The Role of the Early Secretory Pathway in Foot-and-Mouth Disease Virus Replication

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Declaration

I declare that the work carried out in this thesis at the Institute for Animal Health for a research degree at Imperial College London between October 2006 and February 2011 is original. Any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text hererin and have clearly indicated by suitable citation any part of the dissertation that has already appeared in publication.

R. Midgley 08/02/2011
Abstract

Foot-and-Mouth Disease Virus (FMDV) induces rearrangements of host-cell membranes to generate vesicles that are believed to provide platforms for formation of the viral replication complex. The cellular origin of these vesicles and the properties that make them favourable for replication are poorly understood. For some Picornaviruses these vesicles are thought to derive from membranes of the secretory pathway. In this thesis, I have investigated a role for membranes of the secretory pathway in FMDV infection. Key cellular proteins involved in regulating the flow of membranes through the secretory pathway between the ER and Golgi were inhibited using expression of dominant-negative (dn) proteins and small interfering RNA (siRNA) and the effect on FMDV infection determined. Inhibition of ER export using a drug (H89) or Sar1 (the GTPase required for COPII transport vesicle formation at ER exit sites) reduced FMDV infection. In contrast, stabilisation of COPII coats, or inhibition of Arf1 or Rab proteins, that are involved in the secretory pathway after the formation of COPII vesicles, had little or no inhibitory effect on infection. Interestingly inhibition of Arf1, Rab1 or Rab2 enhanced infection. In contrast, Arf1 reduced infection by bovine enterovirus which is inhibited by Brefeldin-A, and therefore likely to be dependent on Arf1 for replication. These results show that Sar1 and/or COPII vesicle formation is necessary for FMDV infection and that inhibiting the formation of COPI coats is in some way advantageous to FMDV infection. These results suggest that FMDV targets COPII vesicles membranes before the COPII/COPI exchange and facilitates FMDV infection and that COPI components are not required.
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Table of Contents

Title page........................................................................................................................................
Abstract........................................................................................................................................... 3
Acknowledgements....................................................................................................................... 4
Table of Contents......................................................................................................................... 6
Table of Figures............................................................................................................................ 11
Abbreviations ............................................................................................................................... 16

Chapter One: Introduction ........................................................................................................ 19
  1.1 Foot-and-mouth disease virus ........................................................................................ 19
  1.2 The Piconaviruses ........................................................................................................ 21
  1.3 The FMDV virion .......................................................................................................... 23
  1.4 FMDV genome organisation ....................................................................................... 25
    1.4.1 The 5’ UTR ......................................................................................................... 25
    1.4.2 S-fragment ....................................................................................................... 25
    1.4.3 Poly (C) Tract and Pseudoknots ....................................................................... 26
    1.4.4 Cis-acting replication element (cre) ............................................................... 27
    1.4.5 Internal Ribosome Entry Site (IRES) ................................................................ 28
  1.5 FMDV polyprotein ......................................................................................................... 30
    1.5.1 Leader Protease ............................................................................................... 30
    1.5.2 The Capsid Precursor P1-2A ........................................................................... 31
    1.5.3 P2 proteins ...................................................................................................... 31
    1.5.4 P3 Proteins ...................................................................................................... 33
    1.5.5 FMDV 3’ UTR ................................................................................................. 35
  1.6 The viral life cycle ............................................................................................................. 37
    1.6.1 Attachment and Entry ....................................................................................... 37
    1.6.2 Translation ........................................................................................................ 39
    1.6.3 Replication ........................................................................................................ 42
    1.6.4 Replication Complexes .................................................................................... 45
  1.7 Particle assembly, maturation and release ..................................................................... 50
  1.8 The early secretory pathway ........................................................................................... 53
    1.8.1 COPII formation .............................................................................................. 55
    1.8.2 COPI formation .............................................................................................. 57
  1.9 Rab proteins ...................................................................................................................... 63
    1.9.1 Effector proteins .............................................................................................. 65
    1.9.2 Rab1 ................................................................................................................ 65
Chapter 2: Materials and Methods ................................................................. 74

2.1 Cell Culture ........................................................................................................ 74

2.2 Virus ..................................................................................................................... 75

2.2.1 Preparation of virus working stocks ............................................................. 75

2.2.2 Virus titration on coverslips ........................................................................ 75

2.2.3 Virus titration by plaque assay .................................................................... 76

2.3 Antibodies and Reagents .................................................................................. 76

2.3.1 Primary Antibodies .................................................................................. 76

2.3.2 Secondary Antibodies ............................................................................. 77

2.3.3 Reagents ................................................................................................ 77

2.4 Virus Infectivity assays .................................................................................... 79

2.4.1 Immunofluorescence assay .................................................................... 79

2.4.2 Infectivity assay ....................................................................................... 79

2.5 DNA Techniques ............................................................................................. 80

2.5.1 Transformation of competent cells .......................................................... 80

2.5.2 Isolation of Plasmid DNA ........................................................................ 80

2.5.3 Restriction digests ................................................................................... 81

2.5.4 DNA gel electrophoresis ......................................................................... 81

2.5.5 Nanodrop – Quantification of DNA concentration ................................... 81

2.5.6 Polymerase chain reaction ...................................................................... 81

2.5.7 DNA purification ...................................................................................... 83

2.5.8 Ligations ................................................................................................. 83

2.5.9 DNA sequencing ..................................................................................... 83

2.5.10 Transient transfection of mammalian cells with plasmid DNA ................. 84

2.6 RNA Techniques ............................................................................................ 84

2.6.1 siRNA transfection ................................................................................ 84

2.6.2 RNA isolation .......................................................................................... 85

2.6.3 RNA extraction ......................................................................................... 85

2.6.4 Reverse transcriptase reaction (RT) ....................................................... 85
2.7 Immunofluorescence ...................................................................................... 86
  2.7.1 Setting up of coverslips ........................................................................... 86
  2.7.2 Detection of intracellular antigen following cell permeabilisation ............ 86
  2.7.3 Treatment of coverslips with pharmacological inhibitors prior to infection .................................................................................................................. 87
2.8 Image Capture ............................................................................................... 87
2.9 Data Analysis ................................................................................................. 88
2.10 Transmission Electronmicroscopy (TEM) ...................................................... 88
2.11 Western Blot................................................................................................... 89

Chapter 3: Investigating the Early Secretory Pathway in FMDV Infection .......... 91
  3.1 Introduction .................................................................................................... 91
  3.2 Membrane rearrangements in FMDV infected cells ....................................... 92
  3.3 The effect of FMDV infection on membranes in the early secretory pathway 92
  3.4 The effect of Brefeldin A on membranes in the early secretory pathway ....... 94
  3.5 The effects of Brefeldin A on FMDV infection .............................................. 101
  3.6 Protein Kinase Inhibitor H89 ........................................................................ 105
    3.6.1 H89 Blocks FMDV Infection .................................................................. 105
  3.7 Endoplasmic Reticulum (ER) Stress ............................................................ 107
    3.7.1 The effect of ER stress on membranes in the early secretory pathway 107
    3.7.2 ER stress does not block FMDV infection ............................................. 110
  3.8 Discussion .................................................................................................... 114

Chapter 4: Investigating the Role of Sar1 in FMDV Infection ............................... 117
  4.1 Introduction .................................................................................................. 117
  4.2 Investigating the role of Sar1 on FMDV infection using Dominant-negative proteins ................................................................................................................... 118
    4.2.1 The effects of dominant-negative Sar1a on membranes in the early secretory pathway ............................................................................................... 118
    4.2.2 The effect of dominant-negative Sar1a on FMDV infection .................. 130
    4.2.3 The effect of dominant-negative Sar1a on BEV-1 infection .................. 140
    4.2.4 The effect of dominant-negative Sar1a on FMDV infection in MAX cells 147
  4.3 Investigating the role of Sec16 on FMDV infection using Dominant-negative proteins ................................................................................................................... 157
    4.3.1 The effects of dominant-negative Sec16 on membranes in the early secretory pathway ................................................................. 158
    4.3.2 The effect of dominant-negative Sec16 on FMDV infection ............... 158
  4.4 Investigating the role of Sar1 in FMDV infection using RNA interference .... 166
    4.4.1 shRNA .................................................................................................. 166
    4.4.2 Sar1 siRNA ........................................................................................... 172
Chapter Five: Investigating the Role of Arf1 in FMDV Infection

5.1 Introduction

5.2 Investigating the role of Arf1 on FMDV infection using Dominant-negative proteins

5.2.1 The effects of dominant-negative Arf1 on membranes in the early secretory pathway

5.2.2 The effect of dominant-negative Arf1 on FMDV infection

5.3 The effect of dominant-negative Arf1 on BEV-1 infection

5.4 The effect of dominant-negative Arf1 on FMDV infection in MAX cells

5.5 Discussion

Chapter 6: Investigating the effect of dominant-negative Rab proteins in FMDV infection

6.1 Introduction

6.2 Rab1

6.2.1 The effects of dominant-negative Rab1 on membranes in the early secretory pathway

6.2.2 The effect of dominant-negative Rab1 on FMDV infection

6.3 Rab2

6.3.1 The effects of dominant-negative Rab2 on membranes in the early secretory pathway

6.3.2 The effect of dominant-negative Rab2 on FMDV infection

6.4 Rab6

6.4.1 The effects of dominant-negative Rab6 on membranes in the early secretory pathway

6.4.2 The effect of dominant-negative Rab6 on FMDV infection

6.5 Rab34

6.5.1 The effects of dominant-negative Rab34 on membranes in the early secretory pathway

6.5.2 The effect of dominant-negative Rab34 on FMDV infection

6.6 TBC1D20

6.6.1 The effects of TBC1D20 on membranes in the early secretory pathway

6.6.2 The effect of TBC1D20 on FMDV infection

6.6.3 The effect of TBC1D20 on BEV infection

6.7 Discussion

Chapter 7: Investigating the effect of Rab RNAi on FMDV infection

7.1 Introduction

7.2 Characterisation of FMDV infection in HeLa cells
Table of Figures

| Figure 1.1 | Picornaviruses | 24 |
| Figure 1.2 | FMDV 5’ untranslated region | 29 |
| Figure 1.3 | FMDV genome organisation and polyprotein processing | 36 |
| Figure 1.4 | FMDV translation | 40 |
| Figure 1.5 | The Picornavirus replication cycle | 44 |
| Figure 1.6 | Replication complexes | 48 |
| Figure 1.7 | Assembly and release | 52 |
| Figure 1.8 | A model of transport between the endoplasmic reticulum (ER) and Golgi compartments | 59 |
| Figure 1.9 | A model for coat protein complex II (COPII)-dependent cargo selection and vesicle formation | 60 |
| Figure 3.1 | FMDV infection induces host cell membrane rearrangements in IBRS-2 cells | 93 |
| Figure 3.2 | Labelling membranes of the secretory pathway | 95 |
| Figure 3.3 | FMDV infection disrupts ER membranes in IBRS-2 cells | 96 |
| Figure 3.4 | FMDV infection disrupts ERGIC membranes in IBRS-2 cells | 97 |
| Figure 3.5 | FMDV infection disrupts Golgi membranes in IBRS-2 cells | 98 |
| Figure 3.6 | Brefeldin A treatment disrupts the Golgi and alters ER membranes in IBRS-2 cells | 100 |
| Figure 3.7 | Brefeldin A blocks BEV-1 but not FMDV infection in IBRS-2 cells | 102 |
| Figure 3.8 | The effect of Brefeldin A treatment on FMDV infection | 103 |
| Figure 3.9 | Brefeldin A treatment blocks BEV-1 infection in IBRS-2 cells | 104 |
| Figure 3.10 | H89 blocks BEV-1 and FMDV infection | 106 |
| Figure 3.11 | FMDV infection is inhibited by H89 | 108 |
| Figure 3.12 | BEV-1 infection is blocked by H89 treatment | 109 |
| Figure 3.13 | Thasparginin (TG) alters membranes in the early secretory pathway | 111 |
| Figure 3.14 | ER stress did not affect the frequency of FMDV infection in IBRS-2 cells | 112 |
| Figure 3.15 | ER stress does not inhibit FMDV infection in IBRS-2 cells | 113 |
| Figure 4.1 | Labelling membranes of the secretory pathway | 120 |
| Figure 4.2 | The effects of wt Sar1a expression on the ER and ERGIC | 121 |
| Figure 4.3 | The effects of wt Sar1a expression on the Golgi | 122 |
Figure 4.4  The effects of dn Sar1a (T39N) expression on the ER and ERGIC .................................................................124
Figure 4.5  The effects of dn Sar1a (T39N) expression on the Golgi (GM130) ................................................................................................................125
Figure 4.6  The effects of dn Sar1a (T39N) expression on the Golgi (23C) .................126
Figure 4.7  The effects of dn Sar1a (H79G) expression on the ER and ERGIC .................................................................................................127
Figure 4.8  The effects of dn Sar1a (H79G) expression on the Golgi (GM130) ................................................................................................................128
Figure 4.9  The effects of dn Sar1a (H79G) expression on the Golgi (23C) ..................129
Figure 4.10 FMDV Infection of cells expressing wt or dn Sar1a (T39N) .................133
Figure 4.11 FMDV Infection of cells expressing dn Sar1a (H79G) .........................134

Figure 4.12 The effects of dominant-negative Sar1 on FMDV infection of IBRS-2 cells .................................................................................................135
Figure 4.13 BEV-1 Infection of cells expressing wt or dn Sar1a (T39N) .................141
Figure 4.14 BEV-1 Infection of cells expressing dn Sar1a (H79G) .........................142
Figure 4.15 The effects of dominant-negative Sar1 on BEV-1 infection of IBRS-2 cells .................................................................................................143
Figure 4.16 Labelling of the Golgi in MAX cells .................................................................148
Figure 4.17 The effects of Sar1 expression on the Golgi in MAX cells .........................149
Figure 4.18 FMDV Infection of MAX cells expressing wt or dn Sar1a (T39N) ........151
Figure 4.19 FMDV Infection of MAX cells expressing dn Sar1a (H79G) .................152
Figure 4.20 The effects of dominant-negative Sar1 on FMDV infection of MAX cells .................................................................................................153
Figure 4.21 The effects of dn Sec16 C-term expression on the ER, ERGIC and Golgi .................................................................................................160
Figure 4.22 FMDV Infection of cells expressing dn Sec16 C-term ...............................161
Figure 4.23 The effects of dominant-negative Sec16 on FMDV infection of IBRS-2 cells .................................................................................................162
Figure 4.24 Knockdown of GAPDH in IBRS-2 cells by shRNA .................................169
Figure 4.25 Knockdown of Sar1 in IBRS-2 cells by shRNA .........................................170
Figure 4.26 Knockdown of Sar1a in IBRS-2 cells by siRNA ........................................174
Figure 4.27 Knockdown of Sar1b in IBRS-2 cells by siRNA ........................................175
Figure 4.28 Knockdown of Sar1a and Sar1b in IBRS-2 cells by siRNA ....................176
Figure 4.29 Optimisation of the knockdown of Sar1 in IBRS-2 cells by siRNA ........178
Figure 4.30 The effects of Sar1 knockdown on the Golgi ............................................179
Figure 4.31 Knockdown of Sar1 by siRNA induces ER swelling.......................181
Figure 4.32 The knockdown of Sar1 by siRNA does not inhibit the formation of
viral replication complexes.................................................................182
Figure 4.33 The effect of Sar1 knockdown by siRNA on FMDV and BEV-1 infection
.............................................................................................................184
Figure 4.34 The effects of Sar1 knockdown on FMDV infection of IBRS-2 cells......185
Figure 4.35 The effects of Sar1 knockdown on BEV infection of IBRS-2 cells........186
Figure 4.36 The effect of Sar1 knockdown on FMDV virus yield........................187
Figure 4.37 The effect of Sar1 knockdown on BEV virus yield...........................188
Figure 5.1 The effects of wt Arf1 expression on the ER, ERGIC and Golgi.............195
Figure 5.2 The effects of dn Arf1 (T31N) expression on the ER, ERGIC and Golgi
.............................................................................................................197
Figure 5.3 The effects of dn Arf1 (Q71L) expression on the ER, ERGIC and Golgi
.............................................................................................................198
Figure 5.4 FMDV Infection of cells expressing wt or dn Q71L Arf1......................200
Figure 5.5 FMDV Infection of cells expressing dn Arf1 T31N................................201
Figure 5.6 The effects of dominant-negative Arf1 (T31N) on FMDV infection
of IBRS-2 cells.........................................................................................202
Figure 5.7 The effects of dominant-negative Arf1 (Q71L) on FMDV infection of IBRS-2
cells...........................................................................................................207
Figure 5.8 BEV Infection of cells expressing wt or dn Arf1 (T31N)......................212
Figure 5.9 BEV Infection of cells expressing dn Arf1 (Q71L)..............................213
Figure 5.10 The effects of dominant-negative Arf1 (T31N) on BEV infection of IBRS-2
cells...........................................................................................................214
Figure 5.11 The effects of dominant-negative Arf1 (Q71L) on BEV infection of IBRS-2
cells...........................................................................................................219
Figure 5.12 The effects of Arf1 expression on the Golgi in MAX cells....................225
Figure 5.13 FMDV Infection of MAX cells expressing wt or dn Arf1 (Q71L)......226
Figure 5.14 FMDV Infection of MAX cells expressing dn Arf1 (T31N).................227
Figure 5.15 The effects of dominant-negative Arf1 on FMDV infection of MAX
cells...........................................................................................................228
Figure 6.1 The effects of Rab1 expression on the Golgi........................................237
Figure 6.2 FMDV Infection of cells expressing wt or dn Rab1a (S25N)...............239
Figure 6.3 The effects of dominant-negative Rab1 on FMDV infection of IBRS-2 cells
.............................................................................................................240
Figure 6.4 The effects of dn Rab2 expression on the Golgi.................................245
Figure 6.5 FMDV Infection of cells expressing wt or dn Rab2 (S20N)...............247
Figure 6.6  The effects of dominant-negative Rab2 on FMDV infection of IBRS-2 cells .............................................................. 249

Figure 6.7 The effects of Rab6 expression on the Golgi.................................................. 254

Figure 6.8 FMDV Infection of cells expressing wt or dn Rab6 (T27N) ...................... 255

Figure 6.9 The effects of dominant-negative Rab6 on FMDV infection of IBRS-2 cells .............................................................. 257

Figure 6.10 The effects of wt Rab34 expression on the ER, ERGIC and Golgi.............. 261

Figure 6.11 The effects of dn Rab34 expression on the ER, ERGIC and Golgi.............. 262

Figure 6.12 FMDV Infection of cells expressing wt or dn Rab34 (T66N) .................... 264

Figure 6.13 The effects of dominant-negative Rab34 on FMDV infection of IBRS-2 cells .............................................................. 265

Figure 6.14 The effects of R105A expression on the ER, ERGIC and Golgi................. 271

Figure 6.15 The effects of TBC1D20 expression on the ER, ERGIC and Golgi............ 272

Figure 6.16 FMDV Infection of cells expressing R105A or TBC1D20......................... 273

Figure 6.17 The effects of TBC1D20 on FMDV infection of IBRS-2 cells .................. 275

Figure 6.18 BEV Infection of cells expressing R105A or TBC1D20......................... 280

Figure 6.19 The effects of TBC1D20 on BEV infection of IBRS-2 cells ..................... 281

