

Picornavirus Assembly in Recombinant Systems

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Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

Imperial College London

Department of Life Sciences

Declaration of originality

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Acknowledgements

Firstly I would like to thank Toby Tuthill for his supervision during the four years of my PhD. His enthusiasm for Picornavirus Molecular Biology is infectious (excuse the pun). His encouragement and confidence in allowing me to discuss, develop and explore new ideas whilst always providing support when problems were encountered, was I feel, invaluable to my development throughout my PhD and in future positions.

I would like to thank my co-supervisor Terry Jackson for his input into my PhD and for providing an environment in which the science of the Picornavirus Structure and Molecular Biology groups could thrive through sharing ideas and providing assistance to each-other. To my university supervisor Stephen Curry who was available to discuss ideas and for support.

Thanks to all past and present members of the Picornavirus groups during my time here (Gareth, Jenny, Pippa, Grace, Lisa, Stephen, Lidia, Emma, Julian, Beck, Hannah and Stuart C) you've been great to work with and great friends throughout my time at the institute. To James, Amin and Alison, thank you for also helping me with various aspects of the work and to Stuart R for constantly correcting my radioactivity calculations! To Anusha and Sarah for constant support throughout and for providing guidance. To my housemate Kyle for a great 4 years; there are not many people that would have put up with me for that long! To Daryl, Ryan, Aravindh, Paul, Tony, Nick, Ali, Mohammad, Martey, Fufa, Murali, Cloudy, Jacqui, Manju, Miki, Sabine, Josie, Jamie, Piers, Jordan and the students and the bike club all for making my time here memorable.

Finally I'd like to thank Caroline, mum, dad, Jamie, Nic (and Aria and Rory!) for their understanding, encouragement and love.

Thank you!

Abstract

Foot-and-mouth disease virus (FMDV) is a member of the picornavirus family of non-enveloped, positive-sense, single-stranded RNA viruses. Picornavirus assembly involves the multimerisation of a capsid subunit (P1 or P1-2A) into pentameric structures, which further assemble into intact capsids containing the viral RNA genome.

The capsid subunit is co-translationally myristoylated and proteolytically cleaved by a viral protease ($3C^{pro}$) to initiate the assembly cascade. A cell-free assay was developed to analyse the requirement for these processes in pentamer assembly. Pentamer assembly was found to be dependent on myristoylation. In these assays, two $3C^{pro}$ recognition sites in the P1 protein could be cleaved independently and were both required for the efficient formation of pentamers. In addition, a system was developed for the production of large quantities of purified recombinant capsid precursor that could be used for future structural studies.

Existing studies have shown that molecular chaperones such as heat-shock protein 90 (Hsp90) are required for capsid assembly of other picornaviruses. Pharmacological inhibition of Hsp90 reduced growth of FMDV in cell culture and prevented pentamer assembly in the cell-free assay. Hsp90 was not required for processing, which contrasted with existing models for this part of the picornavirus life cycle.

Upon RNA encapsidation a maturation cleavage occurs on the inside of the capsid generating the structural protein VP4. Upon virus entry into cells, capsids disassemble into pentamers that no longer contain VP4. Pentamers from the disassembly pathway are therefore thought to have different properties than pentamers found on the assembly pathway. A maturation-like cleavage event was engineered into recombinant pentamers. Cleavage of this site altered pentamer sedimentation from that expected of assembly pentamers (14S) to that expected of disassembly pentamers (12S). This confirmed that loss of VP4 from pentamers controlled the switch in the properties of these pentamers.

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Abbreviations

17DMAG	17-Dimethylaminoethylamino-17-demethoxygeldanamycin
Δ	Deletion
μ (prefix)	micro
μg/μl	microgram(s) per microliter
μg/ml	microgram(s) per millilitre
μl	microliter(s)
μM	micromolar
°C	Degrees Celsius
Å	Ångstrom (10^{-10} m)
Ab	Antibody
ABS	Adult bovine serum
APS	Ammonium persulphate
ATP	Adenosine triphosphate
ATPase	Adenylpyrophosphatase
bp	Base pairs
BrdU	5-Bromo-2'-Deoxyuridine
BSA	Bovine serum albumin
C-	Carboxyl
c (prefix)	centi
Co ²⁺	Cobalt ions
CV	Coxsackie virus
d (prefix)	Deoxy
dH ₂ O	Deionised Water
DMEM	Dulbecco's minimum essential media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ds	Double-stranded
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
ELISA	Enzyme linked immunosorbant assay
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
EV	Enterovirus
FCS	Foetal calf serum
FMDV	Foot-and-mouth disease virus
<i>g</i>	acceleration due to gravity
GA	Geldanamycin
GTP	Guanine triphosphate
GuHCl	Guanidine Hydrochloride
HAV	Hepatitis A virus

HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
His	histidine
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
hr	Hour/s
HRP	Horseradish Peroxidase
HRV	Human Rhinovirus
Hsp	Heat shock protein
IMAC	Immobilised metal affinity chromatography
IP	Immuno-precipitation
IPTG	Isopropyl- β -D-thiogalactopyranoside
IRES	Internal ribosome entry site
k (prefix)	Kilo
kDa	kilodaltons
KOD	kodakaraensis
L	Litre/s
LB	Luria-Bertani culture medium
M	Molar
m (prefix)	Milli
μ M	Micromolar
MCS	Multiple Cloning Site
μ	Microgram/s
mg	Milligram/s
min	Minute/s
ml	Millilitre
MOI	Multiplicity of infection
MW	Molecular Weight
n (prefix)	Nano
N/A	Not applicable
NEB	New England Biolabs
Ni ²⁺	Nickel ions
NMT	N-myristoyltransferase
NTP	Nucleotide Triphosphate
OD	Optical Density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCBP	poly(rC) binding protein
PCR	Polymerase chain reaction
PV	Poliovirus
PVDF	Polyvinylidene difluoride
r (prefix)	Ribo
RdRp	RNA-dependent-RNA-polymerase
RNA	Ribonucleic acid
RRLs	Rabbit reticulocyte lysates

S	Sedimentation coefficient
S ³⁵	Sulphur-35
SDS	Sodium dodecyl sulphate
SDS- PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCID ₅₀	50% Tissue culture infectious dose
TEMED	N, N, N', N'-Tetramethyl ethylene diamine
TK	Thymidine Kinase
TMEV	Theiler's murine encephalomyelitis virus
TNF α	Tumour necrosis factor α
TnT	Transcription and translation
U	Units
UTR	Untranslated region
V	Volts
v / v	Volume per volume
VPg	Viral protein genome-linked
w / v	Weight per volume

Chapter 1 Introduction

1.1 An introduction to the *Picornaviridae*

1.1.1 Classification

The *Picornaviridae* is a family of viruses causing significant human and animal disease. The *Picornaviridae* are located within the order *Picornavirales* in group IV (single stranded, positive sense RNA viruses) of the Baltimore classification system (Baltimore, 1971). The family as of March 2014 consisted of 46 species, organised into 26 genera (table 1.1) (Knowles, 2014). A phylogenetic tree to show the evolutionary relationships between these genera is shown in figure 1.1. The continuous discovery of new picornaviruses means that the classification requires constant refinement to reflect the evolutionary relationships within the family and many new viruses are yet to be assigned a classification (Knowles, 2014, Adams et al., 2013).

Foot-and-mouth disease virus (FMDV) is one of four species of the *Aphthovirus* genus which also includes the *Equine rhinitis A virus* (ERAV), *Bovine rhinitis A virus* (BRAV) and *Bovine rhinitis B virus* (BRBV). Poliovirus (PV) is one of currently 12 species of the *Enterovirus* genus with all three serotypes (PV1, 2 and 3) found in the *Enterovirus C* species along with several Coxsackie A virus (CV-A) and enterovirus A (EV-A) serotypes. Human rhinoviruses (HRVs) are found in three *Rhinovirus* (A-C) species, also in the *Enterovirus* genus (Knowles, 2014).

Picornaviruses are significant both in terms of the impact on the health of the infected host, and in terms of their economic impact. For example; PV can lead to poliomyelitis in humans causing an irreversible paralysis (Whitton et al., 2005), HRV is major cause of the common cold which accounts for an estimated loss of 280 million work days in the United States each year (Garibaldi, 1985). The outbreak of FMDV in a country's livestock can result in the complete restriction of animal movement both within and from that country (EU, 2003).

Table 1.1 List of Picornavirus genera, their number of species and important pathogens. The 26 genera of the *Picornaviridae* are listed alongside the number of viral species that are organised into a specific genus. “*” indicates the type species of the genus. FMDV; *Foot-and-mouth disease virus*, ERAV; *Equine rhinitis A virus*, BRAV and BRBV; *Bovine rhinitis A and B viruses*, DHAV; *Duck hepatitis A virus*, EMCV; *Encephalomyocarditis*, ERBV; *Equine rhinitis B virus*, HAV; *Hepatitis A virus*, HPeV; *Human parechovirus*.

