trafficking of Dil-LDL is not inhibited by expression of wtrab7. However, in the panel D, the rab7-T22N expressing cell has an altered distribution of Dil-LDL when compared to non-expressing or wtrab7 expressing cells. The Dil-LDL is in smaller vesicles near to the periphery of the cell, suggesting that it has become trapped in early compartments. This indicates that rab7-T22N blocks trafficking of ligands from early endosomes to late endosomes.

6.3 Expression of rab7-T22N inhibits trafficking of lysotracker red

The effect of rab7-T22N expression on trafficking of lysotracker red was also investigated. Lysotracker red is a dye which labels highly acidic compartments such
Figure 6.1 Immunofluorescence confocal microscopy showing expression of dominant-negative rab7 (rab7-T22N-EGFP), but not wild-type rab7 (wtrab7-EGFP) inhibits early- to late endosome trafficking of labelled low density lipoprotein (Dil-LDL).

IBRS2 cells were transfected with expression plasmids for wtrab7 and rab7-T22N fused to EGFP. At 6 hours post-transfection Dil-LDL (red) was added to the media and allowed to internalise for 30 minutes. The cells were then fixed with paraformaldehyde. At this time point, the Dil-LDL should have reached late endosomes. Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows the wtrab7 expressing cell (green), and panel C shows Dil-LDL (red). The wtrab7 expressing cell has a similar level of Dil-LDL when compared to non-expressing cells. Panel B shows the rab7-T22N expressing cell (green), and panel D shows Dil-LDL (red). The rab7-T22N expressing cell has a reduced level of Dil-LDL when compared to non-expressing cells, suggesting an inhibition in trafficking from early- to late endosomes when rab7-T22N is expressed.
as late endosomes and lysosomes. Following transfection with an expression plasmid containing wtrab7-EGFP or rab7-T22N-EGFP, lysotracker red was taken up into IBRS2 cells for 30 minutes. At this time point, strongly acidic compartments would be expected to be labelled red. Figure 6.2, panel A shows the wtrab7 expressing cell, and panel B shows the rab7-T22N expressing cell. In panel C, all cells have a similar level of red lysotracker in large vesicles close to the nucleus, in a distribution indicative of late endosomes and lysosomes. This shows that trafficking to late endosomes and lysosomes is not inhibited by expression of wtrab7. However, in the panel D, the rab7-T22N expressing cell has an altered distribution of red lysotracker when compared to non-expressing or wtrab7 expressing cells. The lysotracker is labelling smaller vesicles near to the periphery of the cell, suggesting labelling of mildly acidic early compartments only. This indicates that rab7-T22N inhibits trafficking from early endosomes to late endosomes and lysosomes. This is in agreement with the results seen using rab7-N125I to inhibit trafficking of Dil-LDL and lysotracker described in chapter four.

6.4 Expression of rab7-N125I, but not rab7-T22N inhibits FMDV infection

After showing that both forms of dominant-negative rab7 inhibit trafficking from early endosomes to late endosomes and lysosomes, the next stage was to repeat the FMDV infection assay used in chapter five with rab7-T22N. IBRS2 cells were transfected with expression plasmids for wild-type (wtrab7), dominant-negative rab7-T22N or dominant-negative rab7-N125I fused to EGFP. At 6 hours post-transfection, the cells were infected with FMDV 01Kcad2 for 3 hours. The cells were then fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with a rabbit polyclonal serum recognising the FMDV capsid proteins and
Figure 6.2 Immunofluorescence confocal microscopy showing expression of dominant-negative rab7 (rab7-T22N-EGFP), but not wild-type rab7 (wtrab7-EGFP) inhibits trafficking of lysotracker to late endosomes and lysosomes. IBRS2 cells were transfected with expression plasmids for wtrab7 and rab7-T22N fused to EGFP. At 6 hours post-transfection lysotracker red was added to the media and allowed to internalise for 30 minutes. The cells were then fixed with paraformaldehyde. At this time point, lysotracker red should label highly acidic compartments such as late endosomes and lysosomes. Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panel A shows the wtrab7 expressing cell (green), and panel C shows labelling for lysotracker red. The wtrab7 expressing cell has an equal level of lysotracker red labelling when compared to non-expressing cells. Panel B shows the rab7-T22N expressing cell (green), and panel D shows labelling for lysotracker red. The rab7-T22N expressing cell has a reduced level of lysotracker red labelling when compared to non-expressing cells, suggesting an inhibition in the trafficking of lysotracker into late endosomes and lysosomes when rab7-T22N is expressed.
an Alexa 568nm conjugated secondary antibody (red).

Figure 6.3 shows that although cells expressing wtrab7 (green, panels A and B) or rab7-T22N (green, panels C and D) can be infected by FMDV (red), the cells expressing rab7-N125I (green, panels E and F) are rarely infected.

Figure 6.3, panel G quantifies the effect of expression of each form of rab7 on FMDV infection. This was achieved by counting 1) wtrab7, rab7-T22N or rab7-N125I expressing, FMDV-infected cells, and 2) non-expressing FMDV-infected cells on randomly selected fields of view on the confocal microscope. Expression of wtrab7 results in an ~18% reduction in infection when compared to non-expressing cells on the same coverslip. This suggests that overexpression of wtrab7 has a slight inhibitory effect on FMDV infection (as in chapter five). Overexpression of rab7-T22N inhibits FMDV infection by ~27%. However, the inhibition when rab7-N125I is expressed is much greater, ~82%. If the proportion of wtrab7 expressing cells infected is normalised at 100%, there is an inhibition of only ~9% when rab7-T22N is expressed, but ~79% when rab7-N125I is expressed. This is represented graphically in panel H. This suggests that the two dominant-negative rab7 proteins differ in their ability to inhibit infection by FMDV.

6.5 Expression of rab7-N125I, but not rab7-T22N inhibits intracellular FMDV replication

It is possible that the inhibition of FMDV infection by rab7-N125I is at an intracellular stage of replication rather than entry. This was investigated by expressing wtrab7, dominant-negative rab7-T22N or dominant-negative rab7-N125I
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<th>rab7-N125I</th>
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<tr>
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</tr>
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</tr>
<tr>
<td>% Knockdown</td>
<td>9%</td>
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</tr>
</tbody>
</table>

Figure 6.3 Expression of rab7-N125I inhibits FMDV infection but rab7-T22N does not

IBRS2 cells were transfected with expression plasmids for wtrab7, rab7-T22N and rab7-N125I fused to EGFP. At 6 hours post-transfection, cells were infected with FMDV 01Kcad2 for 3 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and B show wtrab7 and FMDV. Panels C and D show rab7-T22N and FMDV. Panels E and F show rab7-N125I and FMDV. Panel G shows percentage knockdown due to expression of rab7-T22N and rab7-N125I. This is represented graphically in panel H (error bars +/- one standard deviation).
in IBRS2 cells, followed by electroporation of FMDV RNA into the cells. This bypasses endocytosis, delivering the viral RNA directly into the cytoplasm. In this way, any effects of dominant-negative rab7 expression can be assumed to be acting after the stage of virus endocytosis.