Figure 7.1  FMDV a causes cytopathic effect in HeLa cells........................................ 291

Figure 7.2  FMDV produces productive virus in HeLa cells................................. 292

Figure 7.3  Time course of FMDV infection in HeLa cells................................. 294

Figure 7.4  Membrane rearrangements in FMDV infected HeLa cells.................... 295

Figure 7.5  The detection of FMDV vRNA and GAPDH mRNA in IBRS-2 by QPCR .............................................................. 298

Figure 7.6  GAPDH levels in FMDV infected HeLa cells........................................ 302

Figure 7.7  QPCR analysis of FMDV infection HeLa cells........................................ 303

Figure 7.8  Time course of FMDV infection in IBRS-2 cells using QPCR............... 304

Figure 7.9  Time course of FMDV infection in HeLa cells using QPCR.................... 306

Figure 7.10 Knockdown of Rab5a in HeLa cells by siRNA..................................... 308

Figure 7.11. The effects of Rab siRNA on FMDV infection of HeLa cells (A)........... 310

Figure 7.12. The effects of Rab siRNA on FMDV infection of HeLa cells (B).......... 311

Figure 7.13. The effects of Rab1 siRNA on FMDV infection of HeLa cells.............. 312

Figure 7.14 The effects of Rab1 siRNA on FMDV infection (normalised data)... 314

Figure 7.15 The effect of Rab1 knockdown on FMDV virus yield........................... 315
Figure 8.1  Summary: The Role of the Early Secretory Pathway in FMDV Replication
Abbreviations

Arf          ADP-ribosylation factor
ATPase       Adenosine triphosphatase
BEV          Bovine Enterovirus
BfA          Brefeldin A
BIG          Brefeldin-A inhibited guanine nucleotide exchange factor
Ca2+         Calcium
CFP          Cyan fluorescent protein
cre          cis-acting replication element
CPE          Cytopathic effect
COP          Coatomer complex protein
Dn           Dominant-negative
DNA          Deoxyribonucleic acid
EMCV         Encephalomyocarditis virus
ER           Endoplasmic reticulum
ERAV         Equine Rhinitis A virus
ERES         Endoplasmic reticulum exit sites
ERGIC        Endoplasmic reticulum-Golgi intermediate
EV           Enterovirus
FMD           Foot-and-mouth disease
FMDV         Foot-and-mouth disease virus
GAP          GTPase activating protein
GBF1         Golgi-specific Brefeldin-A resistance factor 1
GDI          GDP disassociation inhibitor
GDF          GDI displacement factor
GDP          Guanosine diphosphate
GEF          Guanine nucleotide exchange factor
GFP          Green fluorescent protein
GM130        Golgi matrix protein GM130
<table>
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<tr>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
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<tr>
<td>HPEV</td>
<td>Human parechovirus</td>
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<td>hr</td>
<td>Hours</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HRV</td>
<td>Human Rhinovirus</td>
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<td>IBRS-2</td>
<td>Porcine kidney cells</td>
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<td>ICTV</td>
<td>International committee of the taxonomy of viruses</td>
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<td>Immunofluorescence confocal microscopy</td>
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<tr>
<td>ORF</td>
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<tr>
<td>PABP</td>
<td>Poly (A) binding protein</td>
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<td>PCBP</td>
<td>Poly (C) binding protein</td>
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<td>PDI</td>
<td>Protein disulfide isomerase</td>
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<td>PfM</td>
<td>Paraformaldehyde</td>
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<td>Ribonucleic acid interference</td>
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<td>Secretion associated ras-related</td>
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<td>Short interfering ribonucleic acid</td>
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<td>SNARE</td>
<td>Soluble NSF attachment protein receptor</td>
</tr>
<tr>
<td>TBC</td>
<td>Tre-2/Bub2/Cdc2 domain</td>
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<tr>
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<td>TGN</td>
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<td>UTR</td>
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<td>VTC</td>
<td>Vesicular-tubule clusters</td>
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Chapter One: Introduction

1.1 Foot-and-mouth disease virus

Foot-and-mouth disease virus (FMDV) is the aetiological agent of a highly infectious disease (FMD) of cloven hoofed animals, including cattle, sheep, goats and pigs, and over 70 other species of wildlife. The OIE (Office International des Epizooties) recognise FMDV as an A list disease of major socio-economic importance for the livestock industry, with outbreaks resulting in the implementation of drastic control measures to limit economic damage (OIE Animal health code 2010).

FMDV exists as seven serotypes, known as type A, O, C, South African Territories (SAT) 1, SAT2 and SAT3 and Asia 1. Types A and O are the most prevalent worldwide, whereas there have only been sporadic outbreaks of serotype C in South America, East Africa and Pakistan in recent years (OIE 2008). SAT-1, -2 and -3 are usually found in Africa and members of the Asia 1 are usually restricted to Asia. FMD is endemic in parts of Africa, Asia and South America, while North America, Australia and New Zealand are considered FMD free countries. The majority of Europe is also considered FMD free, including the UK, apart from periodic outbreaks mainly from the Middle East.

FMDV infection is initiated through the upper respiratory tract or lungs, causing infection of the epithelium (Hyslop, 1965; Sellars, 1971) and viraemia. The incubation period can be as little as 2-3 days or longer (up to 2 weeks) (Garland, 1990). Infection is characterised by several vesicular lesions on the coronary band of the hooves and the mucosa of the mouth including the tongue and palate. The disease can vary in severity, from causing death in neonates to
the full recovery of adults mainly due to a neutralising antibody response from 4-5 days peaking at 28 days (Sutmoller and McVicar, 1976).

FMD can be spread via air borne transmission (Donaldson et al., 1987) as infected animals excrete large amounts of virus, especially pigs (Alexandersen and Donaldson, 2002). In addition FMDV can survive for long periods of time at a neutral pH, in low temperatures and may potentially be protected by soil, however, at pH 6.5 and below the virus is destroyed. Infection of cattle, sheep and buffalo, but not pigs, can result in a carrier state where virus can still be isolated from the oropharynx for up to 4-5 months (Sutmoller et al., 1968)

FMD can be controlled by both slaughter and the restriction of animal movements of infected animals, as seen in the 2001 outbreak in the UK, or by vaccination. Current diagnostic techniques include the detection of serum antibodies and virus antigen by ELISA, the growth of virus on primary cell culture and RT-PCR of the highly variable P1 region of the viral genome, which is responsible for serotype variation.

The currently available vaccines are inactivated virus preparations in a semi-purified state. The VP1 protein (VP1 2 and 3) in the vaccine is the immunological component which confers an active immunity. Vaccines can include one or more serotype, but the strain used to vaccinate the animal must be antigenically matched to the current circulating strain that is causing the outbreak, as there is little or no cross-protection between or within serotypes. There are however limitations to the use of vaccines in an outbreak due to a delay between vaccination and protection of up to 14 days, and immunity has only been reported to last for up to 12 months (Cox, 2000). There is also a
reluctance to vaccinate in FMD free countries due to OIE imposing trade embargoes due to the seroconversion of vaccinated animals (OIE Animal Health Code). Currently vaccination does not provide sterile immunity or prevent vaccinated animals from becoming re-infected, in addition clinical signs are masked and current serological tests are unable to distinguish between vaccinated and naturally infected animals. Therefore vaccination is an unpopular choice for the control of outbreaks in disease free countries.

1.2  The Picornaviruses

Picornaviruses are small, non-enveloped RNA viruses that include a wide range of human and animal pathogens e.g. Poliovirus, Human Rhinoviruses, Hepatitis A virus, Swine vesicular disease virus and FMDV. The *Picornaviridae* family comprises of twelve genera (ICTV 2010) which are further divided into one or more virus species that may contain several serotypes. The current taxonomic structure of the Picornavirus family is shown in figure 1.1 (Knowles N. J 2010, private communication). FMDV is the prototype member of the genus *Aphthovirus*, which also includes Equine Rhinitis A virus (ERAV). As previously mentioned (see section 1.1) FMDV consists of seven serotypes which are antigenically distinct from one another and consist of a number of subtypes, (Domingo et al., 2003; Lea et al., 1994).

All Picornaviruses contain a positive-sense single-stranded RNA genome surrounded by a protein capsid. For most picornaviruses, the capsid comprises of 60 copies each of four structural proteins VP1-VP4 whereas for others (e.g. parechoviruses) VP0 is not cleaved into VP2 and VP4. The genomes are approximately 8kb long and share an organisational similarity to eukaryotic cellular mRNA. Like cellular mRNA the viral genome consists of a single open
reading frame (ORF) followed by a 3’ untranslated region (UTR) and a poly (A) tail. However, the viral genome differs from cellular mRNA in that it possesses an extended 5’ UTR, and a 5’ cap structure (normally used to initiate cellular RNA translation) is absent from the 5’ terminus of the genome. In its place is a short virus-encoded peptide VPg (also known as 3B), which is covalently attached to the 5’ end of the genomic RNA. VPg is rapidly lost from the RNA inside the host cell, which exposes a free 5’ end. The open reading frame within the genomic RNA encodes for a polyprotein, which is proteolytically cleaved during synthesis by viral encoded proteases to produce the viral proteins required for virion assembly and genome replication.

None of the viral proteins are required to initiate the infectious cycle, thus picornavirus RNA is infectious when transfected into cells (Belsham and Bostock, 1988). The viral capsid, serves to protect the genome and ensure its delivery into the cell cytoplasm where viral replication occurs. The viral non structural proteins play an important role in genome replication. The first phase of intracellular replication is translation of viral RNA to generate the viral proteins within the cell. Picornaviruses shut down host-cell translation to allow the production of viral proteins to take precedence (see section 1.6.2). The IRES located in the 5’ UTR is the site from which the cap-independent initiation of viral protein synthesis occurs.

Viral RNA is synthesised by the RNA-dependant-RNA-polymerase (3Dpol); additional viral proteins are also required to initiate this process (see section 1.6.3). VPg acts as a primer to initiate RNA synthesis within a membrane associated complex (replication complex [RC]) that contains a number of additional viral proteins (section 1.6.4). Replication takes place on cell membranes after the initial period of translation. A switch in function of the
viral RNA from translation to genome replication occurs. This switch is required as translation of the viral RNA is not compatible with the movement of RNA polymerase from the 3’ end to the 5’ terminus (Gamarnik and Andino, 1998). Replication occurs by the synthesis of negative-strands from the input positive-strand templates. The negative strands are then used as a template to produce multiple positive-sense infectious RNA’s which may be used for production of more viral protein or the packaging into progeny virion particles.

1.3 The FMDV virion

The FMDV capsid is an icosahedral structure, ~25nm in diameter and composed of 60 copies of each of the structural proteins VP1-4. The capsid proteins self assemble from a monomeric protein precursor (one copy each of VP0, VP1 and VP3) which assemble to form intermediate pentamers (Abrams et al., 1995). Twelve pentamers assemble into an icosahedral capsid that encapsidates the vRNA (Grubman et al., 1985; Guttman and Baltimore, 1977) (see section 1.7). Capsid proteins VP1-3 fold into a well characterised 8-stranded β-barrel and form the outer capsid structure (Acharya et al., 1989) whereas VP4 is located inside the capsid with the vRNA. The strands of the β-barrels of VP1-3 are connected by loops which form on the outer surface of the capsid. One such loop (the GH loop of VP1) creates a major antigenic site and contains the Arg-Gly-Asp (RGD) motif required for integrin cell receptor attachment (Jackson et al., 2003).
Figure 1.1 Unrooted Neighbour-joining tree of the Picornaviridae based on a comparison of the P1 capsid region. The 12 currently recognised genera are shown in italics.
The FMDV capsid is different from other picornavirus as it possesses channels at the five-fold axis which allow the entry of small molecules such as CsCl which results in the particle having a high buoyancy density compared to other picornaviruses (Acharya et al., 1989; Jackson et al., 2003). In addition VP1 and VP2 are significantly smaller in FMDV which results in a thinner protein shell, creating a smooth outer surface that lacks canyons or pits (Belnap et al., 2000; Hogle et al., 1985; Kolatkar et al., 1999; Luo et al., 1987; Muckelbauer et al., 1995; Rossmann et al., 1985; Xiao et al., 2001). The capsid dissociates at a low pH (below 6.5) into 12s pentamers due to protonation of His residues at the pentamer/pentamer interfaces (Curry et al., 1995; Ellard et al., 1999).

1.4 FMDV genome organisation

1.4.1 The 5’ UTR

The 5’ UTR is required for both the initiation of viral protein synthesis and viral RNA replication. The FMDV 5’ UTR (1300nt) (Forss et al., 1984) is longer than the majority of other picornaviruses and has five distinct regions including the S-fragment, a poly (C) tract, pseudoknots, a \textit{cis}-acting replication element (\textit{cre}) and an internal ribosome entry site (IRES) (see Figure 1.2)

1.4.2 S-fragment

At the 5’ end of the 5’ UTR of FMDV RNA is a 360 nucleotide sequence termed the S-fragment that has been predicted to form a hairpin loop structure (Escarmis et al., 1992). This structure is believed to protect the genome from exonuclease digestion following the removal of VPg (Grubman and Bachrach, 1979). A similar structure is located at the 5’ end of the poliovirus (PV) genomic RNA (a cloverleaf structure of 80 nt) which has been reported to have a
significant effect on the viral RNA stability by protecting the termini of the viral RNA from degradation (Murray et al., 2001). The PV cloverleaf is thought to interact with both cellular and viral proteins (Parsley et al., 1997) and is required for viral genome replication. The cloverleaf is also thought to play a role in the switch from viral protein synthesis to genome replication (Gamarnik and Andino, 1998) and for circularisation of the viral RNA during replication (Herold and Andino, 2001). Several studies have shown that cellular Poly (C) Binding Protein (PCBP) can, along with other viral and cellular proteins bind the 5’ cloverleaf structure of PV (Gamarnik and Andino, 1997). Recent, evidence indicates that PCBP could play a role in bridging the 5’ and 3’ ends of the poliovirus genome (Barton et al., 2001; Herold and Andino, 2001). Although the function of the S-fragment remains largely unknown it is possible it may play a similar role to the PV cloverleaf in FMDV RNA replication.

1.4.3 Poly (C) Tract and Pseudoknots

Located at the 3’ side of the S-fragment within the FMDV 5’ UTR is a long stretch of C nucleotides (90%), termed the poly (C) tract. The poly C tract varies considerably in length between strains of FMDV, ranging from 80nt-120nt. Laboratory strains appear to contain shorter poly(C) tracts than field viruses, and viruses with fewer than 6 C nucleotides are infectious in cell culture (Harris and Brown, 1977). Retention of the poly (C) tract within the viral RNA is under strong selection pressure as demonstrated by Rieder et al (1993); they produced recombinant FMDV containing 6 C residues. Passage of this virus in cell culture resulted in selection of viruses with poly C tracts up-to 80nt in length thought to be due to recombination events. In comparison, recombinant viruses with just two C residues in the poly (C) tract did not alter in length. The shorter poly C tract still produced viable virus but grew at a reduced titre when
compared to the recombinants with longer poly C tracts, however this virus displayed virulence in mice models equal to that of wild-type virus. This suggested that the poly C tract does not have a role in virulence, but maybe necessary for the virus to grow to high titres (Rieder et al., 1993). In addition, it is possible the FMDV poly (C) tract could be involved in circularisation of the genome by interacting with PCBP.

On the 3’ side of the poly (C) tract, before the IRES in FMDV RNA, is a stretch of 250nts that are predicted to contain multiple pseudoknots. The function of these pseudoknots is unknown, but they may work in conjunction with the poly C tract.

1.4.4 Cis-acting replication element (cre)

Recent studies have shown that a short sequence within the genome, known as the cis-acting replication element, or cre, is vital for viral RNA replication. A cre was first discovered in the P1-coding sequence of Human Rhinovirus (HRV-14) and was shown to be essential for vRNA replication (McKnight and Lemon, 1996). Further study showed that the cre is a stem-loop structure containing an AAACA motif in the loop region. Similar cre structures have since been identified in several other picornaviruses, including FMDV (Gerber et al., 2001; Goodfellow et al., 2000; Lobert et al., 1999; Mason et al., 2002). The cre was found to be position independent and is located at different positions along the genome of different Picornaviruses. FMDV contains a cre in the 5’ UTR upstream of the IRES (Mason et al., 2002) whereas in other picornaviruses it can be found in the protein coding region of the genome. Alteration of the stem loop AAACA motif in PV has been found to inhibit genome replication, but did not seem to have an effect on translation of the RNA (Mason et al., 2002).
The cre has been shown to act as a template to allow the viral RNA polymerase to uridylylate the VPg in vitro (Paul et al., 2000) producing a VPgpUpU. The uridylylation of VPg is an essential step in replication, as the VPgpUpU acts as a primer to initiate viral replication (Nayak et al., 2005; Nayak et al., 2006). It has been suggested that negative strand synthesis is initiated with the uridylylation of the VPg at the 3’poly (A) tail in vRNA and positive strand synthesis is initiated by VPgpUpU synthesised on the cre hairpin (Morasco et al., 2003). However, it has also been suggested that VPgpUpU and the 3D polymerase (3Dpol) are translocated from the cre templates to the 3’ terminus of negative- and positive-strand RNA templates to prime the initiation of RNA synthesis (Goodfellow et al., 2003b; Lyons et al., 2001; Murray and Barton, 2003; van Ooij et al., 2006).

### 1.4.5 Internal Ribosome Entry Site (IRES)

A sequence present at the 3’ end of the FMDV 5’ UTR is known as the IRES and is responsible for initiating viral protein synthesis (Belsham et al., 1990; Martinez-Salas et al., 1993) (see section 1.6.2). The absence of a 7-methyl-G cap at the 5’ end of the viral genome prevents normal cap-dependant translation from occurring. To overcome this, translation is initiated at the IRES, which involves the direct recruitment of the translational machinery (Belsham and Brangwyn, 1990; Kuhn et al., 1990). The advantage of this process is that IRES dependent translation initiation can overcome stressful conditions that would otherwise inhibit cap-dependent translation initiation (Hellen and Sarnow, 2001). The FMDV IRES is a highly ordered region of secondary structure consisting of about 460nt, and is organised into five structural domains. The IRES of both aphthoviruses and cardioviruses share a common secondary
Figure 1.2 FMDV 5' untranslated region

The 5' UTR of FMDV contains the S-fragment, poly(C) tract, pseudoknots (PKs), cre with conserved loop labelled, IRES domains 2, 3, 4 and 5 and poly-pyrimidine tract leading to two stop codons (Mason et al., 2003)
structure and are classified as a type II IRES (Pilipenko et al., 1989) (see Figure 1.2). FMDV translation is aided by a number of cellular trans-acting factors binding to the IRES, such as the polypyrimidine tract-binding protein (PTB) and the proliferation associated factor ITAF45, for the formation of a 48s complex \textit{in vitro} (Pilipenko et al., 2000). The main binding site for PTB is near the 5' end of the IRES which also interacts with the 3' sequence (Luz and Beck, 1991). The FMDV IRES seems to form a modular organisation in which the different domains possess a distinct function but do not act independently.

1.5 FMDV polyprotein

The viral proteins are synthesised as a single polyprotein. The polyprotein is co-translationally cleaved during viral protein synthesis by viral encoded proteases to produce a number of precursors and mature proteins which have important functions in virus replication. In total, 15 different mature proteins including two forms of the leader (L) protein, and three different versions of VPg are produced (see Figure 1.3).