<i>Picornaviridae</i> Genus	Number of species	Notable virus species
<i>Aphthovirus</i>	4	FMDV*, ERAV, BRAV, BRBV
<i>Aquamavirus</i>	1	<i>Aquamavirus A*</i>
<i>Avihepatovirus</i>	1	DHAV*
<i>Avisivirus</i>	1	<i>Avisivirus A*</i>
<i>Cardiovirus</i>	2	EMCV*, <i>Theilovirus</i>
<i>Cosavirus</i>	1	<i>Cosavirus A*</i>
<i>Dicpivirus</i>	1	<i>Cadicivirus A*</i>
<i>Enterovirus</i>	12	<i>Enterovirus A, B, C*, D, E, F, G, H and J</i> <i>Rhinovirus A,B and C</i>
<i>Erbovirus</i>	1	ERBV*
<i>Gallivirus</i>	1	<i>Gallivirus A*</i>
<i>Hepatovirus</i>	1	HAV*
<i>Hunnivirus</i>	1	<i>Hunnivirus A*</i>
<i>Kobuvirus</i>	3	<i>Aichivirus A*</i>
<i>Megrivirus</i>	1	<i>Melegrivirus A*</i>
<i>Mischivirus</i>	1	<i>Mischivirus A*</i>
<i>Mosavirus</i>	1	<i>Mosavirus A*</i>
<i>Oscivirus</i>	1	<i>Oscivirus A*</i>
<i>Parechovirus</i>	2	HPeV*, <i>Ljungan virus</i>
<i>Pasivirus</i>	1	<i>Pasivirus A*</i>
<i>Passerivirus</i>	1	<i>Passerivirus A*</i>
<i>Rosavirus</i>	1	<i>Rosavirus A*</i>
<i>Salivirus</i>	1	<i>Salivirus A*</i>
<i>Sapelovirus</i>	3	<i>Porcine, Simian and Avian sapelovirus</i>
<i>Senecavirus</i>	1	<i>Seneca Valley virus</i>
<i>Teschovirus</i>	1	<i>Porcine teschovirus</i>
<i>Tremovirus</i>	1	<i>Avian encephalomyelitis virus</i>

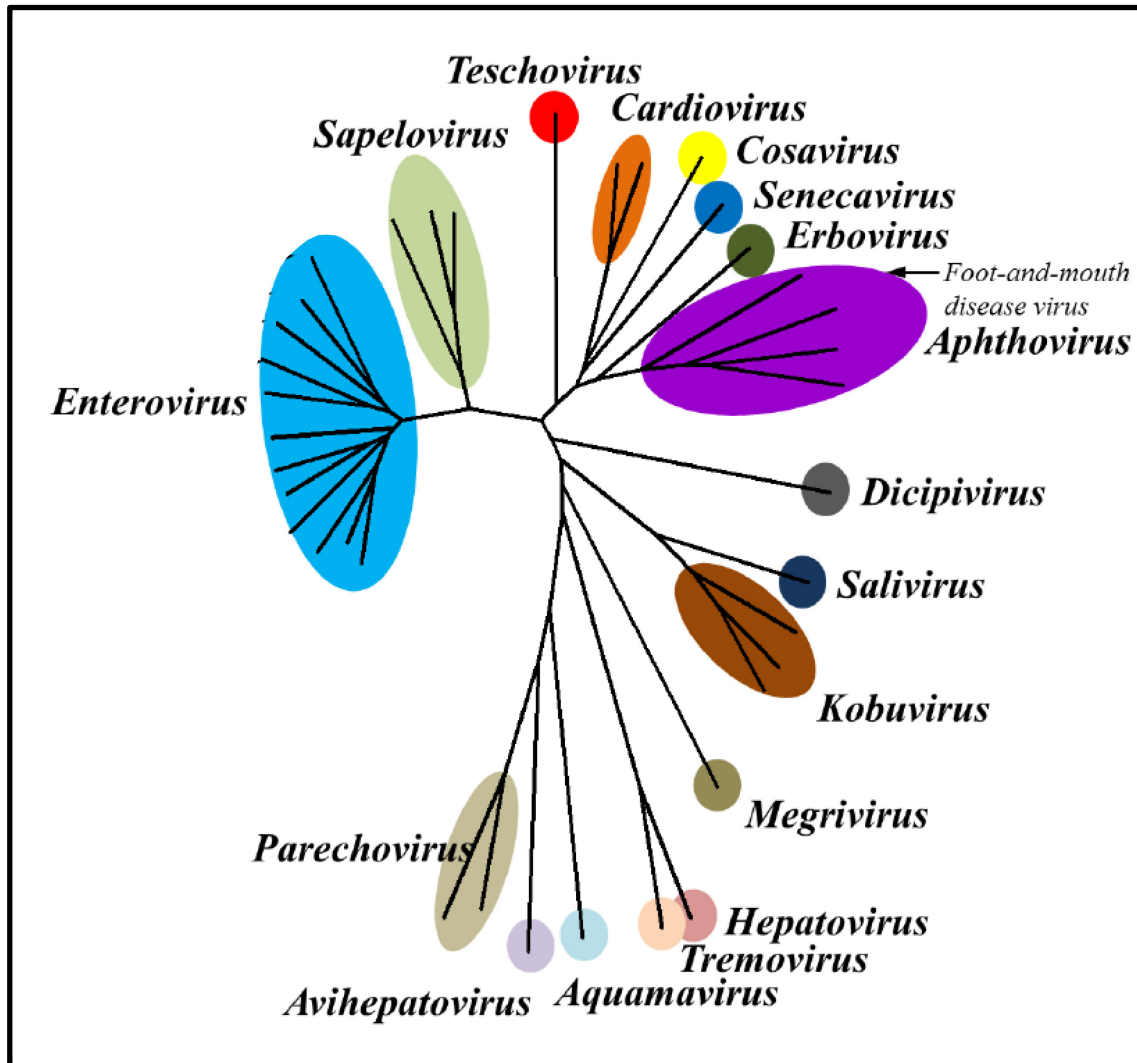


Figure 1.1 Unrooted Phylogentic tree of the *Picornaviridae* family. An unrooted phylogeny showing the evolutionary relationships between the species of 37 of the 46 species of the *Picornaviridae* family depicted as branches and grouped with coloured ellipses into 17 genera. Longer branch lengths indicate greater evolutionary distances from the point of divergence. The location of the *Foot-and-mouth disease virus* species is indicated. Adapted from a figure kindly provided by Dr. Nick Knowles at the Pirbright Institute.