Figure 6.4 shows that consistent with the data shown in figure 6.3, expression of rab7-T22N had little effect on FMDV infection, inhibiting by ~17% when the proportion of wtrab7 expressing infected cells was normalised at 100%. Under these conditions, expression of rab7-N125I inhibited infection by ~90%, again agreeing with the data in figure 6.3. These data show that inhibition of FMDV infection by rab7-N125I is at a stage after cell entry, during intracellular virus replication.

6.6 Expression of dnrab5 inhibits BEV infection

In order to see whether a requirement for rab7 was a feature of all picornaviruses, effects of dnrab5 and dnrab7 on bovine enterovirus (BEV) infection were investigated. BEV is an enterovirus, in the same family as poliovirus. It differs from FMDV in its sensitivity to brefeldin A, being sensitive when FMDV is not (Gazina et al., 2002). Another difference is that enteroviruses form ‘A’ particles and do not require low pH for infection.

BEV infects IBRS2 cells poorly, so for this experiment CHO cells were used. The cells were transfected with expression plasmids for wtrab5 or dnrab5 fused to EGFP. At 6 hours post-transfection, cells were infected with BEV for 5 hours. Cells were fixed with paraformaldehyde, permeabised with Triton X-100 and labelled with 11C5, an anti-BEV antibody and an Alexa 568nm conjugated secondary antibody (red).
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<th>rab7-N125l</th>
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<td><strong>% Expressing/non-expressing</strong></td>
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<td>147%</td>
<td>18%</td>
</tr>
<tr>
<td><strong>Wild Type=100%</strong></td>
<td>100%</td>
<td>83%</td>
<td>10%</td>
</tr>
<tr>
<td><strong>% Knockdown</strong></td>
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</table>

Figure 6.4 Expression of rab7-N125I inhibits FMDV replication but rab7-T22N does not

IBRS2 cells were transfected with expression plasmids for wtrab7, rab7-T22N and rab7-N125I fused to EGFP. At 6 hours post-transfection, cells were electroporated with FMDV RNA and incubated at 37°C for 6 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with rabbit polyclonal serum recognising the FMDV capsid proteins and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and B show wtrab7 and FMDV. Panels C and D show rab7-T22N and FMDV. Panels E and F show rab7-N125I and FMDV. Panel G shows percentage knockdown due to expression of rab7-T22N and rab7-N125I. This is represented graphically in panel H.
Figure 6.5 shows that although cells expressing wtRab5 (green, panels A and B) can be infected by BEV (red), the cells expressing dN-Rab5 (green, panels C and D) are rarely infected. Panel E quantifies the effect of expression of each form of Rab5 on BEV infection. This was achieved by counting 1) wtRab5 or dN-Rab5 expressing, BEV-infected cells, and 2) non-expressing BEV-infected cells on randomly selected fields of view on the confocal microscope (see above). Expression of wtRab5 results in a ~9% reduction in BEV infection when compared to non-expressing cells on the same coverslip. However, the inhibition when dN-Rab5 is expressed is much higher, ~67%. If the proportion of wtRab5 expressing cells infected is normalised at 100%, there is an inhibition of ~63% when dN-Rab5 is expressed. This suggests that BEV infection may require early endosomes.

6.7 Expression of wtRab7 and Rab7-N125I inhibit BEV infection, but Rab7-T22N does not

The effects of dominant-negative Rab7-T22N and Rab7-N125I on BEV infection were also investigated. CHO cells were transfected with expression plasmids for wtRab7, Rab7-T22N or Rab7-N125I fused to EGFP. At 6 hours post-transfection, cells were infected with BEV for 5 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with 11C5, an anti-BEV antibody and an Alexa 568nm conjugated secondary antibody (red).

Figure 6.6 shows that although cells expressing wtRab7 (green, panel A), Rab7-T22N (green, panel B) or Rab7-N125I (green, panel C) can all be infected by BEV (red).
Figure 6.5 Effect of dominant-negative rab5 (dnrab5) on infection by BEV

CHO cells were transfected with expression plasmids for wtrab5 or dnrab5 fused to EGFP. At 6 hours post-transfection, cells were infected with BEV for 5 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with 11C5, an anti-BEV antibody and an Alexa 568 nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and B show wtrab5 and BEV. Panels C and D show dnrab5 and BEV. Panel E shows percentage knockdown due to expression of dnrab5.

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<td>%Expressing/non-expressing</td>
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<td>37%</td>
</tr>
<tr>
<td>% Knockdown</td>
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<td>63%</td>
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</tbody>
</table>

Figure 6.5 Effect of dominant-negative rab5 (dnrab5) on infection by BEV

CHO cells were transfected with expression plasmids for wtrab5 or dnrab5 fused to EGFP. At 6 hours post-transfection, cells were infected with BEV for 5 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with 11C5, an anti-BEV antibody and an Alexa 568 nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10μm.

Panels A and B show wtrab5 and BEV. Panels C and D show dnrab5 and BEV. Panel E shows percentage knockdown due to expression of dnrab5.
**Figure 6.6 Effect of wtrab7, rab7-N125I and rab7-T22N on infection by BEV**

CHO cells were transfected with expression plasmids for wtrab7, rab7-T22N or rab7-N125I fused to EGFP. At 6 hours post-transfection, cells were infected with BEV for 5 hours. Cells were fixed with paraformaldehyde, permeabilised with Triton X-100 and labelled with 11C5, an anti-BEV antibody and an Alexa 568nm conjugated secondary antibody (red). Cell nuclei were stained with DAPI and are shown in blue. Bars=10µm.

Panel A shows wtrab7 and BEV. Panel B shows rab7-T22N and BEV. Panel C shows rab7-N125I and BEV. Panel D shows percentage knockdown due to expression of rab7-T22N or rab7-N125I.
Figure 6.6, panel D quantifies the effect of expression of each form of rab7 on BEV infection. This was achieved by counting 1) wtrab7, rab7-T22N or rab7-N125I expressing, BEV-infected cells, and 2) non-expressing BEV-infected cells on randomly selected fields of view on the confocal microscope. Expression of wtrab7 results in a ~44% reduction when compared to non-expressing cells on the same coverslip. This suggests that overexpression of wtrab7 has a more significant inhibitory effect on BEV infection than FMDV. Overexpression of rab7-N125I inhibits BEV infection by a similar amount, ~45%. This is less than the inhibition seen for FMDV. However, the inhibition when rab7-T22N is expressed is only ~18%. This is similar to FMDV. If the proportion of wtrab7 expressing cells infected is normalised at 100%, there is an enhancement of ~47% when rab7-T22N is expressed, but little difference when rab7-N125I is expressed.