1.5.1 Leader Protease

Two conserved AUG codons (84nt apart) are present within the viral genome (Sangar et al., 1987). Both codons are preceded by polypyrimidine tracts, which is a common characteristic of the 3' end of picornavirus IRES (Meerovitch et al., 1993), and both are used as initiation sites. Therefore the FMDV genome encodes two separate forms of the Leader (L) protein, Lab and Lb that vary only in their N-termini. The L protein (Hinton et al., 2002) is a papain-like cysteine protease. Both forms of the FMDV L protein cleave the L/P1 junction resulting in its detachment (Medina et al., 1993). FMDV Lab and Lb are also known to cleave the eIF4G translation initiation factors (Belsham et
al., 2000) and thereby inhibit cap-dependant translation; consequently there is a promotion of viral protein synthesis and major loss of host cell protein synthesis. One advantage to inhibiting host cell protein synthesis is the reduced ability of the cell to mount an antiviral response such as the interferon response (Chinsangaram et al., 1999).

### 1.5.2 The Capsid Precursor P1-2A

The removal of the L protease from the N-terminus of the polyprotein exposes residues on the N-terminus of VP4, which are recognised by the cellular myristylation machinery. Myristylation is necessary for capsid assembly and is thought to play a key role in vRNA membrane penetration during cell-entry (Abrams et al., 1995; Marc et al., 1991). The capsid proteins are initially separated from the non-structural proteins by ‘cleavage’ after the 2A protein. The FMDV 2A protein itself does not possess any protease activity, ‘cleavage’ occurs as a co-translation event. It is believed that 2A functions by preventing the synthesis of a peptide bond at the 2A/2B junction (Donnelly et al., 2001), releasing the P1 precursor from the polyprotein. In contrast, PV 2A protein possesses its own protease activity, and is known to cleave host cell translation initiation factors including EIF4G (Lloyd et al., 2006). The P1-2A precursor is then processed by viral protease 3C into 1AB (VP0), 1C (VP3) and 1D (VP1). The cleavage of VP0 to VP4 and VP2 occurs when the RNA genome becomes encapsidated.

### 1.5.3 P2 proteins

The other P2 proteins are 2B and 2C and are released from the P3 proteins as the precursor 2BC by the action of 3C. In FMDV infected cells, the 3C protease rapidly cleaves the 2BC precursor to produce 2B and 2C. Studies have identified that 2B contains hydrophobic regions (van Kuppeveld et al.,
1996) and 2B has been observed to co-localise with ER membranes (Moffat et al., 2005). However the role of 2B in replication has not been determined. For PV and Coxsackievirus, 2B is known to form dimers and tetramers which are thought to result in the formation of viroporins. Viroporins are transmembrane proteins that form pores and alter membrane permeability to ions (Sandoval and Carrasco, 1997). For Coxsackievirus B3, 2B is reported to decrease intracellular Ca2+ signalling between cellular organelles and cause an imbalance in Ca2+ levels resulting in the suppression of the cells apoptotic response (Campanella et al., 2004).

The 2C protein is one of the most conserved in picornaviruses and all 2C proteins are predicted to contain a helicase binding motif (Baltera and Tershak, 1989; Klein et al., 2000; Pfister and Wimmer, 1999) which suggests RNA helicase activity. A recent report has demonstrated that FMDV 2C possesses ATPase and RNA binding activity (Sweeney et al., 2010). An amphipathic helix present in the N-terminus of the FMDV 2C protein could provide a membrane anchor, as has been shown for other picornaviruses (Echeverri et al., 1998; Echeverri and Dasgupta, 1995; Kusov et al., 1998; Murray et al., 2009). The 2C of other picornaviruses also bind membranes and have been reported to induce membrane rearrangements and formation of the viral replication complex (RC) (Bienz et al., 1990; Tesar et al., 1989). It has also been reported that picornavirus 2C proteins determine the viral RNAs sensitivity to the replication inhibitor guanidine, although different strains of FMDV display varying sensitivities to this inhibitor (Saunders and King, 1982). PV 2C is thought to bind to the 3’ end of negative strand RNA intermediates and is crucial for vRNA replication (Goodfellow et al., 2003a). Recently, a direct interaction between PV
2C and the capsid protein VP3 suggest 2C plays a role in the formation of PV particles and vRNA encapsidation (Liu et al., 2010).

Recent studies carried out on 2BC protein activity suggest that FMDV 2BC binds to membranes and is able to block the secretory pathway within infected cells, preventing endoplasmic- reticulum-to-Golgi transport (Moffat et al., 2005). Further investigation revealed that the FMDV block in secretion is dependent on both components (2B and 2C), with 2C determining the site of the block (Moffat et al., 2007).

1.5.4 P3 Proteins

In FMDV the P3 region consists of the 3A protein, three tandem copies of 3B, the 3C protease and the 3D RNA-independent RNA-polymerase. The FMDV P3 precursor is also processed by 3C protease. The 3A protein is believed to provide an anchor for attachment to cell membranes at the localisation of RNA replication and is thought to deliver 3B to the RNA replication sites as 3ABBB in the case of FMDV (Belsham, 2005). FMDV 3A also localises to and binds membranes in infected cells and is thought to form part of the replication complex. PV 3A blocks protein secretion, however there is no evidence FMDV 3A blocks membrane traffic or plays a direct role in membrane rearrangements (Moffat et al., 2005). 3B is uridylylated and serves as a primer for synthesis of both positive and negative sense RNA (see section 1.4.4). Recently, it has been shown that neither mutation nor deletion of any one of FMDV’s 3B proteins affects virus replication, or the virus’s ability to infect cattle, suggesting the three 3B proteins of FMDV are functionally interchangeable (Pacheco et al., 2010).
1.5.4.1 3C protease

In FMDV the 3C protease is responsible for the majority of the polyprotein processing. The FMDV 3C functions alone, unlike that of PV which requires the precursor 3CD protease for efficient processing activity of the capsid (Ypma-Wong et al., 1988). The 3C protein is a trypsin-like serine protease (Grubman et al., 1995) and not only cleaves the viral polyprotein but also various cellular proteins. It has been shown that H3 histone (Falk et al., 1990), and the translation initiation factors eIF4A and eIF4G1 are cleaved in cells infected with FMDV by 3C (Belsham et al., 2000). By cleaving eIF4G1, FMDV 3C creates a form of protein that supports IRES function resulting in efficient viral protein synthesis (Belsham et al., 2000).

Nayak et al (Nayak et al., 2006) demonstrated the uridylylation of FMDV VPg could be achieved in vitro with only VPg (3B), 3Dpol, 3C and an RNA template containing the cre, and there was no requirement for the precursor 3CD that had previous been demonstrated as necessary for PV uridylylation. They also identified residues within the 3C protein that interact with the cre and are essential for VPg uridylylation.

1.5.4.2 3D RNA Polymerase

The FMDV 3D protein is a RNA-dependent-RNA-polymerase and is required to produce both positive- and negative-sense viral RNA. An excess of positive sense strands accumulate within infected cells in comparison to negative sense strands. To achieve this, it is presumed that the RNA polymerase differentially identifies the negative sense template over the positive sense template. The 3’ terminus of the positive-sense RNA contains a poly (A) tail while the negative RNA ends with an anti-sense S-fragment. These are significantly different in sequence from each other. Investigation of PV initiation
of negative-strand synthesis produced evidence of a Poly (A) binding protein (PABP)-3CD complex and a PCBP-3CD complex that interacted with each other to create a circular RNP complex (Barton et al., 2001; Herold and Andino, 2001). The 3’ UTR of FMDV has two stem loops which are thought to interact with the S-fragment and the IRES, which infers that the 5’ and 3’ UTRs could be involved in similar process (Serrano et al., 2006).

In the case of FMDV, the 3CD intermediate does not possess RNA polymerase activity. In PV the 3CD binds to the 5’ cloverleaf structure on the PV genome (Gamarnik and Andino, 1998) and is required in the cre-dependant VPg uridylylation assay (Paul et al., 2000). Currently, no specific function has been identified for the FMDV 3CD protein as 3C alone is sufficient for polyprotein processing.

1.5.5 FMDV 3’ UTR

The FMDV 3’ UTR consists of two components: a 100nt heterogeneous sequence that is predicted to fold into a stem loop structure (Pilipenko et al., 1992) and a poly (A) tail. The poly (A) tail is part of the viral genome, which is different to cellular mRNAs where the polyA tail is added post-transcriptionally (Dorsch-Hasler et al., 1975). Little is known about the FMDV 3’ UTR, although it has been shown to be required for infection (Saiz et al., 2001) and stimulates translation by the IRES (Lopez de Quinto et al., 2002). Evidence also suggests that RNA-RNA interactions occur between the 5’ and 3’ UTRs of the genome and that the ends of the genome are bridged via RNA-protein interactions. This circularisation of the genome may be required to maximise virus replication. In respect to this, the poly-A tract would be expected to bind PABP to form the bridge between the 5’ and 3’ ends in the presence of other viral/host proteins (Barton et al., 2001; Herold and Andino, 2001). The terminal A residues could
Figure 1.3 FMDV genome organisation and Polyprotein processing

Translation is initiated via the IRES to produce the FMDV polyprotein that is co-translationally cleaved by virus encoded proteases into structural and non-structural proteins (P1, P2 and P3). These are further processed to produce a number of precursors and mature proteins which have important functions in virus replication. In total, 15 different mature proteins are produced. Thick lines below the genome show partial cleavage products. Open boxes indicate protein encoding regions, lines indicate RNA structure (Mason et al., 2003).
also provide a template for the VPg-pUpU primer to allow negative strand synthesis (Saiz et al., 2001).

1.6 The viral life cycle

(See Figure 1.5)

1.6.1 Attachment and Entry

Picornaviruses employ a variety of strategies to enter cells. For many Picornaviruses, entry requires a major conformational change to the capsid to form an A (altered) particle, as a prerequisite for releasing their viral RNA. For many viruses e.g. Enteroviruses and several major-receptor group human rhinoviruses (HRV), this structural transformation is mediated by binding of specific cellular receptors into the viral canyon (Arita et al., 1998; He et al., 2000; Powell et al., 1997). As mentioned previously, (in sections 1.3 and 1.5.2) there is no conclusive evidence of A particle formation of the FMDV capsid, and FMDV uncoating is believed to proceed straight to 12S pentamer subunits, RNA and VP4 (Baxt and Bachrach, 1980, 1982; Brown and Cartwright, 1961; Curry et al., 1996). FMDV can use several αv integrins (αvβ1, αvβ3, αvβ6, and αvβ8) as receptors to gain entry into cells (Berinstein et al., 1995; Jackson et al., 2004; Jackson et al., 2002; Jackson et al., 2000) The integrin of primary interest is αvβ6, as this integrin has been shown to be expressed on the epithelial cells targeted by FMDV in cattle, and to bind to the RGD motif on the GH loop of VP1 (see section 1.3) with a higher affinity than the other integrin receptors (Burman et al., 2006). The integrin-binding loop of FMDV forms a stable, EDTA-resistant complex with αvβ6 (Dicara et al., 2008) which is believed to trigger internalisation of the virus-integrin complex into the cell.
Berryman et al (Berryman et al., 2005) observed that the virus co-localised with both early- and recycling-endosomes, but not lysosomes, upon entry. In addition, infection was not affected by nocodazole, a reagent that prevents vesicular trafficking between early- and late-endosomes. This was consistent with a study that found FMDV infection levels were significantly reduced in cells expressing dominant-negative mutants to Rab5, a regulator of endosomal traffic (Johns et al., 2009). This evidence combined with the finding of the presence of the integrin receptor αvβ6 in both early and recycling endosomes, shows that FMDV enter cells via clathrin-dependant endocytosis (Berryman et al., 2005; O'Donnell et al., 2005).

It has been observed that tissue culture adaptation of some serotypes of FMDV results in the selection of viruses that bind heparan sulphate and that are consequently attenuated in the host (Sa-Carvalho et al., 1997). The HS-binding FMDV was also found to be taken into the cell by early endosomes, although evidence suggests this is via a caveola-mediated pathway (O'Donnell et al., 2008), suggesting entry of FMDV into cells is dependent on the viral receptor.

Once within endosomes, the prevailing low pH triggers capsid disassembly and the translocation of the viral RNA across the endosomal membrane into the cytosol. To date, the mechanisms of release of the vRNA into the cytoplasm still remain largely unknown. A number of studies have looked at the release of vRNA in Human Rhinoviruses (HRV). There are over 100 serotypes of HRV which are grouped according to their receptor usage (Uncapher et al., 1991). HRV-2, a minor group HRV that uses the low-density lipoprotein receptor [LDLR] as a receptor (Hofer et al., 2004; Marlovits et al., 1998), has been found to have the ability to form a pore in the endosomal membrane through which vRNA could potentially be released (Danthi et al.,
2003; Prchla et al., 1995). In contrast, HRV-14, a major group HRV, which uses the intercellular adhesion molecule 1 [ICAM] as a receptor (Uncapher et al., 1991; Tomassini et al., 1989) was shown to release subviral particles into the cytosol by the total disruption of the endosomal membrane, in a similar manner to Adenoviruses (Prchla et al., 1995). For PV the capsid proteins VP1 and VP4 have been implicated in endosomal penetration. VP4 and the N-termini of VP1 become exposed on A-particle formation. In PV, this rearrangement creates 135S particles that interact with membranes via the hydrophobic N-terminals of VP1, which are able to form ion channels within lipid bilayers (Danthi et al., 2003). It has also been demonstrated that the VP4 of PV is associated with cellular (Danthi et al., 2003) and liposomal membranes (Tuthill et al., 2006) during the initial stages of infection. Mutants lacking VP4 sequences prevent or alter ion channel formation which results in the delay or prevention of the release of the viral genome into the cytosol (Danthi et al., 2003; Knipe et al., 1997). As Aphthoviruses do not form an A particle it remains unknown how these viruses interact with cellular membranes.

1.6.2 Translation

Once within the cytosol, the VPg cap is removed from the 5’ end of the viral genome by cellular host factors and the intracellular phase of replication is initiated. The vRNA functions sequentially as a template for the synthesis of the viral proteins (translation) and then of complementary negative-strand genome copies. The negative strands are then used as a template for the synthesis of new progeny positive-sense genomes (genome replication).

Positive-stranded RNA is translated directly by the cells translation machinery. On infection, most picornaviruses cause the shut-down of host cell translation to allow the production of viral proteins to take precedence. For
Initiation factors are recruited near the IRES 3’ end to guide the ribosome downstream of the IRES. Cellular translation factors including eIF4G, B and A bind to 3’ region of the IRES. The small ribosomal subunit binds to the complex at the IRES. The eIF4G complex is believed to bind with the ribosome bound eIF3 and eIF4B and the 60s ribosomal unit is recruited initiating translation of the viral genome (Saleh et al., 2001)
FMDV this is achieved through the cleavage of the cellular translation factor eIF4G by the viral proteases L (Leader) and 3C (see sections 1.4 and 1.5). Cellular mRNA translation requires a 7-methyl guanine (m7G) 5’ cap which interacts with an intact eIF4F complex for translation initiation (Shatkin, 1976). The eIF4F complex consists of 3 proteins: eIF4E which binds to the 5’ cap, eIF4A which has helicase activity and binds to the mRNA and eIF4G which acts as a bridging protein between the two components (Gingras et al., 1999). The complex also recruits eIF3, which binds to the 40S ribosomal subunit and a tRNA that binds to the complex via eIF2. This creates a 48S pre-initiation complex which acts as a bridge between the mRNA and the 40s ribosomal subunit. The complex scans along mRNA until it reaches an appropriate AUG codon that can be used for the initiation of translation (Kozak, 1989). Once initiated, the 60S ribosomal subunit joins to create the 80S complex, the initiation factors disassociate and translation can begin (Hershey, 2000). In contrast, FMDV translation only requires the L generated C-terminal region of eIF4G which interacts with 40S ribosomal subunit-bound proteins EIF4A and eIF3 (Lopez de Quinto and Martinez-Salas, 2000; Saleh et al., 2001). This complex along with eIF4B, eIF3 and eIF2 bind directly to the FMDV IRES which is responsible for internally initiating the translation of FMDV RNA (Belsham et al., 1990; Martinez-Salas et al., 1993). IRES activity is also modulated by other cellular RNA-binding proteins, such as the polypyrimidine tract-binding protein (PTBP) (Lopez de Quinto and Martinez-Salas, 2000; Meyer et al., 1995; Ochs et al., 1999; Rust et al., 1999) and ITAF45. Both of these proteins are required for the formation of the 48S initiation complex (Martinez-Salas et al., 2001; Pilipenko et al., 1992). Cellular PCBP, is also required for PV RNA translation, however this has not been shown to play a role in FMDV translation.
Nonetheless, the poly (C) tract upstream of the IRES may suggest it plays a part in FMDV translation/replication. PCBP in PV is thought to aid the circularisation of the genome to switch from translation to replication (Barton et al., 2001; Herold and Andino, 2001). The 3’ end of the FMDV genome may also be required for FMDV translation, as deletion of the poly(A) alone or both the 3’ stem loop and poly(A) resulted in non-infectious FMDV RNA with a lower translation in vitro (Lopez de Quinto et al., 2002) (see Figure 1.4).

1.6.3 Replication

FMDV viral RNA replicates with a high efficiency in susceptible cells and a large amount of full-length viral RNA is produced within 4-6 hours post infection. An excess of positive strand RNA is produced in infected cells when compared to negative strands. Viral RNA is synthesised by the RNA-dependant-RNA-polymerase (3Dpol), which is encoded within the viral genome; additional viral proteins are also required to initiate this process. VPg (3B) acts as a primer to initiate RNA synthesis within a membrane associated complex that contains a number of additional viral proteins (see section 1.4.4). A switch in function of the viral RNA inhibits translation to enable RNA replication to proceed. The switch is required as the translation process of the viral RNA is not compatible with the movement of RNA polymerase from the 3’ end to the 5’ terminus (Gamarnik and Andino, 1998). The molecular mechanisms that result in a switch from translation to replication are uncertain but a model has been proposed for Poliovirus. During translation the PV genome is thought to become circularised through an interaction of the 5’ cloverleaf, PCBP2 and 3CD at one end of the genome with PABP bound to the 3’poly(A) at the other (Barton et al., 2001; Herold and Andino, 2001; Lyons et al., 2001; Teterina et al., 2001). The circularisation has been proposed to prevent the binding of
initiation factors and ribosomes to the 5’ of the IRES, therefore the vRNA would eventually become clear of all ribosome’s and the template for negative strand synthesis could be utilised (Barton et al., 2001). A similar model could be possible for FMDV since it has been proposed that FMDV can circularise its genome through the interaction of PCBP and the 5’ UTR poly(C) tract, along with PABP bound to the 3’ poly(A) tail (Mason et al., 2002) (see section 1.5.5).

Both negative- and positive-strand synthesis is initiated by the virus encoded VPg (3B) primer and 3Dpol. It is also thought that several other viral non-structural proteins as well as host proteins might be part of this process. The VPg (3B) peptide acts as a primer for both negative- and positive- strand synthesis. FMDV, unlike other picornaviruses encodes three tandem copies of VPg that are distinguishable from each other but all have been found to interact with vRNA (King et al., 1980). VPg becomes uridylylated by the virus- encoded 3Dpol to create a pool of uridylylated VPg consisting of two forms: VPgpU and VPgpUpU. Uridylylation is reliant on the cre element located at the 5’ UTR of the FMDV, UTP, divalent cations, 3D and its precursor 3CD in vitro (Nayak et al., 2005) (see section 1.4.4) Although all three copies of VPg can be uridylylated in vitro, the reaction is more efficient using the third copy. In addition, the efficiency of uridylylation is enhanced when the entire 5’ UTR is utilised in the reaction as opposed to the cre alone (Nayak et al., 2005).