1.1.2 Characteristics of the picornaviruses

The picornavirus genome is a single stranded and positive sense RNA molecule of between 7,032 bases (*Avian encephalomyelitis virus*) and 8,828 bases (*Erbovirus*). These figures do not include regions of variable length such as the 40-80 base pair poly(A) tail or the poly(C) tract (50-500 bases in some FMDV and *Cardiovirus* species) in the 3' and 5' untranslated (UTR) regions of the genome respectively. Picornaviral RNA genomes have a virally encoded protein, covalently linked to the 5' end of the genome known as VPg (viral protein, genome linked). The 5' and 3' UTRs, and the open reading frame (ORF) of the RNA have structural stem loops which are variable between the viral species and have a range of functions in replication and translation. Several picornaviruses (including FMDV) encode a polyprotein in which the structural proteins are located at the C-terminus of a non-structural protein named "L", and are followed by the non-structural proteins, whereas other viruses do not encode an L-protein. Processing by virally encoded enzymes cleaves the polyprotein into mature proteins. Single genomes are encapsidated in a non-enveloped, pseudo T=3 icosahedral capsid, composed of sixty copies of each structural protein (reviewed in Palmenberg et al., 2010).

1.2 Foot-and-mouth disease virus (FMDV)

1.2.1 Foot and mouth Disease; pathogenesis, host range and transmission

Foot-and-mouth disease (FMD) was the first disease demonstrated to be transmitted by a filterable agent, which was a significant event in the field of virology in that it proved that there were pathogens which were able to cause disease that were smaller than the resolution allowed by light microscopy (reviewed in Racaniello, 2013). FMD is characterised by acute fever, and the development of lesions in the mouth and feet. However, the disease is not easily clinically distinguishable from swine vesicular disease virus (*Enterovirus B* genus of the *Picornaviridae*), vesicular stomatitis virus (*Rhabdoviridae*) and vesiviruses (*Caliciviridae*) infections. The appearance and severity of these symptoms, and the location of lesions, can differ depending on factors such as the strain of the virus and the species of host (Arzt et al., 2014). FMDV mainly infects members of the order Artiodactyla (meaning even-toed ungulates); which includes many species of domestic

livestock and wild animals but can infect a few other species not found within this order (reviewed in Alexandersen and Mowat, 2005).

Despite reports of lesions caused by FMDV in humans (Armstrong et al., 1967), these are very rare cases and the illness caused was only very mild (Donaldson and Knowles, 2001). However, humans are considered important in the passive transmission of the virus between animals on their hands or surfaces, and FMDV can survive in the nasal passage of humans for short periods, suggesting that this could be a further method of transmission (Sellers et al., 1970).

Although most animals recover from the symptoms and can subsequently clear the virus (reviewed in Arzt et al., 2011a, Arzt et al., 2011b), the disease can be fatal in young animals (possibly due to myocarditis (Donaldson et al., 1984, Gulbahar et al., 2007) and can cause abortions in pregnant animals. Clinical disease is most serious in pigs in terms of the amount and severity of lesions (Kitching and Alexandersen, 2002), but cattle can also present with extensive pathology. Pathogenesis in sheep and goats is much less severe than in cattle and pigs with some studies suggesting that up to 25% of animals may be asymptomatic (Gibson et al., 1984). However, sheep are easily infected via aerosol and it is thought that they play a major role in the dissemination of disease in an outbreak. This may have been of particular significance in the 2001 outbreak in the United Kingdom, where sub-clinical infection of sheep allowed the spread of FMDV to other livestock before it could be detected (Kitching and Hughes, 2002).

FMDV in cattle is generally transmitted very efficiently via the inhalation of viral aerosols, whereas the eating of contaminated feed or direct contact are much more important modes of transmission in pigs (Alexandersen et al., 2002a). Virus can also enter the animals through skin abrasions however this mode of transmission is far less efficient. Infected animals shed virus through bodily excretions including milk, semen, urine and faeces as well as via respiratory aerosols (Alexandersen et al.,

2002a, Chase-Topping et al., 2013). Buffaloes, which are persistently infected with FMDV, are thought to be an important reservoir for transmission to cattle in India and Africa (Madhanmohan et al., 2014 and reviewed in Alexandersen, 2005).