As these experiments were carried out in CHO cells, FMDV infection was also quantified in these cells to verify that the effects of rab5 and rab7 on BEV infection were not due to differences in cell type. The results are shown graphically in figure 6.7, with 1) FMDV infection in IBRS2 cells (blue), 2) FMDV infection in CHO cells (red), and 3) BEV infection in CHO cells (yellow). The FMDV results are similar for both cell lines. These data show that the effects of dnrab5 and dnrab7 on FMDV infection can be reproduced in two different cell lines, and the difference in the effects of dnrab5 and dnrab7 on BEV infection when compared to FMDV are most likely due to differences in the viruses and not cell type.
Figure 6.7 Comparison of effects of dnrab5, rab7-T22N and rab7-N125I on infection by FMDV and BEV in CHO and IBRS2 cells

Shown on the graph are percentage infection rates for dnrab5, rab7-T22N and rab7-N125I expressing cells with wtrab5 or wtrab7 normalised at 100%.

1) FMDV infection in IBRS2 cells (blue), 2) FMDV infection in CHO cells (red), and 3) BEV infection in CHO cells (yellow).
6.8 Discussion

Two different dominant-negative mutants of rab7 have been investigated in this chapter. The first mutant, rab7-N125I, is unable to bind nucleotides and is primarily cytosolic. The second, rab7-T22N, has a higher affinity for GDP than GTP. It is inactive and unable to recruit effector proteins, but in its GDP bound form can bind membranes and shows an increased membrane binding in cells when autophagy is activated (Gutierrez et al., 2004). Expression of both dominant-negative rab7 proteins has been shown to inhibit trafficking of labelled LDL from early to late endosomes. They also reduce labelling of late endosomes and lysosomes with lysotracker red. As FMDV is not thought to traffic to late endosomes or lysosomes for infection, it would be predicted that expression of either form of dominant-negative rab7 would have no effect on FMDV entry and infection.

However, although expression of rab7-T22N had little effect on FMDV infection, expression of rab7-N125I inhibited FMDV infection by 78% (similar to the inhibition seen in chapter five). When viral RNA was electroporated into cells, bypassing endocytosis, the effects on virus replication were similar. Compared to cells expressing wild-type rab7, the inhibition of FMDV replication by expression of rab7-N125I was ~90% whereas expression of rab7-T22N again had little effect on virus replication. This suggests that inhibition of FMDV infection by rab7-N125I is at a stage after entry, during intracellular virus replication.

The nature and origin of the membrane vesicles formed during infection with positive-strand RNA viruses has been the subject of much research in recent years. It has been recently suggested that the vesicles formed during polio- and coronavirus
infection have characteristics of autophagosomes (Jackson et al., 2005; Prentice et al., 2004). FMDV is also believed to stimulate autophagy (E. Brooks, personal communication). It has also been shown that rab7 is involved in a late stage of autophagy (Gutierrez et al., 2004; Jager et al., 2004). It has long been believed that there are host factors involved in the formation of the viral replication complex (Ahlquist et al., 2003). It is possible that rab7 is one of these factors, and could be acting as a tethering protein helping to anchor the replication complex to the membrane, or to attract other cellular proteins to the site of replication.

Alternatively, rab7 may be required to maintain the integrity of the replication vesicle. Recent studies have suggested a role for ADP ribosylation factors (ARFs) in anchoring the poliovirus replication complex to the replication vesicles (Belov et al., 2005), and these ARFs are thought to be the targets for brefeldin A inhibition of poliovirus infection (Morinaga et al., 1996). It is tempting to speculate that rab7 has an equivalent role in FMDV infection, and as rab7 is not affected by brefeldin A, this could explain the difference in sensitivity of poliovirus and FMDV to brefeldin A treatment.

The observation that the cytosolic form of dominant-negative rab7-N125I inhibits FMDV infection suggests that the membrane binding ability of rab7 is required for FMDV infection. The observation that a membrane bound yet inactive form of rab7 (rab7-T22N) supports FMDV infection argues against a role for rab7 in formation or movement of the replication vesicles and suggests a more passive role. It may be that rab7 is required on the membrane, but it does not need to be in its active, GTP bound form. It is also interesting to note that the presence of an active form of rab7 on the replication vesicle might promote fusion with acidic lysosomes which would
presumably be detrimental to virus replication. The bacterium *Salmonella typhimurium* replicates in rab7-positive vesicles and encodes a bacterial protein, SifA, which substitutes for the rab7 effector RILP and therefore prevents recruitment of microtubules and thus fusion with lysosomes (Harrison *et al.*, 2004). It may therefore be advantageous for FMDV replication to also maintain rab7 in an inactive form.

The effect of dominant-negative rab5 expression on infection by BEV was similar to that seen for FMDV. Although the inhibition was a little lower, 63% for BEV compared to 77% for FMDV, there still appears to be some requirement for early endosomes. BEV is an enterovirus and therefore is stable to low pH and would not be expected to require exposure to the low pH within endosomes for infection. However, successful infection may require transport of BEV to early endosomes.

Recent evidence has shown that coxsackie B virus forms ‘A’ particles on binding its receptor but viral RNA escape is delayed and occurs following uptake into caveolae (Coyne & Bergelson, 2006). It is possible that for BEV, viral RNA escape takes place from within vesicles following virus uptake.

The effect of dominant-negative rab7-T22N or rab7-N125I expression on infection by BEV was different to that seen for FMDV infection. When compared to cells expressing wtrab7, expression of rab7-T22N appeared to enhance BEV infection, but expression of rab7-N125I had little effect. However, when compared to non-expressing cells, expression of wtrab7 and rab7-N125I appeared to inhibit infection by ~45%. It is difficult to conclude a role for rab7 during BEV infection. Future experiments targeting rab7 by RNA interference would be helpful in clarifying this issue.
Chapter seven: Depletion of rab7 by RNA interference

7.1 Introduction

The results presented in chapter six suggest that two different forms of dominant-negative rab7 have different effects on FMDV infection. Expression of rab7-T22N does not inhibit FMDV infection and the protein is able to bind membranes (Seabra & Wasmeier, 2004), whereas expression of rab7-N125I inhibits FMDV infection and the protein is primarily cytosolic, due to a reduced membrane binding capacity. The purpose of the work carried out in chapter seven is to confirm whether rab7 is required for FMDV infection. This will be investigated using RNA interference, a technique by which a protein can be specifically depleted from cells. Specific depletion of rab7 may have a different effect when compared to overexpression of dominant-negative proteins, as dominant-negative proteins may still be able to bind regulatory proteins. These regulatory proteins include chaperone proteins (such as GDI) and also the two regulatory proteins involved in the GTPase cycle, known as GAPs (GTPase-activating proteins) and GEFs (guanine nucleotide exchange factors, see chapter one, section 1.5).