Poliovirus negative-strand synthesis has been proposed to occur following the translocation of VPgpUpU from the cre to the 3’ poly(A) tail on a positive sense genome (Paul et al., 2000) (see section 1.4.4). Base-pairing of the VPgpUpU with the poly(A) would result in the synthesis of the negative
Figure 1.5 The Picornavirus Replication cycle

Viral entry is by receptor-mediated endocytosis which leads to the release of the positive-sense RNA into the cytoplasm. This RNA is translated into a large polyprotein and cleaved by virus encoded proteases. Viral proteins cause host cell membrane rearrangements to form replication complexes which allow viral replication to proceed. Newly synthesized positive-sense RNA is also translated and the process repeats until sufficient capsid protein precursors are formed, to allow assembly of the procapsid. Procapsids associate with newly synthesized positive-sense RNA still containing VPg at its 5’ end. As the process continues, virions accumulate in the cytoplasm until viral proteins induce cell lysis and virus release occurs (E.K.Wagner, 2004).
strand genome from the positive strand copy by 3Dpol, using VPgpUpU as a primer (Paul et al., 2000). Alternatively, a more recent study showed evidence that negative strand synthesis does not depend on the cre structure in vitro, as mutations within the AAACA motif required for uridylylation did not inhibit negative strand synthesis (Goodfellow et al., 2003b; Morasco et al., 2003; Murray and Barton, 2003). This suggests that the unmodified VPg may prime the 3Dpol, not VPgpUpU (Morasco et al., 2003). This model predicts that the poly(A) acts as a template for uridylylation of VPg by 3Dpol, to create VPg poly(U) which extends to create a full length negative sense-strand copy. This was strengthened by the fact that 3D, VPg, UTP Mg2+ and poly(A) results in VPg-poly(U) formation (Paul et al., 1998). The initiation of positive strand synthesis is believed to occur at the 3’terminus of the RNA template, as an authentic 5’ end of the PV genome is required for positive strand synthesis by 3Dpol (Herold and Andino, 2000).

### 1.6.4 Replication Complexes

Picornavirus infection results in the extensive rearrangement of the host cell membranes, creating replication vesicles that are necessary for viral genome replication (Bienz et al., 1983; Dales, 1965; Gosert et al., 2000; Gosert et al., 2002). These membranes are believed to function as “platforms” to facilitate the assembly of viral replication complexes (RC) containing the correct balance of replicase proteins (see Figure 1.6). The 2C, 2B and 3A proteins of enterovirus are believed to be important components of the RC. The 2C and 2B proteins have been shown to bind membranes via amphipathic helices (Paul et al., 1994), whereas poliovirus 3A and its precursor 3AB interact with membranes through a hydrophobic region of 3A (Towner et al., 1996).
Membrane rearrangements have been extensively studied for poliovirus (Bienz et al., 1983), echovirus (Skinner et al., 1968) and coxsackie virus A (Jezequel and Steiner, 1966), and membranes from several cellular compartments have been associated with formation of replication vesicles. Early studies concluded that, PV-induced vesicles are derived from the endoplasmic reticulum (ER) (Schlegel et al., 1996; Suhy et al., 2000), at sites enriched for the COP II protein complex (Rust et al., 2001). COP II coated vesicles normally play key roles in vesicular transport between the ER and the Golgi (see section 1.8.1). In the later stages of PV infection, membranes from other cellular organelles were implicated in vesicle formation, namely the Golgi and lysosomes (Bolten, 1998). Although different genera of picornaviruses share similar replication strategies, research has highlighted many important variations: specifically, that replication can occur on vesicles derived from different membranes and at varying sites around the cell (Gazina et al., 2002). PV, EV11 and EMCV all appear to induce similar rearrangements of the host cell membranes by forming heterogeneously sized vesicles (200-400nm) arranged in tight clusters. Conversely, HPEV-1 has been observed forming uniform vesicles present in a low number, and in loose arrangements (Gazina et al., 2002). Viral RNA has been shown to be associated with these membranes (Bienz et al., 1980; Butterworth et al., 1976; Caliguri and Tamm, 1970) and replication is thought to take place on the outer cytoplasmic surface (Bienz et al., 1987). Formation of vesicles can be recreated by the expression of poliovirus 2BC and 3A proteins in transfected cells. These vesicles shared similar biochemical and morphological characteristics to naturally infected cells (Suhy et al., 2000). PV vesicles are also noted to share structural similarities with autophagosomes, for example they possess a double membrane and
contain cytoplasmic material (Schlegal et al., 1996), which may suggest a role for autophagy in PV replication.

FMDV infection of BHK-38 cells causes single membrane vesicles, which appear in loose clusters and are fewer in number than in poliovirus infected cells. The entire cellular cytoplasmic contents become condensed and localised to a region on one side of the nucleus. This region contains both viral proteins and vRNA in addition to areas of tightly packed ribosomes and smooth cytoplasmic structures located at perinuclear sites. The viral non-structural protein 2C was found to co-localise with VP1, 3A and 3D within this region, but not proteins associated with the secretory pathway (Knox et al., 2005). This finding suggests that FMDV may not use the secretory vesicles for the formation of replication complexes. Alternatively, FMDV replication complexes may form on secretory vesicles, but the markers for these vesicles may be rapidly excluded from the replication complex (Moffat et al., 2005). As previously mentioned, all Picornaviruses rely on host cell membrane rearrangements (Miller and Krijnse-Locker, 2008; Richards and Ehrenfeld, 1990; Salonen et al., 2005), however it is the properties of the RCs that allow viral replication to proceed. It has been proposed that the membranes may serve to increase the local concentration of viral and host proteins, and vRNA at sites of replication, or that the lipid composition of the membrane maybe required for replication to proceed (Miller and Krijnse-Locker, 2008). The most recent studies on the formation of RC’s in enteroviruses have implicated the recruitment of phosphatidylinositol 4-kinases to RC membranes. Phosphoinositides are cellular phospholipids that are important in the regulation of the recruitment and the activity of signalling proteins on cellular membranes. As mentioned (see section 1.10.3), PV and CVB3 both require the activity of
Figure 1.6 Replication complexes

The above figure shows the ultrastructural changes in cells at mid-stages of infection with FMDV observed at high power. (a) Large numbers of membrane vesicles are present (arrow) within the replication complex. These are often associated with membranes and have the appearance of ribosome-depleted rER. (b) Occasionally double membrane vesicles are seen (see black arrow) (c) A large numbers of ribosomes are present in linear patterns within the replication complexes. (Monaghan et al., 2004).
GBF1, which recruits Arf1 onto host cell membranes. This in turn results in the recruitment of various effectors (Altan-Bonnet et al., 2004; Niu et al., 2005). Arf1 effectors include COPI proteins (see section 1.8.2) that regulate vesicle budding, and phosphatidylinositol-4-kinase IIIβ (PI4KIIIβ) which generate phosphatidyl-inositol-4-phosphate (PI4P) lipids at the membrane bilayer (Altan-Bonnet et al., 2004; Godi et al., 2004; Lee, 2004). PI4P lipids have been reported to regulate ERES biogenesis and autophagy (Blumental-Perry et al., 2006; Yamashita et al., 2006) and alter membrane curvature (Ishiyama et al., 2002; McMahon and Gallop, 2005). Hsu et al (Hsu et al., 2010) have proposed a model showing that enteroviral 3A binds to membranes and recruits host cell proteins GBF1 and Arf1, which in turn preferentially recruit PI4KIIIβ to host cell membranes. The recruitment of PI4KIIIβ results in the production of PI4P leading to the creation of lipid rich membranes, which are distinct from host cell membranes. These lipid rich 'microenvironments' then promote the recruitment of viral proteins involved in viral replication such as 3Dpol, which was shown to preferentially bind to PI4P lipids, to create the RC. The preferential recruitment of PI4KIIIβ onto membranes over COPI would result in a block in transport within the early secretory pathway and disruption of the Golgi, as has been observed in enterovirus infected cells.

1.6.4.1 Brefeldin A

An important difference in picornavirus replication is the sensitivity to brefeldin A (BfA). Brefeldin A is an inhibitor of membrane transport in the secretory pathway between the ER and Golgi. It prevents the formation of COPI vesicles which are necessary for secretory transport (Doedens and Kirkegaard, 1995; Rothman, 1994) (see section 1.8.2). Infection by PV, EV11 and BEV (bovine enterovirus) are all significantly inhibited by brefeldin A (Maynell et al.,
Brefeldin A acts by preventing the activation and function of a small family of GTPases, specifically Arf GTPases, by inhibiting the guanine nucleotide exchange factor (GEF), and recycling Arf1 from an inactive GDP bound state to an active GTP bound form (Mossessova et al., 2003). The ability of BFA to inhibit PV infection suggests that viral replication relies on Arf-dependent membrane trafficking. Components of the COPI coat complex are recruited to membranes by activated Arf1. One of the components has been shown to localise to replication complexes in cells infected by Echovirus 11, which, like Polioviruses, is sensitive to BFA (Maynell et al., 1992). Arf1 was not however, detected on replication complexes of viruses such as EMCV or FMDV which are resistant to BFA (Gazina et al., 2002).

1.7 Particle assembly, maturation and release

The final stages in the replication cycle of FMDV involve encapsidation of the vRNA genome and cleavage of VP0 to create VP2 and VP4 to form the mature virion that is released from the cell (see Figure 1.7).

The building block of the capsid is a protomer formed by one copy of VP0, VP1 and VP3. Pentamers (12S) are formed by the association of five protomers. Then 12 pentamers associate to form the final acid sensitive capsid. On encapsidation of the vRNA, VP0 is cleaved to form the mature particle. Several other possible forms of particles have been observed during infection: RNA containing particles that have not undergone VP0 cleavage (provirion) (Guttman and Baltimore, 1977; Lee et al., 1993) and empty capsids (Grubman et al., 1985; Yafal and Palma, 1979). It remains uncertain what signals are required for RNA encapsidation. Picornaviruses only encapsidate positive strand RNA linked to VPg (Nomoto et al., 1977; Novak and Kirkegaard, 1991;
Wimmer, 1982) and only newly synthesised +ve vRNA is packaged, suggesting there is a link between active replication and encapsidation (Harber et al., 1991; Nugent et al., 1999). Recent studies have shown that PV 2C protein directly interacts with VP3 at RC’s, implying a role for 2C in encapsidation (Liu et al., 2010). There are two models for capsid assembly in picornaviruses. The first suggests pentamers assemble and the RNA is inserted into empty capsids, the second proposes that pentamers interact with RNA directly to form a provirion. FMDV studies showed that radioactive labelled structural proteins were found in protomers, pentamers, empty capsids and virions (Yafal and Palma, 1979), reinforcing the first models proposal. Contrary to this, Verlinden et al (Verlinden et al., 2000) agreed with the second model, producing evidence for pentamers interacting with RNA to produce virions in poliovirus cell-free systems.

Once the vRNA is encapsidated myristylated VP0 (see section 1.5.2) is cleaved to create a mature capsid. It is thought FMDV vRNA plays a role in this process as cleavage is more efficient in natural particles as opposed to artificially formed virions (Curry et al., 1995; Curry et al., 1997). The cleavage is believed to be autocatalytic, and produces a more stable particle by increasing the ordering of the structural protein’s N-termini which is due to the presence of RNA (Curry et al., 1997). Site directed mutagenesis studies of FMDV VP0 produced non-infectious virus particles, but the particle retained its receptor binding and acid sensitivity properties. This provirion’s pentamers appeared to be more hydrophobic after acid dissociation than mature virions, suggesting that VP0 cleavage might be necessary for RNA translocation into the cytosol (Knipe et al., 1997).

Finally, the newly formed virus particle is released from the host cell. Infection of cultured cells results in cell lysis and plaque formation, suggesting
Figure 1.7 Assembly and release

Capsid proteins are cleaved from the polyprotein by 2A protease as a monomer, 3C cleaves the protein further into protomers (VP0, VP3 and VP1). Five protomers assemble to form a pentamer and 12 pentamers form the Procapsid. After RNA encapsidation the VP0 is cleaved into VP2 and VP4 to create the final virion. (E.K.Wagner, 2004)
the release of progeny virus is via cell lysis of infected cells (Belsham, 1993). Poliovirus 3A protein has been implicated in the lysing process (Lama et al., 1998), although the mechanism for this is largely unknown.

1.8 The early secretory pathway

Eukaryotic cells possess a secretory pathway consisting of a complex endomembrane network containing several organelles that function to transport proteins to the plasma membrane and the extracellular environment (Lee, 2004). Newly synthesised proteins enter the system via the rough endoplasmic reticulum (ER) by the action of ribosome docking proteins onto pores on the ER membrane. Docking results in the release the polypeptide into the ER lumen, once inside, chaperones fold proteins into their correct formation and post-translational modification occurs. Transport vesicles bud from the ER-containing cargo at ER exit sites (ERES) and fuse together to form the ER-Golgi intermediate compartment (ERGIC) (Orci et al., 1982). The ERGIC gives rise to the cis-Golgi which accepts incoming transport vesicles, proteins are then processed in the medial-Golgi before being packaged and exported to their required destination via the trans-Golgi network (see Figure 1.8).

Coatomer complex proteins (COP) are essential for transport between organelles. COP II proteins are responsible for the initial anterograde transport from the ER, in vesicles containing cargo and SNARES (soluble NSF attachment protein receptor). It was initially thought that COPII existed as free vesicles that moved along microtubules to the Golgi. However, it is now believed that COPII vesicles rapidly lose their coat after budding from ERES (Oka and Nakano, 1994). Morphological analysis in mammalian cells showed
that COPII vesicles undergo fusion to create vesicular tubular clusters (VTC) also known as the ER-Golgi intermediate compartment (ERGIC). These vesicles are distinct from the ERES but are still interconnected (Bannykh et al., 1996). Both COPI and COPII vesicles have been found associated to the ERGIC (Aridor et al., 1995; Scales et al., 1997). Recently, evidence has shown that COPII components appear to be tightly associated to the ERES and are relatively immobile. COPI is seen to form in close proximity to COPII sites before detaching and moving towards the cis-Golgi, whereas COPII remains at or near the ERES. This suggests a sequential mode of action between the two vesicles (Shima et al., 1999; Stephens and Pepperkok, 2002). COPI transports proteins onto the cis-Golgi and is also involved in the retrograde transport of proteins back to the ER.

The formation and targeting of the transport vesicles relies on a complex sequence of interactions involving regulatory molecules. Cargo marked to leave a compartment is incorporated into a transport vesicle via a direct or indirect interaction with cytoplasmic proteins. These proteins become concentrated on the membranes of the donor compartment to form a coat; assembly of such complexes induce the formation of a transport vesicle on the donor membrane. The docking and fusion of vesicles with the acceptor organelle membrane involves interactions with the actin and microtubule cytoskeleton, recruitment of docking complexes and the specific recognition between v- and t-SNARES. Small GTPases of the ADP-ribosylation factor (Arf) and Rab family have been implicated as regulators of these events (Chavrier and Goud, 1999). Proteins that are secreted from the cell are modified and matured in the Golgi before vesicles transport the proteins to the plasma membrane.
1.8.1 COPII formation

COPII vesicle formation occurs on the smooth ER membranes at ER exit sites (ERES) where cargo for export is concentrated (Bannykh et al., 1996). The ERES are ribosome free subdomains specialised for COPII formation, which are relatively long-lived and distributed throughout the ER (Aridor et al., 2004; Bannykh et al., 1996; Bevis et al., 2002; Stephens, 2003).

Assembly of the COPII coat is initiated through the activation of the small Ras-like GTPase, secretion-associated Ras-related 1 (Sar1) (Nakano et al., 1988). As with all GTPases, Sar1 is bound to GDP in the cytosol, where it is inactive; it is converted to active GTP-bound form by a guanine nucleotide exchange factor (GEF). Sec12 is the known GEF for Sar1 (Barlowe and Schekman, 1993; Nakano et al., 1988) which is localised to the ER, therefore Sar1 activation is restricted to this area of the early secretory system (Sato et al., 1996). The conformational change of Sar1 from the GDP-bound form to the GTP-bound protein exposes the N-terminal, amphipathic α-helix of the protein that inserts into the ER membrane (Bi et al., 2002; Huang et al., 2001). Membrane bound Sar1-GTP recruits a Sec23-Sec24 heterodimer by binding to Sec23, together the complex Sec23/24-Sar1 selects cargo to form a pre-budding complex (Kuehn et al., 1998). Once formed the pre-budding complex recruits Sec13-Sec31 heterotetramer to form an outer coat (Lederkremer et al., 2001). Evidence shows Sec13/31 can assemble into a cage-like structure in the absence of other components of the COPII complex (Stagg et al., 2006). Sec13/31 incorporated into the pre-budding complex is thought to stabilise the coat during polymerisation and drive membrane deformation to form COPII vesicles (60-70 nm in diameter). Several studies have implied a role for the Sec16 protein in vesicle budding. Sec16 is a large peripheral protein found
associated with ER membranes (Espenshade et al., 1995). Sec16 domains make direct contact with the COPII proteins Sec23 and Sec31, which are thought to act as a scaffold for coat assembly, and thus have a role in ERES formation (Gimeno et al., 1996; Supek et al., 2002). However, it remains unclear if Sec16 is required for ERES organisation and/or COPII vesicle formation (see Figure 1.9).

The majority of membrane and soluble cargo proteins in the ER are concentrated in COPII vesicles. COPII efficiently recognises and sorts cargo from ER resident proteins for incorporation into vesicles (Barlowe et al., 1994; Salama et al., 1993). Cargo is selected through export signals within the exposed amino acid sequence of the proteins (Barlowe, 2003). In many cases the export signals of transmembrane proteins are believed to bind directly with the COPII vesicle complex Sec23/24 (Aridor et al., 1998; Kuehn et al., 1998) through the Sec24p subunit (Miller et al., 2003; Mossessova et al., 2003), however some transmembrane and soluble proteins are thought to bind via transmembrane cargo adaptors/receptors.

COPII vesicles shed their coat rapidly after exit from the ER and it is replaced by COPI (see section 1.8.2), before fusing with the ERGIC membrane through Sar1-GTP hydrolysis (Oka and Nakano, 1994). This releases cargo from the sorting subunits along with COPII proteins to be recycled back to the ER. GTP hydrolysis is mediated by GTPase activating proteins (GAPS). GAPs encourage GTP hydrolysis and therefore reverse the Sec23/24-Sar1 interaction. Sec23 of Sec23/24 is the GAP for Sar1 (Yoshihisa et al., 1993); this activity is further stimulated (~10 fold) by the binding of Sec13/31 subunit (Antonny et al., 2001). The Sec23 activity on Sar1 is commonly used by other GAPs of the Ras super-family: an arginine finger interacts with a Sar1 catalytic site to neutralise
the negative charge that occurred in the GTPase transition. In order to maintain stable vesicles Sec23/24 is held on the membrane by the GEF Sec12 which counteracts the GAP activity by constantly supplying Sar1 with GTP (Futai et al., 2004).