Animals infected with FMDV can enter a 'carrier state' in which they are persistently infected with low levels of virus. Virus has been found to persist in the epithelium for up to 10-14 days (Oliver et al., 1988) and for over 28 days in the oesophageal-pharyngeal region (Sutmoller and Gaggero, 1965 infection, Burrows, 1966, Sutmoller et al., 1968). In cattle the carrier state can last for up to 3.5 years and has also been described in sheep and goats. Pigs appear to suffer worse symptoms of disease and do not appear to enter the carrier state (Alexandersen et al., 2002b, Stenfeldt et al., 2014).

1.2.2 Epidemiology and control

The FMDV species has seven serotypes; serotypes O and A have an almost worldwide distribution, serotype C has been found in South America and parts of Asia and Africa (although has not been reported since 2005 and may no longer exist outside of laboratories (Rweyemamu et al., 2008), Asia 1 has been found in parts of Asia and Greece, SAT 1 and 3 have been found in parts of Africa, and SAT 2 has been found in Africa and the middle East (Grubman and Baxt, 2004) (figure 1.2). Some serotypes exist as several topotypes, which are distinct genotypic sub-divisions resulting from geographical isolation of these serotypes (Samuel and Knowles, 2001, and reviewed in Jamal and Belsham, 2013). Viruses with similar genetic and antigenic characteristics appear to reoccur in similar geographical areas (thought to be as a result of patterns of animal movement, trade and FMDV reservoirs in wild animals) and as such, their control requires similar vaccines. However, a vaccine which is effective against one topotype may be less effective against other viruses in different topotypes but in the same serotype, which has resulted in the sub-division of vaccines into pools that are more effective against viruses in that pool (figure 1.2) (OIE/FAO, 2013).