The technique of RNA interference has been developed to selectively inhibit the expression of a specific protein (Hannon, 2002). The aim of the studies described in this chapter are to deplete cells of rab7 using siRNA (short interfering RNA) duplexes specific to rab7. It should then be possible to see if there is an effect on FMDV infection when rab7 is absent from the cells, as compared to overexpression of a dominant-negative form.
Although there have been a number of studies carried out using RNA interference to target viral proteins, fewer have been undertaken targeting cellular proteins required for virus replication and quantifying the effects of their depletion on virus infection. A recent study investigating poliovirus infection used RNA interference to deplete the cells of components required for cellular autophagy (Jackson et al., 2005). These included ATG12 and LC3 (see chapter one, section 1.6.1). Inhibition of intracellular poliovirus infection was quantified as three-fold following a 70% depletion of ATG12 and four-fold following a 90% depletion of LC3. The effects on extracellular poliovirus production were reported to be greater. A recent study depleted cells of polypyrimidine tract-binding protein (PTB), a protein required for translation of picornavirus RNA. Its depletion lead to a decrease in calicivirus infection (Karaskasiliotis et al., 2006). A recent study has been carried out depleting host cellular factors that inhibit FMDV infection. In this study, depletion of double-stranded RNA-dependent protein kinase R (PKR) by RNA interference lead to an increase in FMDV infection (de los Santos et al., 2006).

7.2 Depletion of human rab7 by RNAi

A number of companies produce pre-validated siRNAs directed to commonly studied cellular genes. These companies only develop pre-validated siRNA for the most commonly used species, i.e. human, mouse and rat. Human siRNA and therefore human cells were used in initial experiments for this reason. Dharmaco’s siGLO, a stable, fluorescent, non-targeting siRNA can be transfected into cells and visualised by immunofluorescence confocal microscopy. This was used as a control to determine transfection efficiency and to test a number of transfection reagents.
Figure 7.1 shows transfection of red siGLO siRNA (Dharmacon) in HeLa cells using a variety of transfection reagents. Transfection efficiencies were determined as: Dharmafect 73%, Lipofectamine 69%, siPORTamine 85% and siPORTlipid 0%. The siPORT amine reagent was selected and used in all further experiments in this chapter.

The siPORT amine reagent was used to deliver a pool of four human rab7-specific duplexes into HeLa cells. Cell lysates were prepared for western blotting (see chapter two, section 2.8.1) at 24, 48 and 72 hours post-transfection. A western blot using an anti-rab7 antibody (obtained from S. Pfeffer) is shown in figure 7.2. There is a strong band for rab7 at 24 hours, showing that rab7 protein was still being expressed at this time point. A partial knockdown for rab7 expression was seen at 48 hours, increasing at to near complete at 72 hours. There is some recovery of rab7 expression at 96 hours. This suggested that 72 hours is the optimum time point for further siRNA studies.

The western blots were repeated, including an anti-β-actin control for sample loading. A further control was included to show that rab7 was specifically depleted by the rab7 targeted siRNA. In order to do this, an anti-rab5 blot was included as this protein is similar to rab7, but should not be affected by the rab7-specific siRNA. Cell lysates were prepared at 72 hours post-transfection. Figure 7.3 shows western blots for actin, rab5 and rab7. The first lane shows a mock transfection, the second is the rab7-specific siRNA and the third lane is a non-targeting control siRNA (Dharmacon). This is a pool of siRNA duplexes which have been found to have no significant homology to the human, mouse or rat genomes by BLAST search. Although a good knockdown is seen in the rab7 western blot with rab7-specific
siGLO labelled siRNA (red) was transfected into HeLa cells at 20pmol per coverslip. A variety of transfection reagents were used, each according to the manufacturer's protocol. Cell nuclei were stained with DAPI and are shown in blue. Bars=40µm. Transfection efficiencies are: Dharmafect 73%, Lipofectamine 69%, siPORTamine 85%, siPORTlipid 0%. The most efficient reagent appears to be siPORTamine (Ambion).
Figure 7.2 Time course of rab7 depletion by siRNA in HeLa cells

A pool of four human rab7-specific duplexes were transfected into HeLa cells using siPORTamine. Samples were taken for western blotting at 24, 48 and 72 hours post-transfection. Rab7 was detected by an anti-rab7 antibody and a goat anti-rabbit IgG (H+L) HRP conjugate (Promega). At 24 hours rab7 was still being expressed. A clear knockdown can be seen by 48 hours, increasing at 72 hours and then some recovery of rab7 expression by 96 hours.
A mock transfection with no siRNA, a pool of four human rab7-specific duplexes or a non-targeting siRNA were transfected into HeLa cells using siPORTamine. Samples were taken for western blotting at 72 hours post-transfection. Actin was detected using an anti-actin antibody (Sigma) and a goat anti-mouse IgG (H+L) HRP conjugate (Promega). Rab5 was detected using an anti-rab5 antibody (Stressgen) and a goat anti-rabbit IgG (H+L) HRP conjugate (Promega). Rab7 was detected by an anti-rab7 antibody and a goat anti-rabbit IgG (H+L) HRP conjugate (Promega). Although a good knockdown is seen in the rab7 western blot with rab7-specific siRNA, there is no reduction in rab7 in either control. There is no difference in levels of actin or rab5, showing that the rab7 siRNA is specific.
siRNA when compared to the mock transfection, there is no significant reduction in rab7 in the non-targeting control transfected cells. There is no significant difference in levels of actin or rab5 in any lane, showing that the rab7 siRNA is specific. This means that a non-specific reduction in protein expression induced by the introduction of double-stranded RNA into the cells can be discounted. The introduction of dsRNA into cells can cause activation of a non-specific interferon response, which reduces expression levels of all cellular proteins.

7.3 Transfection with porcine rab7-specific siRNA inhibits intracellular virus replication

As the human rab7-specific siRNA appeared to be working, the next stage was to obtain a porcine rab7-specific siRNA. The facility to design custom siRNA for species other than human, mouse and rat has recently become available. Firstly, IBRS2 cells were tested for their ability to take up the red fluorescent siGLO control siRNA. This is shown in figure 7.4, with a transfection efficiency of approximately 63%, slightly lower than the HeLa cells.