Sar1 is thought to be the component required for the initial stages of membrane deformation and vesicle fission as there is evidence that Sar1 alone is sufficient to deform liposome membranes (Lee et al., 2005). The insertion of the Sar1 N-terminal helix into the ER membrane would act to displace the lipid head-groups causing curvature towards the cytoplasmic side (Farsad and De Camilli, 2003; Sheetz and Singer, 1974). The addition of Sec23/24 and Sec13/31 components results in coat polymerisation; as the coats assemble, the GAP’s hydrolyse the GTP. The energy of polymerisation then causes deformation of the membrane and leads to vesicle scission (Barlowe, 2000).

1.8.2 COPI formation

Coatomer (COPI) is composed of seven conserved subunits and its formation is controlled by the GTPase Arf1. Arf1 has the ability to induce membrane formation at several points along the early secretory pathway by interacting with numerous GEFs which are involved in Arf1 activation and recruitment onto membranes. All of the GEFs known to associate with Arf share a Sec7 domain that is involved with exchange activity. They are present in two forms, either resistant or sensitive to the fungal metabolite brefeldin A (Jackson and Casanova, 2000; Peyroche et al., 1996), which allows the detailed study of GEF activity on Arf1. Different interactions of Arf with GEFs could determine which membrane the protein associates with (Chantalat et al., 2003; Chantalat et al., 2004; Richter et al., 2007; Spang et al., 2001). ARF-GDP has been found to be recruited directly to the membrane by an association
with p23 (an integral membrane protein), where GDP is exchanged for GTP by GEFs (Gommel et al., 2001; Majoul et al., 2001). Once Arf1-GDP has been catalysed by a GEF to Arf-GTP it is able to interact with Arf-GAP and cargo/SNARE proteins. The GAP does not stimulate GTP hydrolysis at this point, but instead plays a role of inducing a conformational change in SNARE proteins which allows a direct interaction with Arf1, this in turn aids the recruitment of the heptameric protein complex called the coatmer (Rein et al., 2002; Schindler and Spang, 2007; Spang, 2002). The coatmer consists of seven subunits that include: α, β, β’, γ, δ, ε, ζ (Waters et al., 1991), Arf and p24 family membrane proteins. Coatmer subunits are directly involved in the recruitment of cargo into the COPI vesicles through dilysine motifs usually containing a KKXX or KXXXX (K = lysine and X = arginine or alanine) sequence at the carboxyl terminus of type 1 transmembrane proteins (Cosson and Letourneur, 1994). The interaction between the motifs and vesicle is necessary for retrograde transport (Letourneur et al., 1994). Three of the coatmer subunits (β, γ and δ) have been identified in cargo recognition and all bind to different motifs (Michelsen et al., 2007). By recruiting these proteins into the COPI vesicle, the membrane deforms to form the vesicle.

Stimulation of Arf1-GAP activity was observed after binding of the coatmer complex (Goldberg, 1999) and the incorporation of specific cargo such as KDEL receptors (Aoe et al., 1997). Like Sar1, Arf-GAP is part of the COPI coat (Lee et al., 2005; Lewis et al., 2004; Yang et al., 2002) and it is therefore likely GTP hydrolysis is regulated in a similar manner. COPI coat disassembly is also thought to stimulate membrane curvature (Bigay et al., 2003; Mesmin et al., 2007) in the Golgi.
Figure 1.8  A model of transport between the endoplasmic reticulum (ER) and Golgi compartments.

After translation and folding of secretory proteins, fully folded cargo and soluble secretory cargo are exported from the ER in (COPII)-formed transport vesicles. COPII vesicles transport proteins in an anterograde direction to fuse with or form the ERGIC. COPI coats provide retrograde transport from the ERGIC and Golgi compartments to recycle ER resident proteins (R) back to the ER (Dancourt and Barlowe, 2010).
Figure 1.9  A model for coat protein complex II (COPII)-dependent cargo selection and vesicle formation.

Sar1 is activated by the nucleotide exchange factor (GEF) Sec12 to Sar1-GTP. Membrane-bound Sar1-GTP recruits Sec23-Sec24 to assemble pre-budding cargo complexes within which the Sec24 subunit binds to specific sorting signals on export cargo. Cargo can link to the Sec24 subunit directly or through transmembrane sorting receptors. Pre-budding cargo complexes recruit the Sec13-Sec31 complex, leading to coat polymerization and vesicle formation (Dancourt and Barlowe, 2010).

Annu. Rev. Biochem. 79:777–802
1.8.2.1 Sar1 and Arf proteins

Sar1 is a unique family within the Ras super-family of small GTPases and only has around a 37% homology to its closest relative Arf1. Sar1 is present as two isoforms, Sar1a and Sar1b, they differ by approximately 20 amino acids at the C-terminus, but no separate functions have been assigned to the different isoforms (Charcosset et al., 2008). The majority of GTPases possess cysteine residues at their c-terminus that are modified and Arf1 has a myristolated N-terminus, in contrast Sar1 does not undergo any posttranslational modifications at either terminus.

Very few studies have been carried out on Sar1 function and interaction when compared to Arf1, however there are some important differences between the two regulatory GTPases. As previously mentioned, Sar1 controls the assembly of proteins coats (COPII) that bud proteins from the ER (see section 1.8.1). Aridor et al (Aridor et al., 2001) found that activation of Sar1 alone leads to the formation of ER-derived tubular domains that resemble the ERGIC, highlighting its key importance in vesicle transport. Sec12 is the only known GEF for Sar1 and it interacts with the protein through a motif of nine hydrophobic amino acids known as STAR (Sar1-NH2-terminal activation recruitment motif) (Huang et al., 2001). The STAR motif along with the NH2-terminal amphipathic helix (see section 1.8.1) facilitates pre-budding complex formation and cargo selection (Huang et al., 2001). Sec12 shares no apparent sequence identity with the Arf-GEFs and possesses a unique transmembrane spanning segment allowing its localisation to the ER membranes (Boehm et al., 1997; Sato et al., 1999; Sato et al., 1996). In contrast Arf1-GEFs are soluble and their localisation is thought to be through interactions with specific receptors (see section 1.8.2). The GAP for Sar1 is the Sec23 (see section 1.8.1) which is
part of the COPII coat complex. This Sec23 subunit stimulates Sar1 GTPase activity which is increased 10 fold by the addition of the Sec13/31 complex. In contrast, Arf GTPase does not appear to be activated by a COPI subunit, instead the addition of Arf-GAP complexes creates GTPase activity (Goldberg, 1999).

Arf1 primarily co-localises to the Golgi complex in mammalian cells (Stearns et al., 1990) and induces the recruitment of COPI coat proteins to the Golgi membranes involved in transport (see above section 1.8.2). All Arfs have a myristylated amino terminus that is required for membrane binding and function. Arfs have been divided into three different classes based on amino acid sequence (Tsuchiya et al., 1991). In total there are six mammalian Arf proteins: Arf1, Arf2 and Arf3 make up Class I; Arf4 and Arf5 comprise Class II; and Arf6 is the only member of Class III. Class I Arfs function at the Golgi complex and it is thought they may have interchangeable functions. Arf5 may also function at the Golgi (Claude et al., 1999; Kawamoto et al., 2002) whereas Arf6 is found localised to the plasma membrane (Al-Awar et al., 2000; Peters et al., 1995).

Arfs are converted into a GTP-bound state after recruitment to the membrane and activation by GEFs (see section 1.8.2). There are four families of Arf-GEFs: the Gea/GBF1, Sec7/BIG1/BIG2, ARNO/cytohesin and EFA6 (Jackson and Casanova, 2000). The Gea/GBF1 and Sec7/BIG families localise to the Golgi and are usually inhibited by BfA (see section 1.6.4.1) whereas the remaining other two families, ARNO/cytohesin and EFA6, are BfA insensitive. In mammalian cells GBF1 is associated with the ERGIC and the Golgi (Garcia-Mata and Sztul, 2003; Kawamoto et al., 2002; Zhao et al., 2002) whereas, Sec7/BIG localises to the trans-Golgi, trans-Golgi network (TGN) and recycling
endosomes (Mansour et al., 2002; Shin et al., 2004; Shinotsuka et al., 2002). GBF1 is known to interact with p115, a Golgi tethering protein (Garcia-Mata and Sztul, 2003), which maybe important for creating a scaffold to aid Arf1 recruitment and activation at the Golgi membranes (Donaldson et al., 2005). GBF1 is also thought to be the GEF responsible for the activation of Arf1 on the Golgi to form COPI vesicles (see section 1.8.2).

More than 16 Arf-GAPs have been identified in the human genome (Nie et al., 2003) which all contain a zinc-finger and catalytic domain. The Arf-GAP (Arf-GAP1) was the first to be identified (Cukierman et al., 1995) and is localised to the Golgi via an interaction with carboxyl terminal of the protein (Huber et al., 1998; Yu and Roth, 2002). Over-expression of Arf-GAP1 disassembles the Golgi, a phenotype very similar to BfA treatment or expression of a dominant negative Arf1 T31N (Dascher and Balch, 1994). Over-expression of other Arf-GAPs does not have this effect, therefore suggesting that Arf-GAP1 influences Arf1 activity in the early secretory pathway (see section 1.8.2).

1.9 Rab proteins

Rab proteins exist in all eukaryotic cells and form the largest group within the Ras super family (ras-related in brain) of GTPases, with over 70 different Rab proteins identified in the human genome (Bock et al., 2001). Rab proteins are localised to the cytoplasmic surface of all organelles and regulate discrete transport steps along the secretory and endocytic pathways. Here they are involved in all stages of membrane trafficking including budding, transport, tethering and fusion events. Rab1, 2 and 6 act at the ER and Golgi and are
involved in the secretory pathway (Gonzalez and Scheller, 1999; Martinez and Goud, 1998).

Rabs are soluble proteins present in the cytosol sequestered by Rab escort proteins (REPs) that chaperone the Rab protein around the cell. Initially the REPS deliver the Rab protein to the geranylgeranyl transferase enzyme for prenylation where one or two hydrophobic geranylgeranyl groups are added to enable insertion into the cellular membrane (Anant et al., 1998). The Rabs are then delivered to the appropriate membrane where their lipid attachment allows them to insert into the bilayer (Alexandrov et al., 1994).

Rabs go through an insertion-extraction cycle from the membrane. Rabs, like other Ras-like proteins exist in GDP-bound inactive form in the cytosol. GDP dissociation inhibitor (GDI) binds to prenylated Rabs in their GDP bound form, maintaining Rabs in the cytosol (Ullrich et al., 1993). Therefore membrane attachment of Rabs, requires a GDI displacement factor (GDF) which dissociates both GDI and REPs from the Rab (Dirac-Svejstrup et al., 1997). Once GDI has been dissociated, the Rab specific GEFs can stimulate GTP-binding and membrane binding through the Rab prenylated residues (Yang et al., 1998). As with all Ras proteins the exchange of GDP for GTP is mediated by specific GEFs. GEFs trigger the binding of GTP to the Rab resulting in it binding to the membrane and this in turn activates the protein. Active Rabs are now able to bind to their specific effectors and carry out their roles in membrane trafficking. GAP proteins hydrolyse GTP thereby releasing the Rab from the membrane where GDI sequesters the Rab in the cytosol. Over 51 Rab specific GAPs have been identified to date all of which contain a conserved TBC (Tre-2/Bub2/Cdc6) domain (Bernards, 2003).
1.9.1 Effector proteins

Effector proteins bind to specific Rab proteins to initiate one or more downstream effects (Munro, 2002; Pfeffer, 2001; Zerial and McBride, 2001). They bind specifically to Rabs in their GTP bound state (Harrison et al. 2003). Rabs can signal through a variety of different effectors that act together to translate the signal from one Rab protein to multiple aspects of membrane transport (de Renzis et al., 2002; Segev, 2001). Effectors create specific membrane domains and these domains can mature in the form of a Rab cascade mechanism, which allows the GEF of one Rab to serve as an effector for another Rab (Grosshans et al., 2006). Effectors involved in membrane fusion are termed SNAREs (Brodsky et al., 2001; Jahn and Grubmuller, 2002; Sollner, 2004). SNARE proteins interact to form stable complexes. It is thought two SNARE proteins attached to different membranes may be able to form a “zipper”, driving fusion between the two membranes (Melia et al., 2002).

1.9.2 Rab1

The Rab1 GTPase is present in two isoforms A and B, which have both been implicated in the regulation of anterograde transport of cargo between the ER and Golgi (Plutner et al., 1991; Tisdale et al., 1992). Rab1 localises to the ERGIC membrane (Saraste et al., 1995) and has been seen to associate with the ERGIC that accumulates in transport arrested cells absent of cargo proteins (Palokangas et al., 1998). More recently, studies have now shown that Rab1 is the only single GTPase essential for anterograde transport through the Golgi and maintenance of the Golgi structure (Haas et al., 2007; Itoh et al., 2006). Rab1 regulation has been linked to the recruitment of COPI protein coats. The dominant-negative mutant of Rab1 is able to block forward transport and disrupt the Golgi, causing Golgi proteins to relocate to the ER (Haas et al., 2007).
Inactive Rab1 also causes the redistribution of the ERGIC to a punctate structure. It was also observed that while COPII exit machinery remained functional, COPI was compromised, as β-COP was released into the cytosol. It can be concluded that Rab1 influences COPI recruitment and may play a role in Arf and its GEF (GBF) mediated COPI recruitment (Alvarez et al., 2003).

Rab1 activity is also involved in the regulation of vesicle tethering and docking on acceptor membranes. Tethering factors consist of long coiled-coil proteins including p115, golgins and EEA1 (Allan et al., 2000). Coiled-coil tethers are thought to act as bridge between the vesicle and target membrane. For example, vesicle associated protein p115 interacts with GM130 (a Golgi residing protein) and GRASP65, producing a complex that is thought to tether ER derived vesicles to the Golgi complex (Moyer et al., 2001; Plutner et al., 1990; Tisdale et al., 1992). The role of Rab1 maybe to regulate the assembly and/or activity of GM130/GRASP65 complex ensuring the specific activation of p115, and prevent its mis-targeting (Alvarez et al., 2001).

1.9.3 Rab2

Rab2 has been shown to be essential for the maturation of the ERGIC. It is resident of the ERGIC, and is required for protein transport from the ER to the Golgi complex (Tisdale et al., 1992). Rab2 binds to the ERGIC to promote the recruitment of soluble components that aid membrane fusion and cytoskeletal interaction. This results in the release of retrograde-directed transport vesicles (Tisdale, 1999; Tisdale and Jackson, 1998). Dominant-negative GTP-binding mutants of Rab2 inhibit vesicular transport between the ER and Golgi complex. This suggests that along with Rab1, they are essential for regulating the secretory pathway by being involved in vesicle fusion between early compartments of the pathway (Tisdale et al., 1992). In addition to regulation of
membrane flow from the ERGIC to the Golgi, Rab2 is also involved in the transport from the Golgi back to the ER (Tisdale, 1999; Tisdale et al., 1992; Tisdale and Jackson, 1998). This is thought to be achieved by Rab2 recruiting PKCl/x to ERGIC membranes, which promotes the recruitment of COPI to generate retrograde transport vesicles. Therefore, Rab2 plays a critical role in maintaining the ERGIC in a steady state. (Tisdale, 1999, 2000; Tisdale and Jackson, 1998).

1.9.4 Rab6

Rab6 is known to form a number of effector complexes mostly with motor proteins. This is required for microtubule transport between organelles. The Rab6 protein is believed to directly link transport vesicles to the cytoskeleton. Rab6 is a Golgi associated protein, which is shown to interact with a kinesin-like protein, Rabkinesin-6 (Echard et al., 1998), and is connected to tubular structures moving along microtubules from the Golgi to the cell membrane. Rab6 could aid the transport of these structures to their acceptor organelles, most likely the ER. In addition, Rab6 and Bicaudal-D have been seen to combine to tether transport vesicles destined for the Golgi to the cytoskeleton and once again collect them at the perinuclear region (Girod et al., 1999; Mallard et al., 2002; Young et al., 2005).

1.9.5 Other Rabs involved in the early secretory pathway

Rab34 localises to the Golgi and is implicated in manoeuvring lysosomes to the cell centre (Wang and Hong, 2002) It is also believed to be involved in intra-Golgi transport with the early secretory pathway (Goldenberg et al., 2007). Rab8 is thought to form an effector complex with Rab8ip, involving it in trans-Golgi to plasma membrane transport (Huber et al., 1993). Similarly, Rab11 has been reported in the late recycling of transferrin receptors (Ren et al., 1998).
from the trans-Golgi network (TGN) to plasma membrane transport (Chen et al., 1998). This list of Rab proteins however is likely to be incomplete, and the involvement of other Rabs in the early secretory system remains to be determined.

1.10 The early secretory pathway and viruses

Many pathogens replicate in specialised compartments including bacteria and viruses. All positive-strand RNA viruses, along with several DNA viruses (e.g. Vaccinia virus and African horse sickness virus) induce host cell membrane rearrangements required for the formation of replication complexes. The majority of these viruses have implicated membranes from the early secretory pathway in this process, with the ER playing a major role in vesicle formation. The formation of membrane structures by viruses is believed to allow the accumulation of components required for replication. This would not only provide a platform for the replication complex, but would also allow evasion of the immune response which would normally be triggered by the presence of dsRNA.

1.10.1 Membrane rearrangements

Several studies have shown that the individual expression of viral proteins causes membrane rearrangements similar to those observed in infected cells. Enterovirus non structural protein 2BC in conjunction with 3A induces formation of membrane vesicles with the same morphology as cells infected with the full length genome (Barco and Carrasco, 1995; Bienz et al., 1983; Cho et al., 1994; Suhy et al., 2000). Similarly, Hepatitis C transmembrane protein NS4B was shown by EM to alter host cell membranes into a membranous web that is usually observed during infection and in cells expressing subgenomic replicon.
Flavivirus non-structural protein 4A appeared to locate to RC’s in infected cells (Mackenzie et al., 1998; Miller et al., 2007), and when expressed alone induced membrane vesicle formation, which is thought to support vRNA replication (Miller et al., 2007; Roosendaal et al., 2006). More recently Bailey et al found feline calicivirus proteins p32, p39 and p30 all localised to ER and possessed potential transmembrane domains. Further investigation found that expression of these proteins lead to the reorganisation of the ER membranes, suggesting they play a role in replication complex formation (Bailey et al., 2010).

1.10.2 Blocking of ER to Golgi transport

In addition to individual viral proteins inducing host cell membrane rearrangements, several viral proteins have been identified as possessing the ability to block transport through the early secretory system. Arresting transport in this way may possibly be a strategy to evade the immune response. A consequence of the block of ER to Golgi transport could prevent the presentation of the major histocompatibility complex (MHCI) on the cell surface; the cell would not be marked for degradation, allowing infection to continue undetected. The block in secretion by PV appears to be mediated by 3A which when expressed alone in cells is able to reduce the secretion of components of the immune response such as β-interferon, interleukin 6 and 8, this also results in the reduction of MHCI expression on the cells surface (Choe et al., 2005; Deitz et al., 2000; Dodd et al., 2001). FMDV infection was also found to reduce MHCI cell surface expression (Sanz-Parra et al., 1998), however unlike PV, the co-expression of FMDV 2B and 2C viral proteins or the precursor 2BC is required to block secretion within the cell (Moffat et al., 2007). Another enterovirus, Coxsackie B3 is believed to utilise a number of viral proteins to block secretion at the Golgi; it was observed that non structural proteins 2B, 2C
and 3A are all able to block secretion, with 3A showing the strongest block
(Cornell et al., 2006).