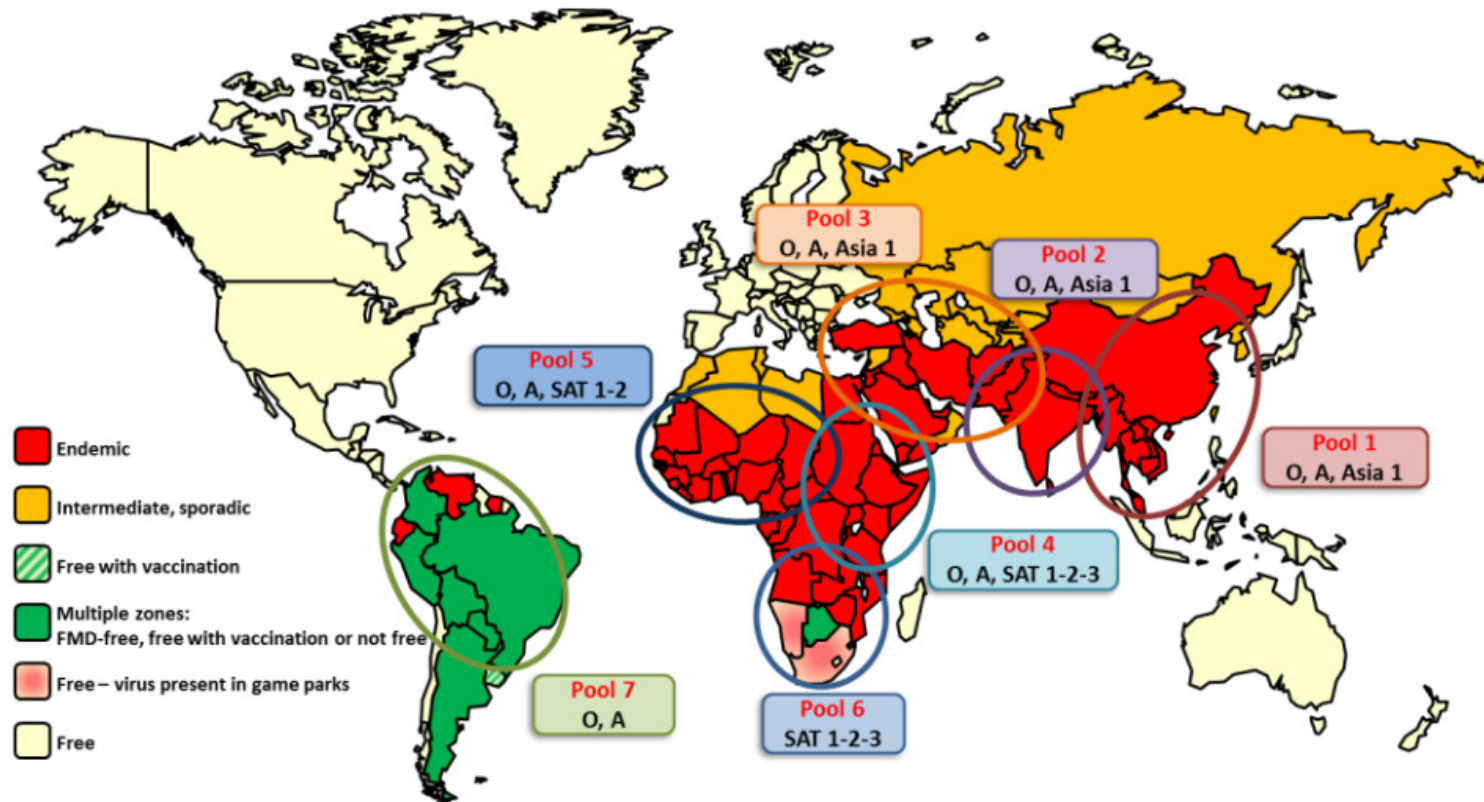


Figure 1.2. World map to show endemicity and the distribution of antigenically similar pools within serotypes of FMDV. Pools are identified with ellipses that cover the antigenic pools circulating in specific countries. Countries are colour coded according to the key on the left-hand side to reflect the assumed FMDV status within each country in 2013. Overlapping ellipses indicate that the countries covered by these regions experience outbreaks caused by a virus from different pools.

Figure taken from the OIE and FAO FMDV reference network annual report 2013 (OIE/FAO, 2013).

Historically FMDV also caused a significant number of outbreaks in Western Europe and North America in the 20th Century. These were largely controlled by restrictions in animal movement, the slaughter of infected animals and the disinfection of infected premises which resulted in the last outbreak of FMDV in the United States having occurred in 1929 (reviewed in Grubman and Baxt, 2004). A mixture of the above measures and the use of an inactivated vaccine achieved a disease-free status in Western Europe and in many South American countries. The current inactivated vaccines are produced by large-scale culture of viruses grown in BHK-21 cells that are subsequently inactivated using binary ethylenimine (BEI) and mixed with adjuvants (Bahnemann, 1975). The vaccination program in Western Europe was stopped in 1992 owing to the success of the programs, the cost of producing the vaccines (including the danger of a virus leak from vaccine production facilities) and the fact that while vaccination is successful in greatly reducing the levels of virus replication in infected animals, it does not prevent the carrier state (reviewed in Grubman and Baxt, 2004). Since then, only a few sporadic outbreaks have occurred resulting in huge losses for the countries involved, for example, in the 2001 outbreak of FMDV serotype O in the UK which resulted in the cull of millions of animals and a cost to the UK economy estimated to be £8 billion in compensation to farmers, controlling the epidemic and losses to trade and tourism (Bourn, 2002). There are, therefore, huge financial incentives for countries to retain their disease free status and obtaining FMDV disease-free status without vaccination is the goal of many countries. The vaccination strategy in Europe was successful in the eradication of FMDV from many countries, but the implementation of this strategy globally has not been feasible due to a lack of available veterinary facilities and resources, which would be required to coordinate such a strategy. Also, as described above, current vaccination is serotype specific and unable to prevent the carrier state occurring in a significant proportion of animals (reviewed in Grubman and Baxt, 2004). While natural infection results in a high neutralising antibody response and long-lived protection from challenge (reviewed in Habiela et al., 2014), vaccines may need to be re-administered every 6 months (reviewed in Doel, 1996). The reasons for why this is the case are unclear but several studies in

mouse models (Lopez et al., 1990, Piatti et al., 1991, Wigdorovitz et al., 1997) have speculated that the amount of antigen that comes into contact with the immune system is the important factor. The use of inactivated vaccines also suffers from the fact that large quantities of virus have to be made before vaccination, which requires expensive high containment facilities. This brings the risk of an outbreak caused by a laboratory strain of virus, such as in the 2007 United Kingdom outbreak, and is the reason that countries such as the United States ban this kind of vaccine production on their mainland. Other problems with current inactivated vaccines are their short shelf-life (particularly a problem in countries with a limited cold chain), the difficulty of growing certain strains well in culture and the difficulty of differentiating infected from vaccinated animals (DIVA) (reviewed in Rodriguez and Grubman, 2009 and Parida, 2009). The induction of neutralising antibodies by the vaccine is also reduced by the presence of maternal antibodies to FMDV (Bucafusco et al., 2014).

Several recent and on-going approaches are looking at tackling the problems posed by the current FMDV vaccines using, for example, antigens generated in recombinant systems as vaccines (Moraes et al., 2011, Pacheco et al., 2005, Porta et al., 2013a, Rodriguez et al., 2003); virus vectored vaccines (Chai et al., 2013, Chu et al., 2013, Romanutti et al., 2013) and antivirals targeting FMDV proteins responsible for parts of its replication cycle (Roque Rosell et al., 2014) with findings which appear promising for the better control of the virus.