No complete sequence for porcine rab7 is available. A number of partial sequences were combined in order to design porcine rab7-specific siRNA. A pool of four porcine rab7-specific siRNAs (Dharmacon) was obtained and was transfected into IBRS2 cells in a 96-well plate. The rab7 antibody used for western blot assay in the HeLa cells did not cross-react with porcine rab7, meaning that the level of rab7 depletion could not be quantified. At 48 or 72 hours post-transfection with rab7-specific siRNA, cells were infected with FMDV 01Kcad2 and processed for ELIspot assay as previously described in chapter three. The numbers of infected cells were
Figure 7.4 Transfection efficiencies for siGLO siRNA delivery in IBRS2 cells

siGLO labelled siRNA (red) was transfected into IBRS2 cells using siPORTamine (Ambion) at 20pmol per coverslip. Cell nuclei were stained with DAPI and are shown in blue. Bars=20μm. Transfection efficiency is ~63%.
averaged over 6 wells. The results are represented graphically in figure 7.5. At 48 hours post-transfection, the number of FMDV infected cells is reduced by ~28% when compared to mock transfected cells. Transfection with the non-targeting control siRNA had little or no effect on the number of FMDV infected cells. At 72 hours post-transfection, the time point at which rab7 levels should be at their lowest, the number of FMDV infected cells is reduced by ~42% when compared to mock transfected cells. Transfection with the non-targeting control siRNA did not reduce the number of FMDV infected cells. The experiment was repeated once more, looking only at 72 hours post-transfection, but this time using two control siRNAs; the non-targeting siRNA used in the previous experiment and a RISC-free siRNA. This siRNA cannot be processed by the RNA-induced Silencing Complex (RISC) and therefore discounts effects on the cell induced by the presence of the siRNA. Figure 7.6 shows that again, transfection with rab7-targeting siRNA caused the number of FMDV infected cells to be reduced by ~44% at 72 hours post-transfection. This is in agreement with the inhibition of ~42% at 72 hours post-transfection seen in figure 7.5.

7.4 Transfection with porcine rab7-specific siRNA reduces virus yield

It seems that transfection with rab7-targeting siRNA inhibits the number of FMDV infected cells by ~42%-44%. The effect of this depletion on FMDV yield was also assayed. IBRS2 cells in a 24-well plate were transfected with no siRNA (mock transfection) or with a pool of four porcine rab7-specific siRNAs. At 72 hours post-transfection, cells were infected with FMDV 01Kcad2 and incubated at 37°C for 1 hour. Cells were then acid washed to remove virus that had not yet been internalised. After washing with serum-free media to restore pH, the cells were
Figure 7.5 Transfection with rab7 targeting siRNA causes a reduction in the number of FMDV infected cells.

IBRS2 cells in a 96-well plate were transfected with no siRNA (mock), porcine rab7-specific siRNA, or a non-targeting siRNA. After 48 or 72 hours, cells were incubated with FMDV 01K.ead2 for 1 hour at 37°C. The cells were washed to remove excess virus and infection was allowed to continue for a further 4 hours. An anti-3A antibody was used to label infected cells (see chapter two), followed by a biotinylated secondary antibody and streptavidin-conjugated alkaline phosphatase. On addition of the enzyme substrate, infected cells are stained blue/black. Numbers of infected cells were quantified using an ELIspot plate reader (see chapter two).

The graph shows the number of infected cells +/- one standard deviation. Infection is expressed as a percentage of the number of virus infected cells in mock transfected wells.

At 48 hours post-transfection, transfection with porcine rab7-specific siRNA leads to a 28% inhibition in the number of FMDV infected cells when compared to mock transfected cells. By 72 hours, this inhibition is 42%.
Figure 7.6 Transfection with rab7 targeting siRNA causes a reduction in the number of FMDV infected cells

IBRS2 cells in a 96-well plate were transfected with no siRNA (mock), porcine rab7-specific siRNA, a RISC-free siRNA or a non-targeting siRNA. After 72 hours, cells were incubated with FMDV 01Kcad2 for 1 hour at 37°C. The cells were washed to remove excess virus and infection was allowed to continue for a further 4 hours. An anti-3A antibody was used to label infected cells (see chapter two), followed by a biotinylated secondary antibody and streptavidin-conjugated alkaline phosphatase. On addition of the enzyme substrate, infected cells are stained blue/black. Numbers of infected cells were quantified using an ELIspot plate reader (see chapter two).

The graph shows the number of infected cells +/- one standard deviation. Infection is expressed as a percentage of the number of virus infected cells in mock transfected wells.

At 72 hours post-transfection, transfection with porcine rab7-specific siRNA causes a 44% reduction in the number of FMDV infected cells when compared to mock transfected cells.
again incubated at 37°C. Samples of cell media were taken at two hour intervals and
titrated on BHK cells to determine virus yield from the infected cells. Figure 7.7
shows plaque assays for samples taken 6 hours after the acid washing step. The
figure shows dilutions of $10^{-3}$ and $10^{-4}$. The samples from mock transfected cells
have produced more virus than the rab7 siRNA transfected cells. This suggests an
inhibition in virus yield when cells are transfected with rab7-targeting siRNA.

7.5 Discussion

Due to time constraints, the results in this chapter are incomplete and should be
considered as preliminary. The system for depletion of cellular rab7 by RNA
interference was set up using a pool of four human rab7 specific siRNA duplexes
(Dharmacon). When these were transfected into HeLa cells, a clear knockdown of
cellular rab7 could be seen by western blot analysis at 72 hours post-transfection. A
similar pool of four porcine rab7 specific siRNA duplexes were obtained in order to
carry out studies in IBRS2 cells, the cell line used in the majority of experiments
carried out in this thesis. Using these cells, a knockdown in cellular rab7 could not
be determined as the anti-rab7 antibody used with the HeLa cells did not cross-react
with porcine rab7. However, it appears that transfection with rab7-targeting siRNA
in IBRS2 cells has an inhibitory effect on FMDV infection, reducing both the
number of infected cells and virus yield. In two separate experiments, the reduction
in the number of infected cells as seen by ELISpot was 42% and 44% at 72 hours
post-transfection. This is compared to the ~79% inhibition seen when dominant-
negative rab7-N125I was expressed (see chapter five, figure 5.4 and chapter six,
figure 6.3). One possible explanation for this difference is that not all the cellular
rab7 was depleted. The inhibition of infection in the rab7-N125I expressing cells
Figure 7.7 Transfection with rab7 targeting siRNA causes a reduction in FMDV yield

IBRS2 cells were transfected with no siRNA (mock) or a pool of porcine rab7-specific siRNAs. After 72 hours, cells were infected with FMDV 01Kcad2. After 1 hour incubation at 37°C, cells were washed with citric acid to remove virus not internalised. Samples of media were taken at intervals and assayed for virus yield by plaque assay on BHK cells. This figure shows samples taken 6 hours post-infection. Two virus dilutions are shown.