1.10.3 Host cell protein interactions

To enable a block in protein secretion, disruption of the Golgi apparatus
and host cell membrane rearrangements, viral proteins interact with many host
cell proteins, many of which are linked to the early secretory pathway. The
prime example of this is the work done on PV. COPII vesicle budding from the
ER has been suggested to occur at the beginning of PV infection to form the
replication vesicles (see section 1.6.4). Formation of PV vesicles appeared
morphologically similar to the formation of COPII vesicles, with Sec13/31, a
component of COPII vesicles co-localised on PV vesicles surface. The
precursor 2BC or the entire P2 and P3 coding region appeared to be able to re-
produce vesicles containing a COPII coat. This was observed early in infection
and did not appear to involve the Golgi at this point (Rust et al., 2001).
However, the observation that PV is sensitive to BfA treatment (Gazina et al.,
2002; Maynell et al., 1992), which only effects Arf1 GEFs and not effect COPII
formation (Ward et al., 2001), lead to several studies producing evidence that
Arf1 may be involved in PV RC formation. Initially Belov et al (Belov et al.,
2005) observed that Arf1 translocated to PV replication complexes, and PV 3A
and 3CD bound Arf to membranes due to the translocation of Arf GEF’s GBF1
and BIG1/2 to membranes (Belov et al., 2008; Belov et al., 2005; Belov et al.,
2007b). The recruitment of Arf onto membranes would normally result in the
formation of the COPI complex suggesting the recruitment of these proteins
may result in the formation of RC complexes in PV infected cells. However
evidence shows that 3A alone does not result in vesicle formation and BfA
treatment does not prevent the formation of vesicles in PV infected cells,
perhaps indicating Arf-GEF activity is not actually required for RC formation (Belov et al., 2008). One explanation could be that RC’s could be formed early on in infection from COPII vesicles; 3A and 3CD may then divert the GEFs and Arf from the early secretory pathway to the already formed RC’s to aid replication (Belov and Ehrenfeld, 2007). In contradiction, Cherry et al produced a study indicating PV vesicle formation was only inhibited when COPI was depleted from cells, whereas loss of COPII had no effect on the vesicle structure (Cherry et al., 2006). Interestingly PV 2C was found to be a GTPase (Rodriguez and Carrasco, 1993, 1995), and when a single point mutation was introduced to the protein it resulted in PV becoming resistant to BfA (Crotty et al., 2004). This may suggest a role for 2C in vesicle formation and/or stabilisation. Coxsackie B3, another BfA sensitive virus also uses 3A to bind to Arf GEF GBF1, however 3A was found to inhibit Arf GEF activity and reduces Arf-GTP in the cells (Wessels et al., 2007; Wessels et al., 2006a; Wessels et al., 2006b). The action of Coxsackie 3A is thought to block secretion by stabilising Arf1-GDP-GBF1 complexes therefore reducing the amount of Arf-GTP for COPI formation. Echovirus 11 infection has been shown to redistribute COPI to viral replication complexes; this virus is also sensitive to BfA (Gazina et al., 2002) which may indicate a direct role for COPI in virus replication. Parechovirus infection also resulted in COPI being observed in the replication vesicles. In addition it was also dispersed through the cytoplasm (Gazina et al., 2002) which showed a similar pattern observed in BfA treated cells. Currently, there is no evidence for the use of either COPI or COPII vesicles in BfA insensitive viruses such as EMCV and FMDV (Gazina et al., 2002).

Hepatitis C virus NS4B creates a membranous web used for replication of vRNA (Gosert et al., 2003). HCV was shown by a yeast-two hybrid system to
interact with the cellular GAP TBC1D20, this protein was also found to be required for efficient replication (Sklan et al., 2007b). TBC1D20 regulates the early secretory pathway through its interaction with Rab1, which plays a role in regulating traffic between the ER and Golgi. Over-expression of TBC1D20 was found to block transport from the ER, whereas depletion of Rab1 reduced HCV RNA levels. This suggests a role for Rab1 in HCV replication through an interaction with GAP TBC1D20 (Sklan et al., 2007a).

Coronavirus nsp 3, 4 and 6 are thought to form replication complexes from the ER of infected cells (Knoops et al., 2008). Coronavirus Mouse Hepatitis virus (MHV) is believed to rely on the early secretory pathway to form replication complexes, as when ER export machinery was blocked by either the kinase inhibitor H89 or by expression of the mutant Sar1 H79G, replication was inhibited (Oostra et al., 2007). It was also noted by IF that replication sites themselves did not form, indicated by the lack of dsRNA present after infection in the presence of H89 (Oostra et al., 2007). MHV was also found to be BfA sensitive as both the drug and expression of the dominant negative Arf1 T31N significantly reduced infection. MHV replication was however not affected by BfA in MDCK cells. MDCK are resistant to BfA treatment due to a mutation in the cells GBF1 preventing the drug from binding to the GEF. Therefore, it was concluded that GBF1 activation of Arf1 was required for MHV replication (Verheije et al., 2008). In contrast, a study on SARS-coronavirus showed that although BfA treatment of infected cells reduced infection by around 80%, formation of the vesicles and viral replication were not blocked completely. Confocal microscopy also showed that components from the early secretory pathway did not localise with the SARS-CoV replication complexes. This evidence allowed them to conclude that the early secretory pathway did not play
a direct role in replication of the virus and that the effects of BfA treatment on the cell, such as a loss of membrane integrity, may cause the loss of replication observed (Knoops et al., 2010).

1.11 Aims

To date very little is known about how Foot-and-Mouth Disease Virus (FMDV) rearranges host cell membranes to form its replication complexes (RCs). This PhD thesis aims to gain a better understanding of how FMDV utilises regulatory proteins in the early secretory pathway to help form replication complexes that are believed to act as platforms for viral replication.
Chapter 2: Materials and Methods

2.1 Cell Culture

Baby Hamster Kidney cells, strain 13 (BHK-21) were purchased from the European Cell Culture Collection. The porcine kidney cell line IBRS-2 was provided by the central services unit (CSU) at the Institute for Animal Health (IAH). An alternative porcine kidney cell line, MAX, were a kind gift from Julian Seago at IAH. HeLa cells were obtained from Imperial College, London.

BHK-21 cells were maintained in Glasgow Minimum Essential Medium (GMEM) (Sigma) containing 10% foetal bovine serum (FBS) (Autogen Bioclear), 2 mM glutamine (IAH), 100 U/ml penicillin (IAH), 100 μg/ml streptomycin (IAH) and 5% tryptose phosphate broth solution (Sigma).

IBRS-2 cells were cultured in GMEM containing 10% Adult Bovine Serum (ABS) (Gibco), 100 U/ml penicillin (IAH), and 100 μg/ml streptomycin (IAH).

MAX cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) plus L-Glutamine, 25mM Hepes (Gibco) containing 10% FBS (Autogen Bioclear), 100 U/ml penicillin (IAH), 100 μg/ml streptomycin (IAH).

HeLa cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Sigma) containing 10% FBS (Autogen Bioclear), 100 U/ml penicillin (IAH) and 100 μg/ml streptomycin (IAH).

All cells were grown at 37°C, 5% CO₂ and routinely passaged three times a week.
2.2 Virus

Virus stocks were stored at -80°C. Experiments utilise the non-heparin binding FMDV strain O1Kcad2, the heparin binding FMDV laboratory strain, O₁BFS and Bovine Enterovirus type 1 (BEV-1). Working stocks were prepared using BHK-21 cells for FMDV and IBRS-2 cells for BEV-1.

2.2.1 Preparation of virus working stocks

Either BHK-21 (FMDV) or IBRS-2 (BEV-1) were grown in 175cm² tissue culture flasks until confluent. The cells were then washed with Phosphate Buffered Saline (PBS) and a 1ml 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 at -80°C. Working stocks of virus were centrifuged to remove cell debris and the supernatant aliquoted into 1.5ml screw capped eppendorf tubes and stored at -80°C.

2.2.2 Virus titration on coverslips

Cells were plated onto 13mm coverslips (VWR International) in a 24 well tissue culture plate and incubated over night at 37°C until cells were 80-90% confluent. Virus working stocks were diluted in a ten-fold serial dilution. Cells were washed once in serum free media before adding 200μl of each dilution and incubating for 1 hour at 37°C. After uptake, the virus was removed from the cells and replaced with VGM, before a further 3hrs at 37°C. Coverslips were fixed in 4% paraformaldehyde (4% PFM) dissolved in PBS (Sigma), for 40 minutes at room temperature, before being processed for confocal microscopy.
2.2.3 Virus titration by plaque assay

Six well tissue culture plates of BHK cells were cultured overnight to reach approximately 80% confluency. Virus working stocks were diluted with PBS in a ten-fold serial dilution; the cells were washed with PBS and 100μl of the appropriate dilution added to the cells. The dishes were incubated at 37°C for 15 minutes before adding 3ml Eagle’s overlay (see appendix) at 45°C. The overlay was allowed to set at room temperature and the cells returned to 37°C for the required time. Plaques were visualised by fixing and staining with methylene blue and PFM in PBS for 12-24 hours.

2.3 Antibodies and Reagents

2.3.1 Primary Antibodies

To detect FMDV infection by immunofluorescence, an antibody raised in rabbit against the FMDV-O capsid was used, (kindly donated by Nigel Ferris, IAH). Anti-double stranded RNA antibody was obtained from English & Scientific Consulting Bt. Bovine Enterovirus-1 (BEV-1) was detected through the use of a Guinea-pig anti-BEV-1 antibody (IAH). Anti-c-myc 9E10 (Developmental Studies Hybridoma Bank, University of Iowa) and high affinity anti- Haemagglutinin (HA) (Roche) enabled detection of plasmid expression. GM130 (BD Transduction Laboratories), 23C (a kind gift from Dr Harrison-Lavoie), ERGIC-53 (Sigma) and Protein Disulphide Isomerase (PDI - Bioquote) labelled for the Golgi, Endoplasmic Reticulum-Golgi Intermediate Compartment (ERGIC) and Endoplasmic Reticulum (ER) respectively.

Western blots were labelled with either anti-Sar1 (Millipore), anti-Rab5A (Santa-Cruz), or anti-Actin (Sigma) as a loading control.
2.3.2 Secondary Antibodies

Species specific Alexa-Fluor conjugated secondary antibodies used for confocal microscopy were from Invitrogen (Molecular Probes). Anti-mouse/rabbit HRP secondary antibodies were used for western blot (Promega).

2.3.3 Reagents

Pharmacological reagents were made up into stock concentrations and stored according to manufacturers guidelines. Brefeldin A (BfA) (10mg/ml) ready-made solution and Thapsigargin (1mg/ml in DMSO) were both from Sigma. H89 Dihydrochloride (1mg/ml in DMSO) was from Calbiochem.

2.3.3.1 Plasmids

Plasmids for the expression of wild-type Cyan-fluorescent protein (CFP) Sar1a, the dominant negative CFP-Sar1a (T39N) and the dominant active CFP-Sar1a (H79G) was from A. Townley, University of Bristol. Plasmid expression of the wild-type Green-fluorescent protein (GFP) - Arf1 was donated by E. Ehrenfeld (NIAID, NIH, Bethesda, MD 20892, USA). Dominant negative HA - Arf1 (T31N) and dominant active GFP- Arf1 (Q71L) and were both obtained from J. Lippincott-Schwartz (National Institutes for Health, Maryland, USA). Plasmid expression of wild-type myc–Rab1a, dominant negative myc-Rab1a (S25N), wild-type myc-Rab2, dominant negative myc-Rab2 (S20N), wild-type myc-Rab6 and dominant negative myc-Rab6 (T27N) were from T. Herbert (McGill University, Montreal, Canada). Wild-type GFP-Rab34 and the dominant negative GFP-Rab34 (T66N) were obtained from N. Goldenberg (University of Toronto, Ontario, Canada). The plasmids expressing GFP-TBC1D20 and the mutant version GFP-R105A were obtained from J. Glenn (University of Stanford,
California, USA). Finally, GFP-empty vector, GFP-Sec16L and GFP-Cterm-Sec16L were from B.Glick (University of Chicago, Chicago, USA).

2.3.3.2 siRNA

siRNA targeting Sar1a and b (1a 005 sense:CUACAAGAAAUCCCGGAAAUUU, anti-sense: UUUUCCCGGAUUUCUUGAGUU, 1a 007 sense: AGUCAAGCUUAAUGCUUUAUU, anti-sense: UAA AGCAAUAAAGCUCGACUUU, 1b 009 sense: CAUGAAAGGCUGUUAGAAUUU, anti-sense: AUUCUA ACAG CCU UUCAUUU, 1b 013 sense: GCUCGGAGAGUGGGAAAUUU, anti-sense: UUUUCCACACUCUCGGAGCUU ) were custom designed from Dharmacon. Allstars non-target siRNA used as a negative control was from Qiagen. SIGLO (Thermoscientific) allowed detection of transfection efficiencies. The Human Rab custom siRNA library from Ambion (see appendix III) was a generous gift from Ian Goodfellow at Imperial College, London.

2.3.3.3 Primers

Sar1a and b primers used to sequence Sar1 from IBRS-2 cells were from Sigma, (Sar1a forward: ATGTCCTTCATATTTGACTGG, Sar1a reverse: TCAGTCAATATACTGGGAGACCAGCGG AGCCCTCG, Sar1b reverse: TTAATCTATGTACTGGGCCATCCAGCGGAAGCTTT). The primers used to sequence p-GEM-T easy plasmid (Promega) with Sar1a and b inserts were the T7 promoter and SP6 promoter primers from Promega.

GAPDH and FMDV primers and probes (Sigma) used for quantitative PCR included: (GAPDH forward: GCATCGTGGAGGGACTGATGAC, GAPDH reverse: AGCCCCTCGGCCRTCA, GAPDH probe: [6FAM]CCATCACTGCCCACCCAGAAGACTGTG[TAM], FMD 3D forward: ACTGGGTTTTACAAA CCTGTA, FMD 3D reverse: GCGAGTCCTGCCACGGA, FMD 3D probe: [6FAM]CCTTTGCACGCCGTGGGACTAGG[TAM].

The primers used to sequence p-GEM-T easy plasmid (Promega) with Sar1a and b inserts were the T7 promoter and SP6 promoter primers from Promega.
2.4 Virus Infectivity assays

Infection was quantified using three methods: Immunofluorescence; titration of viral supernatant and Quantitative Polymerase Chain Reaction (QPCR).

2.4.1 Immunofluorescence assay

Cells were seeded on 13mm coverslips (VWR International) in 24 well tissue culture plates so as to reach 40-50% confluency overnight. Cells were transiently transfected (see section 2.5.10) with mammalian expression plasmids (see section 2.3.3.1), using Lipofectamine 2000 (Invitrogen). After incubation at 37°C for 14 hours, cells were washed and infected with FMDV O1Kcad2 for a total of 3.5 hours at MOI = 0.5. The same procedure was used for bovine enterovirus (BEV). The cells were then fixed with 4% PFM for 40 minutes at room temperature, and washed three times with PBS. Coverslips were either processed for confocal microscopy or stored at 4°C under PBS.

2.4.2 Infectivity assay

For the collection of viral supernatants and QPCR samples, cells were seeded in a 24 well tissue culture plate overnight to reach around 40-50% confluency. Transient transfection of the cells (see section 2.6.1.) was performed using Lipofectamine 2000 (Invitrogen) with siRNA (see section 2.3.3.2). After incubation at 37°C for 48hrs, cells were washed with serum free media and virus was added at an MOI =0.5 for 40 minutes at 37°C. An acid wash (see appendix I) was added for 3 minutes at room temperature to remove any unbound virus. The cells pH was neutralised with serum free media, before 1ml VGM was added. A time zero sample of either supernatant or lysed cells was collected in screw capped eppendorfs and stored at -80°C. The remaining
samples were incubated for a further 3 hrs at 37°C. After incubation the remaining supernatants were collected in screw capped eppendorfs and stored at -80°C until titrated (see section 2.2.3). The remaining cells were lysed in Qiagen AVL buffer for 10 minutes at room temperature and RNA extracted using a QiaAMP viral RNA mini kit (Qiagen).

2.5 DNA Techniques

2.5.1 Transformation of competent cells

Competent cells were thawed for 5 minutes on ice. An aliquot of DNA was added to 50μl of competent cells and the mixture incubated on ice for 30 minutes. The samples were then heat shocked for 50 seconds at 42°C, and incubated for 2 minutes on ice. 1ml of LB was added and the culture incubated for 60 minutes at 37°C. The transformed bacteria were then plated onto LB/agar plates containing a suitable antibiotic (e.g. Ampicillin [100μg/ml]).

2.5.2 Isolation of Plasmid DNA

2.5.2.1 Small scale plasmid purification (mini-preps)

Between 3-5ml of LB broth was inoculated with a single bacterial colony and incubated overnight at 37°C with continuous shaking at 200 rpm. 1.5ml of the culture was transferred to a fresh eppendorf tube and centrifuged at 13,000rpm for 1 minute. DNA was extracted using the Qiagen mini-prep system following manufacturer’s instructions. DNA was analysed on a 1% agarose gel.

2.5.2.2 Large scale plasmid purification (Maxi-preps)

Around 50-100ml of LB media was inoculated with 50μl-1ml of starter culture and grown up overnight at 37°C, 200rpm. DNA was extracted using the
Qiagen endo-toxin free maxi-prep system as per the manufacturer’s instructions, and DNA analysed after restriction digestion on a 1% agarose gel.

2.5.3 Restriction digests

Digests were carried out in a total volume of 20μl according to manufacturer’s instructions supplied with each enzyme (Promega).

2.5.4 DNA gel electrophoresis

DNA was analysed by gel electrophoresis using 1-1.2 % agarose (Invitrogen) gels containing a final concentration of 0.5μg/ml ethidium bromide solution (Promega). Gels were run at a constant 100V.

2.5.5 Nanodrop – Quantification of DNA concentration

To determine DNA concentration, one microlitre (μl) of DNA was applied to the Nanodrop (1000) spectrophotometer on the DNA-50 setting to produce a reading in nanograms per μl.

2.5.6 Polymerase chain reaction

The polymerase chain reaction (PCR) was carried out with the use of a Taq DNA polymerase kit (Invitrogen). A master-mix was created (see table below) and added to 2μl cDNA in an RNase-free tube. The Taq enzyme was added last (0.25μl/reaction). The reactions were run on a pre-programmed thermocycler (see appendix II).
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (per reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer</td>
<td>5μl</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>1.5μl</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>1μl</td>
</tr>
<tr>
<td>Forward primer (10pmol)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Reverse primer (10pmol)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>39.25μl</td>
</tr>
</tbody>
</table>

### 2.5.6.1 Quantitative PCR

Quantitative PCR reactions were carried out as follows using the Taqman PCR Master-mix from Roche, forward and reverse primers and 6FAM-TAM labelled probes (Sigma) (see section 2.3.3.3). 20μl of master-mix was added to 5μl of cDNA, produced from prior reverse transcriptase reactions (see section 2.6.3), and loaded into a 96 well plate (Abgene). Reactions were run on a MX3005 quantitative PCR machine (Stratagene)(appendix II).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taqman master-mix</td>
<td>12.5μl</td>
</tr>
<tr>
<td>Forward primer (10pmol)</td>
<td>2.25μl</td>
</tr>
<tr>
<td>Reverse primer (10pmol)</td>
<td>2.25μl</td>
</tr>
<tr>
<td>Probe (5pmol)</td>
<td>1.5μl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>1.5μl</td>
</tr>
</tbody>
</table>
2.5.7 DNA purification

DNA was purified using the Wizard PCR purification kit (Promega) as per the manufacturer’s instructions.