1.3 Poliovirus (PV)

PV is the best known and best characterised member of the *Picornaviridae*. The three serotypes of PV are found in the *Human enterovirus C* cluster. This cluster also consists of several serotypes of Coxsackie A Viruses also thought to cause CNS and muscular disease in humans, and acute flaccid paralysis in mice (Hyypia and Stanway, 1993). PV causes disease in humans and non-human primates, is transmitted via the faecal-oral route and children under five are the main group affected. Most patients (about 95%) develop a mild and usually asymptomatic viraemia, however, in

1-2% of PV infections the virus invades the central nervous system (CNS) and either causes an aseptic meningitis or paralytic poliomyelitis (reviewed in De Jesus, 2007). The presentation of paralytic poliomyelitis (0.1-1% of all PV infections) differs depending on the strain and/or host factors and causes the destruction of motor neurones in the spinal cord (spinal poliomyelitis) resulting in skeletal muscle paralysis and/or, destruction of parts of the brain stem involved in controlling the respiratory muscles (bulbar poliomyelitis), resulting in the inability to breathe (Bodian, 1972).

Two types of PV vaccination were introduced in the mid-20th Century; the inactivated and oral poliovirus vaccines (IPV and OPV respectively). IPV or Salk vaccine (Salk et al., 1954a, Salk et al., 1954b) is an inactivated mixture of the three serotypes which is safe and protective against poliomyelitis but does not prevent gastrointestinal infection of wild type polio in vaccinated individuals. OPV has the ability to replicate in the gut and induce immunity without invasion of the CNS, and as such, protects against infection with wild-type virus. However, it has been shown in a small number of cases, the vaccine can revert back to virulence and cause poliomyelitis. Despite this, the use of OPV resulted in the eradication of wild type PV infection in the Americas and most developed countries by 1991. In 1988 the World Health Organisation (WHO) launched a campaign to globally eradicate PV by the year 2000. This campaign succeeded in reducing the reported cases of polio from a reported 60,000 in 1980 to fewer than 1,000 in 2001 (reviewed in Nathanson and Kew, 2011). The IPV replaced the OPV in developed countries in the year 2000 amid fears that unvaccinated individuals could be infected by individuals who had received the OPV (Aylward et al., 2005). Since the year 2000 the cases of PV have been at a steady level of 500-2000 mainly due to the continued endemicity of PV1 in Nigeria, Afghanistan and Pakistan, and subsequent incursion of these viruses into non-endemic countries (WHO, 2014).

1.4 Diseases caused by other important and related *Picornaviridae*

Picornaviruses cause a wide range of diseases in humans and both domesticated and wild animals.

While the investigation in this thesis focuses on FMDV, comparisons are made to other species found within the picornaviruses, especially PV. Several examples of which are described in further detail below.

1.4.1 Non-FMDV aphthoviruses

FMDV is the most well characterised virus of the *Aphthovirus* genus. Among the other three viruses of the genus, the disease caused by ERAV is the best described. ERAV is probably underdiagnosed in equids with respiratory disease (Carman et al., 1997, Diaz-Mendez et al., 2010) and causes fever, nasal discharge and impairs respiratory tract cilia function, which can render animals more susceptible to secondary bacterial infections (Diaz-Méndez et al., 2014, Plummer, 1962, Hoffman et al., 1993). As with FMDV, high neutralising antibody titres are protective against the virus, although the virus may still be shed in urine for at least 37 days (Lynch et al., 2013). Characterisation of the molecular biology of ERAV has resulted in findings to indicate that the virus structure and cell entry mechanism is similar to that of other picornavirus genera (Tuthill et al., 2009, Warner et al., 2001, Groppelli et al., 2010).

The bovine rhinoviruses BRAV and BRBV share a high sequence similarity to FMDV (Hollister et al., 2008, Knowles, 2014) and antibodies to the viruses appear to be widespread in domesticated cattle. Similarly to ERAV, they cause a respiratory disease but the disease caused is reported to be mild or sub-clinical (Ide and Darbyshire, 1972, Mohanty et al., 1969, Betts et al., 1971).

1.4.2 Non-polio enteroviruses

The *Enterovirus* genus contains the largest number of distinct viral serotypes characterised into the largest number of species within the *Picornaviridae* and contains a large number of important human and animal pathogens. Several important examples are given.

Human rhinoviruses (HRV) are characterised into three species (*Rhinovirus A*, *B* and *C*) of the *Enterovirus* genus and are a major cause of the common cold, one of the most common respiratory infections in humans. Infection with one of the many HRV serotypes can lead to fever, coughs, nasal congestion and are considered a nuisance in terms of their high economic impact due to work days lost. Infection with HRV has been linked with exacerbation of respiratory conditions such as asthma, severe bronchiolitis and even fatal pneumonia in immune-compromised individuals (reviewed in Jacobs et al., 2013).