Panels A and B are at a dilution of 10^-3. Panel A shows virus yield from mock transfected cells. Panel B shows a reduction in virus yield from porcine rab7-specific siRNA transfected cells.

Panels C and D are at a dilution of 10^-4. Panel C shows virus yield from mock transfected cells. Panel D again shows a reduction in virus yield from porcine rab7-specific siRNA transfected cells. The estimated titres are \(3.1 \times 10^3\) in the mock transfected cells and \(9 \times 10^4\) in the porcine rab7-specific siRNA transfected cells. This is 3.4-fold reduction.
was calculated using only cells expressing the dominant-negative protein, therefore assuming 100% inhibition. A real-time PCR assay could be used to quantify reduction in rab7 mRNA levels. Total mRNA has been extracted from IBRS2 cells transfected with rab7 siRNA and cDNA has been made from this, but the assays have not yet been carried out due to lack of time.

The effect on virus yield was also investigated, with a time course experiment being carried out at the same time as the ELIspot. When cells were transfected with rab7-targeting siRNA, there appears to be a reduction in FMDV yield at 6 hours post-infection.

The preliminary data presented in this chapter supports the theory that FMDV requires rab7 for infection. Transfection with rab7 targeting siRNA reduces the number of FMDV infected cells, and virus yield. However, the plaque assays and ELIspot assays need to be repeated, and the extent of the porcine rab7 knockdown must be quantified. If more time could have been devoted to this investigation, a good starting point would have been to clone porcine rab7. The sequence used for the siRNA design was based on partial sequences available on the internet which need to be confirmed. It would also be useful to obtain or to make an anti-rab7 antibody that cross-reacts with porcine rab7 for use in western blot assays. This could then be used to quantify knockdown of the rab7 protein, supporting real-time PCR assays showing knockdown of rab7 mRNA.
Chapter eight: Conclusions

The data presented in chapter three confirmed that αVβ8-mediated infection of IBRS2 cells by FMDV proceeds via clathrin-dependent endocytosis and is dependent on the low pH within endosomes. This was shown using specific inhibition of clathrin-dependent endocytosis by expression of AP180C (figure 3.1), and by inhibition of the vacuolar ATPase responsible for maintaining low endosomal pH by treatment with concanamycin A (figure 3.2). These data are in agreement with those presented for αVβ6-mediated FMDV entry into SW480 cells (Berryman et al., 2005).

To further investigate which compartments of the acidic endosomal system are required by FMDV during infection, dominant-negative rab GTPases were used. Although the exact site of infection by FMDV (defined here as translocation of the viral RNA across an endosomal membrane into the cytoplasm) is unknown, likely candidates are early- or recycling endosomes as viral capsid proteins accumulate in these vesicles during cell entry. However, FMDV is not thought to require trafficking to late endosomes or lysosomes for infection. The effects of expression of each dominant-negative rab GTPase on FMDV infection of IBRS2 cells are summarised in figure 8.1.

Rab5 is required for the fusion of early endocytic vesicles to form early endosomes. Expression of dominant-negative rab5 (dnrab5) was shown to inhibit both the formation of early endosomes and uptake of labelled transferrin through these compartments (figures 4.3 and 4.9). As seen in figure 8.1, expression of dnrab5 inhibited FMDV infection by ~77% when compared to wild-type rab5 (wtrab5),
Figure 8.1 Summary of effects of dnrab expression on FMDV infection

Shown is the percentage of FMDV infected cells expressing dnrab protein with the percentage of infected cells expressing the wtrab protein normalised to 100%. Error bars represent one standard deviation. The knockdown of dnrab5 compared to wtrab5 is significant at $P=0.01$ according to the student’s T-Test. The knockdown of rab7-N125I as compared to wtrab7 is significant at $P=0.001$. 

Conclusions
indicating that FMDV requires intact early endosomes for infection. This inhibition is not due to reduced receptor expression (figure 5.6) or virus binding (figure 5.8). However, delivery of FMDV to early endosomes was inhibited in dnrab5 expressing cells (figure 5.10), consistent with a depletion of early endosomes by dnrab5. Virus was seen to enter cells expressing dnrab5 but remains in small puncta at the cell periphery, suggesting that rab5 is not required for formation of incoming endocytic vesicles. Expression of constitutively active rab5 (carab5) resulted in the formation of ‘giant’ early endosomes but had little effect on FMDV infection (figure 5.1).

Rab4 is involved in the rapid recycling pathway from early endosomes back to the plasma membrane. Expression of dnrab4 had little effect on the integrity of early- or recycling endosomes (figures 4.10 and 4.12). Expression of dnrab4 had little effect on FMDV infection, indicating that the steps of the recycling pathway regulated by rab4 are not required for FMDV infection.

Rab11 is involved in the slower recycling pathway from early endosomes back to the plasma membrane via recycling endosomes. Expression of dnrab11 resulted in a change in the pattern of labelling of transferrin receptor-positive compartments (figure 4.16). In dnrab11 expressing cells, labelled transferrin was not recycled in the normal way and became trapped in compartments with a similar distribution to early endosomes (figure 4.18). These data suggest that the number of recycling endosomes may be reduced in dnrab11 expressing cells. The abundance and distribution of early endosomes was not reduced in dnrab11 expressing cells (figure 4.15). Expression of dnrab11 inhibited FMDV infection by ~35%, indicating that recycling endosomes may play a role in FMDV infection. However, the virus is less reliant on these than on early endosomes for infection. This inhibition did not appear
to be due to reduced receptor expression (figure 5.7) or virus binding to the cells (figure 5.9). It may be that some virus particles do not uncoat in early endosomes and are carried into the recycling pathway where the virus experiences a prolonged exposure to acidic pH.

Rab9 regulates trafficking from late endosomes to the Golgi. Expression of dnrab9 had little effect on the pattern of labelling for all cellular endocytic compartments investigated (figures 4.24 and 4.25). Similarly, expression of dnrab9 had little effect on FMDV infection, indicating that the virus does not require trafficking from late endosomes to the Golgi.

These results in this thesis therefore provide further evidence for FMDV cell entry occurring by clathrin-dependent endocytosis, followed by uptake into early endosomes, where the low pH in this compartment causes capsid uncoating and translocation of the viral RNA genome into the cytoplasm. The mechanism by which this translocation occurs is unknown, and would be a subject for future work.