2.5.8 Ligations

Ligation Reactions were performed using p-GEM-T easy ligation kit (Promega) according to the manufacturer’s instructions. Ligations were performed using a vector : insert ratio of 3:1 in a total volume of 10μl. Ligation mixes were incubated overnight at 4°C, and were subsequently transformed into JM109 cells.

2.5.9 DNA sequencing

DNA sequencing was carried out using a ABI sequencing kit (Applied Biosystems) as indicated below.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (per reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer</td>
<td>1.88μl</td>
</tr>
<tr>
<td>Reaction Mix</td>
<td>0.25μl</td>
</tr>
<tr>
<td>Primer</td>
<td>1μl at 10pmol</td>
</tr>
<tr>
<td>DNA template</td>
<td>250ng plasmid DNA</td>
</tr>
<tr>
<td>Water</td>
<td>To final volume 10μl</td>
</tr>
</tbody>
</table>

The reaction was run on a thermocycler for the required length of time (see appendix II). The DNA was then ethanol precipitated, re-suspended in 40μl
of sample loading solution, and loaded on to the 96-well sequencing plate. The samples were run on a 48 capillary, 3730 DNA Analyzer (Applied Biosystems) and data analysed using Bioedit software.

2.5.10 Transient transfection of mammalian cells with plasmid DNA

Cells were seeded on sterile coverslips in transfection medium (cell culture medium without 100 U/ml penicillin and 100 μg/ml streptomycin), aiming to be around 50% confluent the next day.

Two master mixes were made; the first contained 1 μg plasmid DNA in 50 μl opti-MEM (Invitrogen) per coverslip, and the second 1 μl Lipofectamine 2000 (Invitrogen) in 50 μl opti-MEM per coverslip. Each master mix was incubated for 5 minutes at room temperature. The two were then combined, mixed and incubated at room temperature for 20 minutes.

Cells were washed once with 1 ml opti-MEM, and then 500 μl of fresh opti-MEM was added to each coverslip. One hundred microlitres of the transfection complex was added drop-wise to the 500 μl opti-MEM, and the plate agitated to mix. Coverslips were incubated at 37°C, 5% CO₂ for 4-6 hours, the opti-MEM was then removed and 1 ml transfection medium added. Each transfection was run for 12-14 hrs.

2.6 RNA Techniques

2.6.1 siRNA transfection

Cells were seeded on sterile coverslips in transfection medium so as to reach 40-50% confluency overnight. Transfection follows the method previously described in section 2.5.10, with the exception of the first master-mix which contained 10pmol of each siRNA in opt-MEM (Invitrogen) (see section2.3.3.2). All isoforms were targeted in one reaction, equalling between 30-40pmol in total
per coverslip. Both non-target siRNA (Qiagen) and SIGLO (Thermoscientific) were added at equivalent concentrations.

Cells were washed once with 1 ml opti-MEM, then 400 μl fresh opti-MEM added to each coverslip. One hundred microlitres of the transfection complex was added to the opti-MEM. Coverslips were incubated at 37°C, 5% CO₂ for 6 hours, then the opti-MEM removed and 1 ml transfection medium added. All transfected cells were incubated for a total of 48 hrs.

2.6.2 RNA isolation

Total RNA was isolated from cell lysate using the Olido-dT bead kit (Invitrogen) following manufacturer’s instructions. mRNA was separated from the oligo-dt beads by elution and denatured as protocol indicates, before proceeding onto reverse transcription reactions (RT) (see section 2.6.4).

2.6.3 RNA extraction

Cells were lysed in AVL buffer (Qiagen) at room temperature for 10 minutes and RNA extracted using QiaAMP viral RNA mini kit (Qiagen) as per manufactures instructions.

2.6.4 Reverse transcriptase reaction (RT)

A master-mix of the RT reaction was made using the Taqman reverse transcriptase reagents (Roche) and nuclease-free water, to give each reaction a final volume of 30μl. One microlitre of RNA was added per reaction prior to running on a thermocycler (see appendix II).
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (per reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer</td>
<td>3µl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>6.6µl</td>
</tr>
<tr>
<td>dNTP’s</td>
<td>6µl</td>
</tr>
<tr>
<td>Random Hexamers</td>
<td>1.5µl</td>
</tr>
<tr>
<td>RNasin</td>
<td>0.6µl</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>0.75µl</td>
</tr>
</tbody>
</table>

2.7 Immunofluorescence

2.7.1 Setting up of coverslips

Cells were plated onto borosilicate 13 mm coverslips (VWR International) in a 24 well plate, and incubated for a minimum of 16 hours at 37°C, 5% CO₂, to be between 50-80% confluent the next day.

2.7.2 Detection of intracellular antigen following cell permeabilisation

After transfection (see section 2.5.10 and 2.6.1) and/or infection (see section 2.4.1-2), coverslips were fixed with 4% PFM at room temperature for 40 minutes, then washed three times with PBS. Coverslips were either labelled immediately or stored at 4°C under PBS.

All subsequent steps were carried out at room temperature. Cells were permeabilised with 0.1% Triton X-100 (Sigma) in PBS (Sigma) for 20 minutes. Cells were then washed twice with PBS and blocked with block buffer (Tris buffered saline, gelatin from cold water fish and goat serum) for 30 minutes to
prevent any unspecific binding. Cells were incubated with a primary antibody
diluted in block buffer to detect the antigen of interest (200 μl per coverslip), for
1 hour. Coverslips were then washed three times for 5 minutes with PBS before
incubation with a species specific Alexa-Fluor labelled secondary conjugate
antibody (Molecular Probes) in block buffer at 1/200 (200 μl per coverslip), for 1
hour. Coverslips were washed as before and the nucleus stained with either 4',
6'-Diamidino-2-Phenyldinole (DAPI) (Sigma) at 1:10,000 made fresh in H₂O, for
10 minutes at room temperature, or ToPro3 (Invitrogen) at 1:8000 dilution in
PBS, for 5 minutes. Each coverslip was then washed three times with PBS
before being dipped in deionised H₂O and the excess water blotted off.
Coverslips were mounted in vectashield mounting medium for fluorescence
(Vector Laboratories), on a glass slide (Agar Scientific), sealed with clear nail
varnish, and stored at 4°C.

2.7.3 Treatment of coverslips with pharmacological inhibitors prior to
infection

Cells were prepared on coverslips as in method 2.7.1. The following day
cells were washed twice with serum-free medium, and pre-treated with 200 μl
drug at pre-determined concentrations or mock-treated (including the solvent for
the drug) for 30 minutes at 37°C, 5% CO₂. Cells were then washed with serum-
free medium and virus (MOI = 0.5) added diluted in drug or solvent (for mock-
treatment) for 1 hour at 37°C, 5% CO₂. Infection was continued in the presence
of the drug as in method 2.4.1.

2.8 Image Capture

All coverslips were viewed on a Leica SP2 Confocal Scanning Laser
Microscope and images were taken (n= ≥500 cells) using either the 63 x or 40 x
lens. All data was collected sequentially to minimise cross-talk between
fluorescent antibodies. Images were processed using Adobe Photoshop software.

2.9 Data Analysis

Student’s $t$ test was used to determine statistical significance ($P$ values: * $<0.05$, ** $<0.01$, *** $<0.001$), $n= >500$ cells.

2.10 Transmission Electronmicroscopy (TEM)

Cells were seeded on Thermanox coverslips (VWR International) in 24 well plates so cells would reach 50-80% confluency overnight. Coverslips were infected with FMDV-O for 3.5 hours in total (see section 2.4.1) before the addition of EM fixative (see appendix I) to chemically fix the samples.

Samples were left in EM fixative for 90 minutes on a rotator at room temperature then fixed in 1% osmium tetroxide (Agar Scientific) for 60 minutes at room temperature. The cells were then dehydrated in an ethanol series:

<table>
<thead>
<tr>
<th>Ethanol</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>30 minutes</td>
</tr>
<tr>
<td>90% ethanol</td>
<td>15 minutes</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3x10 minutes</td>
</tr>
</tbody>
</table>

Coverslips were transferred to polythene cups (TAAB, UK) and wash in propylene oxide (Agar Scientific) for 10 minutes prior to the addition of a 1:1 mix of propylene oxide and epoxy resin (Agar Scientific, made up as manufacturer’s instructions indicate) for 60 minutes on a rotatory mixer at room temperature. The above mix was replaced with 100% resin and incubated for 60 minutes at room temperature.
Coverslips were then transferred into fresh polythene cups with fresh resin ensuring the cells were facing upwards, and allowed to polymerise overnight at 60°C. Approximately 20 hours later, the Thermanox coverslips were peeled from the blocks. Sections (70nm) of the resin blocks were collected onto copper grids and stained with uranyl acetate and lead citrate in a Leica EM AC20 staining machine. Images were taken using the Phillips Technai 12 TEM with TVIPS F214 2Kx2K digital camera.

2.11 Western Blot

Samples were prepared by washing cells in PBS before lysing with Cellytic M cell lysis reagent (Sigma) for 2 minutes at room temperature. Lysates were collected in 1.5ml eppendorfs and spun at 13,000 rpm for 5 minutes at room temperature to remove cellular debris. The supernatant was either diluted in PBS (1 in 30) for protein quantification or added to reducing lane marker sample buffer (5X) (Thermoscientific) for western blot. Western blot samples were heated to 90°C for 5 minutes and allowed to cool to room temperature.

A micro Bioinchroninic (BCA) assay (Thermoscientific-Pierce) was carried out as per manufacturer's instructions on the PBS diluted samples, to quantify the concentration of protein per sample, allowing the equal loading of protein onto the SDS-PAGE gels.

Samples were run on an 10% acrylamide resolving gel (including 1.5 M Tris pH 8.8) and a 2.5% acrylamide stacking gel (including 1 m Tris pH 6.8) (see appendix I ). Samples were loaded onto the gel using a Hamilton syringe, and run at 100-200 V. The proteins were transferred onto Hybond-C extra membrane (Amersham) at 100 V for 1.5 hour.
The membrane was incubated in block buffer (5% semi-skimmed milk (marvel) in 1x PBS-Tween), with continuous rotation overnight at 4°C. Labelling was carried out at room temperature. The membrane was then incubated with primary antibody in block buffer for 1 hour and then washed in 1 x PBS-Tween for 1 hour, changing the wash every 15 minutes. The membrane was then incubated with the appropriate secondary HRP-conjugated antibody in block buffer for 1 hour and then washed as above. The membrane was finally incubated in ECL reagents (Pierce), made up as per manufacturer’s instructions, for 5 minutes. The bands were visualised on Kodak film developed manually using Polycon Manual X-ray Developer (Champion Photochemistry) and B&W Amfix, Film and Paper Fixer (Champion Photochemistry).
Chapter 3: Investigating the Early Secretory Pathway in FMDV Infection

3.1 Introduction

Picornaviruses induce host cell membrane rearrangements to facilitate their replication. These membranes are believed to act as platforms for formation of viral replication complexes (RC). Membranes of the early secretory pathway have been implicated in the formation of RC’s by several picornaviruses. Early studies with poliovirus (PV) have implicated the ER (Rust et al., 2001) as the source of membranes for RC formation whereas more recent studies have identified a role for proteins associated with the Golgi membranes (Belov et al., 2005; Belov et al., 2007b; Cho et al., 1994; Hsu et al., 2010) (see section 1.6.4).

For both poliovirus and coxsackie B3 the 3A protein has been shown to block protein transport through the secretory pathway. In contrast, for FMDV 2BC and not 3A was shown to block protein transport (Moffat et al., 2005). This block may indicate a role for 2BC and the early secretory pathway in FMDV replication and formation of RCs. However, the interaction of FMDV 2BC with host cellular factors within the early secretory pathway has yet to be established.

The IBRS-2 kidney cell line derived from the natural porcine host of FMDV was used to investigate the role of the early secretory pathway in FMDV replication.
3.2 Membrane rearrangements in FMDV infected cells

Membrane rearrangements have been observed in BHK21 cells infected with FMDV (Monaghan et al., 2004) (see section 1.6.4). To determine if host cell membrane rearrangements also occur in IBRS-2 cells, infected and uninfected cells were compared by TEM. Cells were infected with FMDV O1Kcad2 (MOI 0.8) for 3.5 hours. Infected and uninfected cells were then processed for TEM (see methods, 2.9). Figure 3.1 shows uninfected (panels A-B) or infected cells (panels C-D). Uninfected cells show a normal complement of organelles in the cytosol (Fig 3.1, A-B), whereas infection with FMDV resulted in the formation of membrane vesicles (Fig 3.1, C-D). In infected cells remnants of the ER and a large number of membranous vesicles are present in the cytosol. The majority of these vesicles appear to possess a single membrane (Fig 3.1, panel C-D) although on occasion double membrane vesicles are seen. These results are similar to those seen previously for BHK cells and confirm that extensive membrane rearrangements are induced by FMDV infection of IBRS-2 cells.

3.3 The effect of FMDV infection on membranes in the early secretory pathway

Next I investigated the effects of FMDV infection on the membranes in the early secretory pathway. IBRS-2 cells were mock-infected or infected with FMDV O1Kcad2 (MOI 0.5) for 3.5 hours. The cells were then fixed with 4% pfm and permeabilised using 0.1% Triton-X and then blocked for 1 hour in blocking buffer (see methods 2.7). The cells were then labelled for either the ER, the ERGIC or the Golgi using mouse antibodies to PDI, ERGIC53 or GM130 respectively and a goat anti-mouse Alexa-488 conjugated secondary antibody.
Figure 3.1 FMDV infection induces host cell membrane rearrangements in IBRS-2 cells
IBRS-2 cells were infected with FMDV (MOI 0.8) for 3.5 hours. Cells were fixed and processed for TEM. Panels (A-B) show uninfected IBRS-2 cells and panels (C-D) show FMDV infected cells containing vesicles (black arrow). Scale bars shown at the bottom of each panel.
The cells were co-labelled for FMDV infection using a rabbit polyclonal antibody to type O virus and a goat anti-rabbit Alexa-568 conjugated secondary antibody.

Figure 3.2 shows labelling for mock-infected cells. Panel A shows a normal labelling pattern characteristic of the ER. Panel B shows labelling for the ERGIC; labelling was dispersed throughout the cytosol but most cells also showed a peri-nuclear area where labelling appeared more concentrated. Panel C shows a typical labelling pattern expected for the Golgi which is normally located in a peri-nuclear region.

Figure 3.3 shows cells infected with FMDV (shown in red, panels B and D) and labelled for the ER (PDI shown in green). The ER labelling was greatly reduced in infected cells and some cells showed localised areas of labelling in the cytosol. Figure 3.4 shows cells infected with FMDV (shown in red, panels B and D) and labelled for the ERGIC (shown in green). ERGIC labelling is greatly reduced in cells that appear at an advanced stage of infection (as judged by the intense labelling for FMDV) (Fig 3.4, panels A-B) while in cells that appeared to be at an early stage of infection the ERGIC was still present but fragmented and concentrated into large puncta (Fig 3.4 panels C-D). Figure 3.5 shows cells infected with FMDV (shown in red, panels B and D) and labelled for the Golgi (GM130, shown in green). The Golgi is clearly dispersed throughout the cytoplasm in cells infected with FMDV. These results show that FMDV infection results in major disruption of membranes of the secretory pathway.

3.4 The effect of Brefeldin A on membranes in the early secretory pathway

Brefeldin A (BfA) is a fungal metabolite that blocks protein transport through the secretory pathway and causes a redistribution of Golgi proteins
Figure 3.2 Labelling membranes of the secretory pathway
IBRS-2 cells on coverslips were fixed, permeabilized and labelled for (A) the ER (PDI), (B) the ERGIC (ERGIC53) or (C) the Golgi (GM130) using compartment specific primary antibodies and an appropriate Alexa-488 conjugated secondary antibody (shown as green on the figure). The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 µm.
Figure 3.3 FMDV infection disrupts ER membranes in IBRS-2 cells
IBRS-2 cells were infected with FMDV and labelled for the ER (PDI) and FMDV infection. FMDV infection is labelled in red. ER labelling is shown in green. Panels A and C show the signal for PDI. Panels B and D showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.
Figure 3.4 FMDV infection disrupts ERGIC membranes in IBRS-2 cells

IBRS-2 cells were infected with FMDV and labelled for the ERGIC (ERGIC53) and FMDV infection. FMDV infection is labelled in red, ERGIC labelling is shown in green. Panels A and C show the signal for ERGIC53. Panels B and D showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.
Figure 3.5 FMDV infection disrupts Golgi membranes in IBRS-2 cells
IBRS-2 cells were infected with FMDV and labelled for the Golgi (GM130) and FMDV infection. FMDV infection is labelled in red. Golgi labelling is shown in green. Panels A and C show the signal for GM130. Panels B and D showed merged images for red and green fluorescence. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.
back to the ER (Doms et al., 1989; Lippincott-Schwartz et al., 1990; Lippincott-Schwartz et al., 1989) (see section 1.6.4.1). Brefeldin-A inhibits nucleotide exchange by Arf1 (Donaldson et al., 1992; Helms and Rothman, 1992) by trapping the Sec7 domain of GBF1 in a non-productive complex with GDP and therefore preventing Arf activation (Mossessova et al., 2003; Renault et al., 2003) and the recruitment of COPI components onto target membranes (Klausner et al., 1992; Orci et al., 1991). Hence BfA inhibits both protein secretion and PV replication. In contrast FMDV is insensitive to BfA. Some cells (e.g. Madin-Darby Canine Kidney cells) carry mutations in the Sec7 domain of GBF1 which results in a resistance to BfA (Niu et al., 2005). Therefore I characterised the effects of BfA on the membranes in the early secretory pathway in IBRS-2 cells.

Cells were treated with DMSO (mock treated cells) or BfA (5μg/ml) for 30 minutes prior to being fixed in 4% pfm and processed for immunofluorescence confocal microscopy (IFCM). Cells were labelled for the ER (using an antibody to PDI), the ERGIC (using an antibody to ERGIC53) or the Golgi (using an antibody to GM130) as described above. Figure 3.6 shows labelling for mock and BfA treated cells. Characteristic labelling was seen in mock treated cells for the ER (Fig 3.6, panel A) the ERGIC (Fig 3.6, panel C) and the Golgi (Fig 3.6, panel E). Figure 3.6 also shows the effect of BfA on membrane labelling. In comparison to the mock treated cells, BfA appears to cause a concentration of ER labelling (red) around the peri-nuclear region (blue) (Fig 3.6 shown as red, panel B), although this effect was not strongly observed in all cells. Labelling for the Golgi was greatly reduced and dispersed by BfA (Fig 3.6, panels F). Changes in the ERGIC were less dramatic than those in the Golgi;
Figure 3.6 Brefeldin A treatment disrupts the Golgi and alters ER membranes in IBRS-2 cells
IBRS-2 cells were mock treated with DMSO (panels A, C & E) or Brefeldin-A (5μg/ml) (panels B, D & F) for 30 minutes and labelled for the ER (PDI; panels A-B), the ERGIC (ERGIC53; panels C-D) or the Golgi (GM130; panels E-F). The ER and ERGIC are shown in red and the Golgi shown in green. The cell nuclei were stained with DAPI and are shown as blue. Arrow indicates concentration of ER labelling around nuclei. Scale bar = 10 μm.
however, the ERGIC labelling in the peri-nuclear region was dispersed in virtually all cells (Fig 3.6, panel D).

3.5 The effects of Brefeldin A on FMDV infection

The above results confirm that BfA disrupts membranes of the secretory pathway in IBRS-2 cells. Previous published experiments have shown that FMDV infection is insensitive to BfA as virus yield is not reduced by exposure of cells to this reagent (Monaghan et al., 2004; O'Donnell et al., 2005). Next I confirmed the insensitivity of FMDV to BfA using IBRS-2 cells and BEV as a control virus that is known to be BfA sensitive (Monaghan et al., 2004).

IBRS-2 cells were mock treated with DMSO or incubated with BfA (5µg/ml) for 30 minutes prior to infection with either FMDV O1Kcad2 or BEV-1 (MOI 0.5) for 3.5 hours. The cells were incubated with BfA (or DMSO) for the duration of the experiment. The cells were fixed in 4% pfm, permeabilised with 0.1% Triton-X and blocked in goat serum prior to labelling with anti-FMDV or anti-BEV-1 antibodies and the appropriate Alexa Fluor-conjugated secondary antibody and processed for IFCM.

Figure 3.7 shows cells treated with DMSO (panels A and C) or BfA (panels B and D) and infected with FMDV (infected cells are shown in red on panels A-B) or BEV-1 (infected cells are shown in red on panels C-D). Brefeldin A treatment does not appear to have an effect on FMDV infection (A-B). In contrast, BEV-1 labelling was not seen in BfA treated cells indicating that infection was inhibited. The above experiments confirm that FMDV infection of IBRS-2 cells is insensitive to BfA treatment.

The proportion of mock- or BfA-treated cells that were infected was determined. Approximately ~50% of DMSO treated cells and ~60% of BfA treated cells were
Figure 3.7 Brefeldin A blocks BEV-1 but not FMDV infection in IBRS-2 cells
IBRS-2 cells were treated with BfA (5μg/ml) (B & D) or DMSO (A & C) for 30 minutes and infected with either FMDV or BEV-1. Infected cells are shown as red. Panels A and B show labelling for FMDV. Panels C and D show labelling for BEV-1. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.
Figure 3.8 The effect of Brefeldin A treatment on FMDV infection
IBRS-2 cells were treated with DMSO or Brefeldin A (5ug/ml) for 30 minutes prior to infection with FMDV for 3.5 hours. The proportion of cells infected is shown as the mean +/- SD of triplicate samples.
Figure 3.9 Brefeldin A treatment blocks BEV-1 infection in IBRS-2 cells
IBRS-2 cells were treated with DMSO or Brefeldin A (5ug/ml) for 30 minutes prior to infection with BEV for 3.5 hours. The proportion of cells infected is shown as the mean +/- SD of triplicate samples.
infected with FMDV (Fig 3.8) while for BEV, ~50% of mock treated cells were infected whereas infection of the BfA treated cells was completely inhibited (Fig 3.9). These results confirm that FMDV infection of IBRS-2 cells is insensitive to BfA.

3.6 Protein Kinase Inhibitor H89

The above experiments show that FMDV infection is not inhibited by BfA and therefore unlikely to require Golgi membranes for replication. Therefore it is possible that FMDV replication may require membranes derived from an earlier stage of the secretory pathway (e.g. the ER or ERGIC).

The isoquinolinesulfonamide H89 is a protein kinase inhibitor and when used in the micro molar range it blocks ER exit without affecting retrograde transport from the ERGIC or Golgi to the ER. The block to ER exit is believed to results from a failure of SEC13 to be recruited to ERES (Lee and Linstedt, 2000).

3.6.1 H89 Blocks FMDV Infection

To determine if FMDV replication was inhibited by a block to ER export, cells were treated with H89 (50 or 100µm) or mock treated with DMSO for 30 minutes prior to infection with either FMDV O1Kcad2 or BEV-1 (MOI 0.5) for 3.5 hours. The cells were incubated with H89 for the duration of the experiment. Cells were fixed in 4% pfm and labelled with either anti-FMDV or anti-BEV-1 antibodies (as described above). Figure 3.10 shows cells treated with DMSO (panels A and D) or H89 (100µm panels B and E or 50µm panel C) and infected with FMDV (shown in red, A-C) or BEV-1 (shown in red, D-E). Infection of both FMDV and BEV-1 was completely inhibited by treatment with 100µm H89.
**Figure 3.10 H89 blocks BEV-1 and FMDV infection**

IBRS-2 cells were pre-treated with DMSO (panels A & D), 50 μm H89 (panel C) or 100μm H89 (panels B & E) and infected with FMDV or BEV-1. Infection is shown in red. Panels A-C show FMDV infected cells. Panels D-E show BEV-1 infected cells. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.
Fig 3.10 B and E). A reduction in FMDV infection was also observed in cells treated with 50μm H89 (Fig 3.10 C). The proportion of H89 or mock treated cells that were infected with FMDV or BEV was determined and is shown in figure 3.11. Approximately 50% of mock treated cells (DMSO) were infected with FMDV, whereas H89 treatment dramatically inhibited infection (Fig 3.11, panel A-B). Figure 3.12 shows that H89 (100μm) also inhibited BEV infection. The block of FMDV and BEV-1 infection by H89 suggests that both viruses rely on the early secretory pathway for replication.

3.7 Endoplasmic Reticulum (ER) Stress

The previous experiment suggested FMDV requires the early secretory pathway for replication. An imbalance in protein homeostasis in the ER activates the Unfolded Protein Response (UPR) (also known as the ER Stress Response [ERSR]) (Patil and Walter, 2001; Schroder and Kaufman, 2005a, b). It has been reported that induction of ER stress results in a reduction in ER export and therefore COPII vesicle formation (Amodio et al., 2009). To determine if FMDV infection is effected by ER stress, I investigated the effect of Thapsigargin (TG) on FMDV infection. Thapsigargin is a specific inhibitor of endoplasmic Ca (2+)-adenosine 5'-triphosphatase (Ca (2+)-ATPase) and induces ER stress.

3.7.1 The effect of ER stress on membranes in the early secretory pathway

Initially I investigated the effects of ER stress on membranes in the early secretory pathway using confocal microscopy. TG (300nM) was used to induce ER stress in IBRS-2 cells. Cells were incubated with TG for 4 hours, fixed in 4% pfm and processed for IFCM. Figure 3.13 shows the effects of ER stress on the ER (labelling for PDI, panel B) the ERGIC (labelling for ERGIC53, panel D)
Figure 3.11 FMDV infection is inhibited by H89
IBRS-2 cells were treated with DMSO and 50 or 100 μm H89 for 30 minutes prior to infection with FMDV for 3.5 hours. The proportion of cells infected is shown. Panel (A) shows the effect of 50 μm H89 on FMDV infection. Panel (B) shows the effect of 100 μm H89 on FMDV infection.
Figure 3.12 BEV-1 infection is blocked by H89 treatment
IBRS-2 cells were treated with DMSO or H89 (100µm) for 30 minutes prior to infection with BEV-1 for 3.5 hours. The proportion of cells infected is shown.
and the Golgi (labelling for GM130, panel F) in comparison to mock treated cells (DMSO, panels A, C and E). The labelling for the ER in TG treated cells appears to become condensed around the nucleus in comparison to mock treated cells although this effect was marginal in some cells (Fig 3.13, panels A-B). The ERGIC labelling appears to be dispersed or completely absent (Fig 3.13, panels C-D). In contrast, the Golgi labelling was still detected, however the Golgi did appear fragmented (Fig 3.13, panels E-F). These observations suggest that ER stress is causing an accumulation of protein at the ER due to reduced ER export and this in turn is preventing the formation of the ERGIC. However, these results suggest that TG may have an effect on the Golgi which prevents complete disruption of this organelle.

### 3.7.2 ER stress does not block FMDV infection

Next TG treated cells were infected with FMDV to establish if ER stress would have any effect on FMDV infection. Cells were treated with TG as described above (section 3.7.1) and infected with FMDV O1Kcad₂ for 3.5 hours. The cells were fixed in 4% pfm and processed for IFCM using the anti-FMDV antibody as described above. Figure 3.14 shows mock treated cells (panel A) or TG treated cells (panel B) infected with FMDV. The proportion of mock- or TG-treated cells that were infected was determined. Approximately ~50% of DMSO treated cells and ~60% of TG treated cells were infected with FMDV (Fig 3.15). These results suggest that ER Stress does not block FMDV infection (see discussion, section 3.8).
Figure 3.13 Thasparginin (TG) alters membranes in the early secretory pathway
IBRS-2 cells were pre-treated with DMSO (panels A, C & E) or 300nM TG (panels B, D & F) for
4 hours and labelled for the ER (PDI; panels A-B), the ERGIC (ERGIC53; panels C-D) and the
Golgi (GM130; panels E-F). The ER, ERGIC and Golgi are shown in green. The cell nuclei were
stained with DAPI and are shown as blue. Arrow indicates concentarted ER labelling around the
nucleus (panel B). Scale bar = 10 μm.
Figure 3.14 ER stress did not effect the frequency of FMDV infection in cells
iBRS-2 cells were pre-treated with DMSO (panel A) or 300nM Thapsigargin (panel B) for 4 hours and infected with FMDV for 3.5 hours. Cells labelled for FMDV are shown in red. The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.
Figure 3.15 ER stress does not inhibit FMDV infection in IBRS-2 cells
IBRS-2 cells were pre-treated with DMSO or Thapsigargin (TG) (300nM) for 4 hrs before infection with FMDV for 3.5 hrs. The proportion of cells infected is shown as the mean +/- SD of triplicate samples.
3.8 Discussion

In this chapter I have examined the feasibility of using IBRS-2 cells to investigate FMDV induced membrane rearrangements using drugs that inhibit the secretory pathway and immunofluorescent confocal microscopy and Transmission Electron Microscopy (TEM).

All positive strand RNA viruses are known to induce host cell membrane rearrangements. Figure 3.1 shows evidence of membrane rearrangements and the formation of large membrane vesicles in FMDV infected IBRS-2 cells. The clearing of the cytoplasm and re-distribution of the cells cytoplasmic contents to one side of the nucleus was similar but not as extensive as seen in BHK21 cells (Monaghan et al., 2004). These differences indicate that subtle differences in membrane rearrangements may occur in different cell lines.

The data in figure 3.3 shows that FMDV infection results in a major disruption of membranes in the early secretory pathway. The observed disruption to membranes in the early secretory pathway may be due to the action of the precursor 2BC which is reported to block protein secretion (Moffat et al., 2005; Moffat et al., 2007). From these experiments it is not clear if FMDV has a direct effect on the ERGIC and Golgi or if disruption of the ER results in a reduced flow of membranes from the ER to the ERGIC/Golgi, hence the disruption of these membrane compartments.

Brefeldin A inhibits protein transport by preventing the activation of Arf1. Brefeldin A treatment caused a loss of labelling for the Golgi marker GM130 and the slight concentration of labelling for ER proteins. These observations agree with published data that shows that BfA causes disruption of the Golgi and the re-absorption of protein back into the ER (Sciaky et al., 1997).
Brefeldin A is not thought to stop ER export and therefore does not collapse the ERGIC (Lippincott-Schwartz et al., 1990); however our data saw a slight loss to the peri-nuclear region of the ERGIC labelling. This difference may be due to the loss of the cis-Golgi that is thought to be continuous with the ERGIC and labelled with ERGIC53. However, the data appears to agree with another study that saw the redistribution of the ERGIC and Golgi to peripheral structures (Amodio 2009).

The results of the experiment shown in figure 3.7 confirm that BfA blocks BEV-1 but not FMDV infection and that IBRS-2 cells are susceptible to BfA treatment. Quantification of these results shows a complete loss to BEV infection, whereas a 10% increase to FMDV infection was observed. The increase in FMDV infection may suggest that BfA is enhancing FMDV replication; however such a small increase is not conclusive evidence.

The protein kinase inhibitor H89 (100μM) blocks FMDV and BEV infection completely when added immediately after virus entry, which suggests that H89 is blocking replication. When used at 50μM, H89 treatment is reported to cause a slow redistribution of Golgi proteins back to the ER (GM130 ~2hr) supporting that anterograde, but not retrograde transport is blocked. As FMDV replication does not require an intact Golgi, this suggests that FMDV may require membranes of the secretory pathway before the Golgi complex. The block to BEV infection may be due to the fact that anterograde transport is required for infection or that H89 treatment is in fact interfering with retrograde transport at the Golgi.

Endoplasmic reticulum stress did not block infection; in fact around a 10% increase was observed. The observation that ER stress did not have any
detrimental effect on FMDV infection could be due to an incomplete block to COPII transport as Amodio et al (Amodio et al., 2009) showed only a 50% reduction in ER export in cells treated with Thapsigargin (300nM).

In summary, FMDV infection appears to disrupt membranes in the early secretory pathway and cause the formation of large membrane vesicles. In addition, infection is inhibited by a block to ER export by H89 but not by BfA which affects retrograde transport. Overall, it appears FMDV requires the early secretory pathway before the BfA sensitive step and does not appear to require an intact Golgi.
Chapter 4: Investigating the Role of Sar1 in FMDV Infection

4.1 Introduction

The experiments of the previous chapter confirmed the insensitivity of FMDV to BfA and suggested that FMDV infection requires the early secretory pathway. The sensitivity of PV to BfA results from its inhibitory effect on GBF1, the GEF of Arf1 (Donaldson et al., 1992; Helms and Rothman, 1992) (see section 1.6.4.1). One of the major differences between FMDV and PV is that FMDV is insensitive to BfA (Gazina et al., 2002; Martin-Acebes et al., 2008; Monaghan et al., 2004; O'Donnell et al., 2001). Therefore, it can be assumed that FMDV does not require GBF1 or Arf1 for replication, and that FMDV is likely to be utilising other components of the early secretory pathway (before a BfA sensitive step) during infection. COPII formation is mediated by Sar1 and its GEF, Sec12; it occurs before a GBF1/Arf1 mediated step and is unaffected by BfA treatment (Lippincott-Schwartz, 2001; Orci et al., 1993; Ward et al., 2001). In this chapter a role for Sar1 in FMDV infection is investigated.

Sar1 is bound to GDP in the cytosol and converted to a GTP-bound (or active) form by its GEF, Sec12. Conversion to a GTP-bound form results in an insertion of Sar1 into the ER membrane and the recruitment of the COPII complex proteins that are required to form COPII transport vesicles. Sar1-GTP is then hydrolysed by its GAP and disassociates from the membrane which promotes disassembly of COPII coats (see section 1.8.1).

The involvement of Sar1 in FMDV infection was investigated using dominant-negative (dn) mutants of the protein, Sar1a T39N-CFP and Sar1a H79G-CFP. Dominant-negative Sar1a T39N-CFP is GTP-restricted and
remains bound to GDP, which prevents Sar1 from interacting with its GEF, Sec12 (Kuge et al., 1994) and hence, Sar1 activation. The dn activity of Sar1 T39N results from a block to COPII vesicle formation and budding, and leads to the retention of transport proteins in the ER, ultimately blocking anterograde transport (Aridor et al., 1995). Dominant-negative Sar1a H79G-CFP remains constitutively active (GTP-bound) and stabilises COPII coats (Dascher and Balch, 1994; Kuge et al., 1994; Oka and Nakano, 1994; Tanigawa et al., 1993). It does not interfere with coat assembly, or COPII vesicle formation or budding, however, COPII coat stabilisation prevents the disassociation of COPII and the subsequent acquisition of COPI (i.e. COPII/COPI exchange). In cells expressing dn Sar1a H79G, COPII vesicles appear to accumulate close to the ER in structures that resemble VTCs (Aridor et al., 1995; Ward et al., 2001). Although dn Sar1a H79G-CFP is locked in its GTP-bound form and therefore constitutively active, the fact that Sar1 cannot be released from the COPII complex results in an arrested transport at the ERES (Altan-Bonnet et al., 2004). Therefore Sar1a H79G-CFP is considered to be a dn protein.

4.2 Investigating the role of Sar1 on FMDV infection using Dominant-negative proteins

4.2.1 The effects of dominant-negative Sar1a on membranes in the early secretory pathway

Initially, the effect of dn Sar1 on membranes of the early secretory pathway was investigated by immunofluorescence confocal microscopy (see methods 2.7) using IBRS-2 cells. IBRS-2 cells were transfected to express either wt Sar1a, dn Sar1a T39N or dn Sar1a H79G as fusions with CFP (which allows for detection of transfected cells) for 14 hours. The cells were fixed with PFM and permeabilised with 0.1% Triton-X. They were then blocked in goat
serum prior to labelling with antibodies to the ER (PDI), the ERGIC (ERGIC-53) or the Golgi (GM130 and 23C) and the appropriate Alexa-Fluor labelled secondary antibody. Cells expressing a transgene (i.e. a Sar1 protein) are shown as Cyan on the figures.

Untransfected cells were similarly labelled for the ER, ERGIC and the Golgi (shown in red). Figure 4.1 shows the typical labelling patterns for these compartments. As expected, labelling for the ER was seen throughout the cytoplasm (Fig 4.1, panel A) and labelling for the Golgi was confined to a peri-nuclear region (Fig 4.1, panel B). Labelling for the ERGIC was also predominantly located to the peri-nuclear region but also showed some puncta dispersed through the cytosol (Fig 4.1, panel C).

Figure 4.2 shows cells expressing wt Sar1a-CFP (shown as cyan in panels B and D) and labelled for the ER (shown as red on panels A-B) or the ERGIC (shown as red on panels C-D). A slight reduction in ER labelling was observed in cells expressing the wt Sar1a-CFP (Fig 4.2 A-B). However the ERGIC showed no obvious loss of labelling or integrity (Fig 4.2 C-D). Figure 4.3 shows cells expressing wt Sar1-CFP (shown as cyan, panels B and D) labelled for the Golgi with either GM130 (shown as red, panels A-B) or 23C (shown as red, panels C-D). Transfection of wt Sar1a-CFP caused no noticeable effect to either of the Golgi proteins. In general, the expression of wt Sar1-CFP did not appear to have any major effect on the membranes of the ERGIC and Golgi but did cause a slight loss to ER labelling. The loss of ER labelling may result from a dn effect of over-expression of wt Sar1. However, overall, it appears that wt Sar1a-CFP does not alter the ERGIC or Golgi membranes and therefore is unlikely to have a major inhibitory effect on the secretory pathway.
Figure 4.1 Labelling membranes of the secretory pathway
iBRS-2 cells on coverslips were fixed, permeabilized and labelled for (A) the ER (PDI), (B) the ERGIC (ERGIC53) or (C) the Golgi (GM130) using compartment specific primary antibodies and an appropriate Alexa-568 conjugated secondary antibody (shown as red on the figure). The cell nuclei were stained with DAPI and are shown as blue. Scale bar = 10 μm.