A number of viruses require a low pH step for capsid uncoating and infection. For some viruses, early endosomes are not sufficiently acidic and they require the lower pH found in late endosomes and lysosomes (Prchla et al., 1994). There is no evidence that FMDV infection requires conditions any more acidic than those found in early endosomes, as the FMDV capsid is extremely sensitive to acid and uncoats at pH 6.5, just below neutral pH (Curry et al., 1995). The FMDV capsid proteins have not been co-localised with markers for late endosomes or lysosomes at early time points during infection (Berryman et al., 2005; O'Donnell et al., 2005). Furthermore, treatment of SW480 cells with nocodazole, a drug which disrupts the
microtubule network and prevents trafficking from early to late endosomes, has no effect on FMDV infection (Berryman et al., 2005). This was also shown in chapter three to be the case for FMDV infection of IBRS2 cells (figure 3.3). All evidence therefore suggests that FMDV does not traffic to late endosomes during cell entry.

The observations above predict that as expression of dominant-negative rab7 inhibits trafficking from early- to late endosomes and lysosomes, it would not be expected to have an effect on FMDV infection. Expression of dominant-negative rab7 was shown to inhibit trafficking of labelled LDL and lysotracker (figures 4.22 and 4.23), both of which are known to traffic from early- to late endosomes. Surprisingly, expression of the N125I version of dnrab7 inhibited FMDV infection by ~78% compared to cells expressing wtrab7, suggesting a requirement for rab7 during FMDV infection. Cellular uptake of FMDV was not inhibited in dnrab7 expressing cells when compared to non-expressing cells (figure 5.10). In addition, early endosome formation was not inhibited in cells expressing dnrab7 (figure 4.19).

These observations suggest that FMDV is internalised into early endosomes in the normal way and that rab7 could be required for FMDV infection at a stage after endocytosis, i.e. during intracellular virus replication.

In common with dnrab7-N125I, expression of dnrab7-T22N was shown to inhibit trafficking of labelled LDL and lysotracker (figures 6.1 and 6.2). However, rab7-T22N had a different effect on FMDV infection. When expressed in IBRS2 cells, rab7-N125I had little effect on infection (figure 6.3). Rab7-N125I is unable to bind GTP or GDP and therefore has a reduced capacity to bind membranes and is primarily cytosolic. Rab7-T22N is predominantly GDP bound and retains the capacity to bind membranes. It has also been shown to have increased membrane
binding during autophagy (Gutierrez et al., 2004). This suggests that a form of rab7 able to bind membranes may be required for FMDV infection.

To verify that the inhibition of FMDV infection by rab7-N125I is at a stage after endocytosis, viral RNA was electroporated into cells expressing wtrab7, rab7-T22N or rab7-N125I. This method of introducing the viral RNA into the cells bypasses endocytosis and delivers the RNA directly into the cytoplasm. Figure 6.4 shows that expression of rab7-T22N had little effect on intracellular FMDV replication, whereas the rab7-N125I inhibited by 90%. This verifies that the inhibition of FMDV infection by rab7-N125I is at a stage after endocytosis, during intracellular virus replication.

In cells infected by FMDV, extensive membrane rearrangements occur, resulting in the formation of membrane vesicles on which the viral replication complex is assembled (Monaghan et al., 2004). Studies on poliovirus replication vesicles have suggested that the double-membraned vesicles induced on infection are similar to autophagosomes (Jackson et al., 2005; Suhy et al., 2000). Autophagy is a cellular response to amino acid starvation and autophagosomes are the only cellular vesicles surrounded by a double membrane. Rab7 is required for the late maturation of autophagic vacuoles, and can be found on autophagosomes from an early stage of autophagy (Gutierrez et al., 2004; Jager et al., 2004). FMDV infection stimulates autophagy (E. Brooks, personal communication), which suggests a link between rab7 and FMDV replication. However, it must be noted that FMDV replication vesicles are predominantly single membraned (Monaghan et al., 2004).
In chapter six, experiments were carried out using bovine enterovirus (BEV). Like poliovirus, BEV is an enterovirus and induces the formation of vesicles morphologically similar to those seen in poliovirus infected cells (Monaghan et al., 2004). Expression of dnrab5 inhibited BEV infection by ~63% when compared to wtrab5 (figure 6.5). This suggests that BEV may be taken up into early endosomes during infection, although this virus is stable to low pH and does not require exposure to acid for infection. However, the requirement for rab7 appears different to that observed for FMDV. When compared to cells expressing wtrab7, expression of rab7-T22N appeared to enhance BEV infection, whereas expression of rab7-N125I had little effect. However, when compared to non-expressing cells, expression of wtrab7 and rab7-N125I appeared to inhibit infection by ~45% (figure 6.6). These differences are not due to differences in cell type (figure 6.7). It is difficult to conclude a role for rab7 during BEV infection, but further studies using rab7-specific RNA interference may help to clarify whether there is a role for rab7 in enterovirus infection.

Two forms of dominant-negative rab7 had different effects on FMDV infection. To further define the role of rab7 during FMDV infection, experiments were designed using RNA interference targeted to cellular rab7. The results presented in chapter seven show that transfection with porcine rab7-targeted siRNA in IBRS2 cells reduced both the number of FMDV infected cells (figures 7.5 and 7.6) and virus yield (figure 7.7). These experiments must be considered preliminary, as a cross-reactive antibody to rab7 was not identified and a knockdown of rab7 in IBRS2 cells could not be demonstrated. An alternative to western blotting could be using real
time PCR to quantify levels of rab7 mRNA after transfection with rab7-targeted siRNA.

The results presented in this thesis suggest a requirement for cellular rab7 during FMDV infection. The electroporation results show that the inhibition of FMDV infection by expression of rab7-N125I is at a stage after translocation of the vRNA into the cytoplasm. Stages at which the inhibition could occur include polyprotein synthesis and processing, host cell membrane rearrangements and formation of the replication complex, negative-strand vRNA synthesis, positive-strand vRNA synthesis and virus assembly. In future experiments, the effects of rab7-N125I expression or depletion of rab7 by RNAi on the different stages of the FMDV life cycle could be investigated in order to determine precisely where the inhibition occurs.

It is believed that host factors are involved in the formation of the viral replication complex (Ahlquist et al., 2003). It is possible that rab7 could be one such factor. Rab7 could be required for formation of the replication complex or to act as a tethering protein to anchor it to the membrane. In addition, rab7 could be needed to attract other cellular proteins to the replication complex. Alternatively, it may be required to maintain the integrity of the replication vesicle. Recent studies have suggested a role for ADP ribosylation factors (ARFs) in anchoring the poliovirus replication complex to the replication vesicles (Belov et al., 2005). These ARFs are thought to be the targets for brefeldin A inhibition of poliovirus infection (Morinaga et al., 1996). As FMDV infection is not affected by brefeldin A, it is tempting to speculate that rab7 has an equivalent role in FMDV infection to the ARFs in poliovirus infection.
These possibilities could be further explored in future experiments to demonstrate co-localisation of rab7 with the replication complex. This could be shown by co-localisation of rab7 with FMDV non-structural proteins or isolation of the replication membranes and western blotting for rab7. Alternatively, the replication membranes could be visualised by electron microscopy (EM) and labelled with rab7 antibodies. However, if the interaction of rab7 with the replication vesicles is transient, this may not be possible. To confirm whether the membrane binding ability of rab7 is required for infection, the membrane binding domain of rab7-T22N could be mutated to see if this protein would then be able to support infection.

The observation that a membrane bound but inactive form of rab7 (rab7-T22N) can support infection argues against a role for rab7 in the formation of replication vesicles as only an active, GTP-bound rab protein can recruit effectors and drive vesicle formation. It would be interesting to see whether the replication vesicles are formed in dnrab7 expressing cells, or cells depleted of rab7 by siRNA.

Alternatively, rab7 could be acting as an anchoring factor, stabilising the replication complex on the membrane vesicle. Demonstrating a direct interaction between rab7 and FMDV non-structural proteins could be done by FRET/FRAP in infected cells or in vitro by direct binding assays or pull-down assays.

To conclude, the results in this thesis show that FMDV enters IBRS2 cells expressing αVβ8 via clathrin-dependent endocytosis. FMDV requires low pH and intact early endosomes for infection. It may also enter recycling endosomes, but these compartments appear less important for infection. FMDV does not require intact microtubules and therefore is unlikely to traffic to late endosomes. These observations identify the early endosome as the critical compartment for FMDV
infection. Although rab7 is not required for trafficking of FMDV to late endosomes or lysosomes for infection, it appears to be required for intracellular virus replication. These observations lay the foundation for future experiments to explore the role of rab7 in intracellular FMDV replication.


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Appendix I

T-Test Calculations
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standard deviations

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variance

<p>| | | | | | |</p>
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sum of variances

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divide both by number of samples (3)

<p>| | | | | | |</p>
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</table>

add both together

288.3937

square root

16.98216

divide difference of means (6.1) by this number

0.357343

degrees of freedom = (3+3)-2

=4

from t-table not significant
expt1    expt2    expt3    expt4    mean    diff of mean
wtrab5   74.93878  81.98246  62.05449  64.26047  70.8    55.1

standard deviations
wtrab5   4.13    11.17   -8.75     6.5
dnrab5   5.61    -6.39   -2.46     -3.2

variance
wtrab5   17   125   77   43
dnrab5   32   41   6   10

sum of variances
wtrab5   261
dnrab5   89

divide both by number of samples (4)
wtrab5   65.35651
dnrab5   22.21375

add both together
87.57026

square root
9.357898

divide difference of means (55.1) by this number
5.889109

degrees of freedom = (4+4)-2
=6

from t-table significant at 0.01
### Experiment Results

<table>
<thead>
<tr>
<th></th>
<th>exp1</th>
<th>exp2</th>
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<th>mean</th>
<th>diff of mean</th>
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<td>81.98246</td>
<td>62.05449</td>
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<td>carab5</td>
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<td>81.44953</td>
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#### Standard Deviations

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#### Variance

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#### Sum of Variances

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#### Degrees of Freedom

Divide both by number of samples (3)

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</thead>
<tbody>
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<td>wtrab5</td>
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<td>carab5</td>
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Add both together

227.1729

Square root

15.07226

Divide difference of means (1.9) by this number

0.126465

Degrees of freedom = (3+3)-2

=4

From t-table, not significant
<table>
<thead>
<tr>
<th></th>
<th>expt1</th>
<th>expt2</th>
<th>expt3</th>
<th>mean</th>
<th>diff of mean</th>
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<tbody>
<tr>
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<td>71.42</td>
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standard deviations

<table>
<thead>
<tr>
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<th>dnrab7</th>
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<tbody>
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variance

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th>dnrab7</th>
<th></th>
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sum of variances

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<tr>
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<th></th>
<th>dnrab7</th>
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<tbody>
<tr>
<td></td>
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divide both by number of samples (3)

<table>
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<th>wtrab7</th>
<th></th>
<th></th>
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<tbody>
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<td>247.6249</td>
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add both together

275.3408

square root

16.5934

divide difference of means (57.6) by this number

3.471199

degrees of freedom = (3+3)-2

=4

from t-table significant at 0.05
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<tr>
<th></th>
<th>expt1</th>
<th>expt2</th>
<th>expt3</th>
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<th>diff of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>non expressing cells</td>
<td>8.5</td>
<td>9.6</td>
<td>10.1</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>dnrab9</td>
<td>9.4</td>
<td>8.1</td>
<td>7.7</td>
<td>8.4</td>
<td>1.0</td>
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**standard deviations**

<table>
<thead>
<tr>
<th></th>
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<th>dnrab9</th>
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<tbody>
<tr>
<td></td>
<td>-0.86</td>
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**variance**

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**sum of variances**

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divide both by number of samples (3)

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add both together

0.969557

square root

0.984661

divide difference of means (1.0) by this number

1.053848

degrees of freedom = (3+3)-2

= 4

from t-table not significant
<table>
<thead>
<tr>
<th></th>
<th>expt1</th>
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<th>expt3</th>
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standard deviations

<table>
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<tr>
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variance

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sum of variances

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divide both by number of samples (3)

<table>
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add both together

211.7678

square root

14.55224

divide difference of means (30.3) by this number

2.078991

degrees of freedom = (3+3)-2

= 4

from t-table not significant
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<tr>
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<th>exp2</th>
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standard deviations

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variance

<p>| | | | | | |</p>
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sum of variances

<p>| | | | | | |</p>
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divide both by number of samples (2)

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add both together

194.7562

square root

13.95551

divide difference of means (7.6) by this number

0.541825

degrees of freedom = (2+2)-2

=2

from t-table not significant
<table>
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<tr>
<th></th>
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<th>expt2</th>
<th>expt3</th>
<th>mean</th>
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**standard deviations**

<table>
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<th>rab7N125l</th>
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**variance**

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**sum of variances**

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divide both by number of samples (2/3)

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</thead>
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<td>1.40333</td>
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</tbody>
</table>

add both together

15.46256

square root

3.932246

divide difference of means (64.9) by this number

16.50498

**degrees of freedom** = (2+3)-2

=3

from t-table significant at 0.001