Hand, Foot, and Mouth disease (HFMD) is a human disease caused primarily by enteroviruses of the *Enterovirus A* and *Enterovirus B* species. The most common viruses associated with HFMD are EV-A71 and CV-A16. CV-A6 is increasingly associated with HFMD and several other CV strains (A5, A7, A9, A10, B2 and B5) have also been linked with the disease (Repass et al., 2014). The disease is most common in infants but can also affect adults who come into contact with them. HFMD is typically described by fever, stomatitis and mouth ulcers, and a rash on the hands soles of the feet and elsewhere on the body which can last for a week. While these findings are common during most HFMD cases, infection with EV-A71 can lead to more serious neurological and cardio-pulmonary consequences such as poliovirus-like aseptic meningitis and paralysis, brainstem encephalitis, and pulmonary oedema among others (McMinn et al., 2001, Zeng et al., 2012, Chang et al., 1998). Infection with CV-A6 causes an atypical HFMD with a widespread rash which can progress to skin lesions, but no oral lesions, and rare cases of myocardial damage, encephalitis and meningitis (Yang et al., 2014a, Feder Jr et al., 2014). Infections caused by echoviruses in the *Enterovirus B* genus also have the potential to cause paralysis, aseptic meningitis and myocarditis and are more commonly found in men and children (Dalwai et al., 2010, Grimwood et al., 2003).

Examples of the animal diseases caused by viruses of the *Enterovirus* genus are swine vesicular disease (SVDV) (a strain of the human pathogen CV-B5 in the *Enterovirus B* genus) and bovine

enteroviruses that have been renamed EV-E1 to E4 and EV-F1 to F4 in the *Enterovirus E* and *Enterovirus F* (Knowles, 2014). The symptoms of SVDV can be confused with FMDV and so its early diagnosis is crucial (Fernández et al., 2008). Bovine enterovirus diseases are widespread and the viruses have proved useful as models e.g. virus assembly (Li et al., 2012).

Many diseases of pigs, monkeys and other animals are also found in the *Enterovirus* genus demonstrating the wide-ranging impact that diseases of viruses in the same evolutionary lineage can have.

1.4.3 Parechoviruses

The *Ljungan virus* (LV) species of the *Parechovirus* genus was first identified in Swedish bank voles and is thought to cause myocarditis, encephalitis, diabetes and pregnancy disorders in rodents (Niklasson et al., 1999, Niklasson et al., 2006). LV is being investigated as a cause of diabetes, myocarditis and Guillain-Barré in humans because the incidence of these diseases correlates with the 3-4 year cycle of bank vole abundance (Niklasson et al., 1998). The virus has also been indicated in intrauterine foetal death and malformation in humans (Niklasson et al., 2007, Niklasson et al., 2009). Infections by the Human parechovirus (HPeV) species have long been associated with gastroenteritis and diarrhoea in children (Reviewed in Harvala and Simmonds, 2009).

1.4.4 Cardioviruses

The two species of the *Cardiovirus*; *Encephalomyocarditis virus* and *Theilovirus* can cause gastrointestinal and respiratory infections, myocarditis, type I diabetes and encephalitis in rodents. Several species of the *Theilovirus* have also recently been implicated in human disease. For example, Saffold viruses have been isolated from children with gastrointestinal and respiratory diseases, non-polio flaccid paralysis and aseptic meningitis but epidemiological investigations are on-going to determine the link between Saffold virus and the symptoms (reviewed in Blinkova et al., 2009 and Himeda and Ohara, 2012). Vilyuisk human encephalomyelitis virus (VHEV) is a further *Theilovirus* and is thought to cause encephalomyelitis in human populations in Siberia. The virus had been isolated

after extensive passage in mice in the 1950's, but, whether this virus is indeed the cause, or whether the isolates are TMEV or a recombinant of TMEV and VHEV due to their passage in mice, is the subject of debate (reviewed in Himeda and Ohara, 2012). Theiloviruses have also served as a useful mouse model in the investigation of multiple sclerosis (Tsunoda and Fujinami, 2010).

1.4.5 Hepatovirus

Hepatitis A virus is the only species of the *Hepatovirus* genus and causes acute and fulminant hepatitis that can be fatal, however, infections with HAV do not lead to the chronic liver diseases seen in cases of *Hepatitis C virus* (HCV; a member of the *Flaviviridae* family) infection. The HAV vaccine is almost 100% effective against the virus but the disease still results in an estimated 60,000 cases each year in the United States (reviewed in Martin and Lemon, 2006). HAV exhibits some unique features in its lifecycle compared with other picornaviruses (used for comparison in sections 1.5 to 1.7) and appears to be the only picornavirus to acquire an envelope (Feng et al., 2013).

1.5 Picornavirus genome organisation and molecular biology

A large amount of research effort has gone into understanding the molecular biology of picornaviruses. They are amongst the smallest of mammalian viruses with a compact genome that encodes proteins with little, or no, redundancy in their function. The structure of the RNA has also been finely tuned through evolution to aid in the replication of the viruses. Most information about picornavirus molecular biology has been demonstrated in the prototype picornavirus, PV, but differences occur in nearly all of the encoded proteins and features of the genome, which confer important changes to the characteristics of different family members. The diagram shown in figure 1.3 depicts the order of structural RNA elements, and of proteins that are produced after the translation of the RNA genome (section 1.5.4) and all proteolytic processing events (section 1.5.5) in PV (figure 1.3A) and FMDV (figure 1.3B).

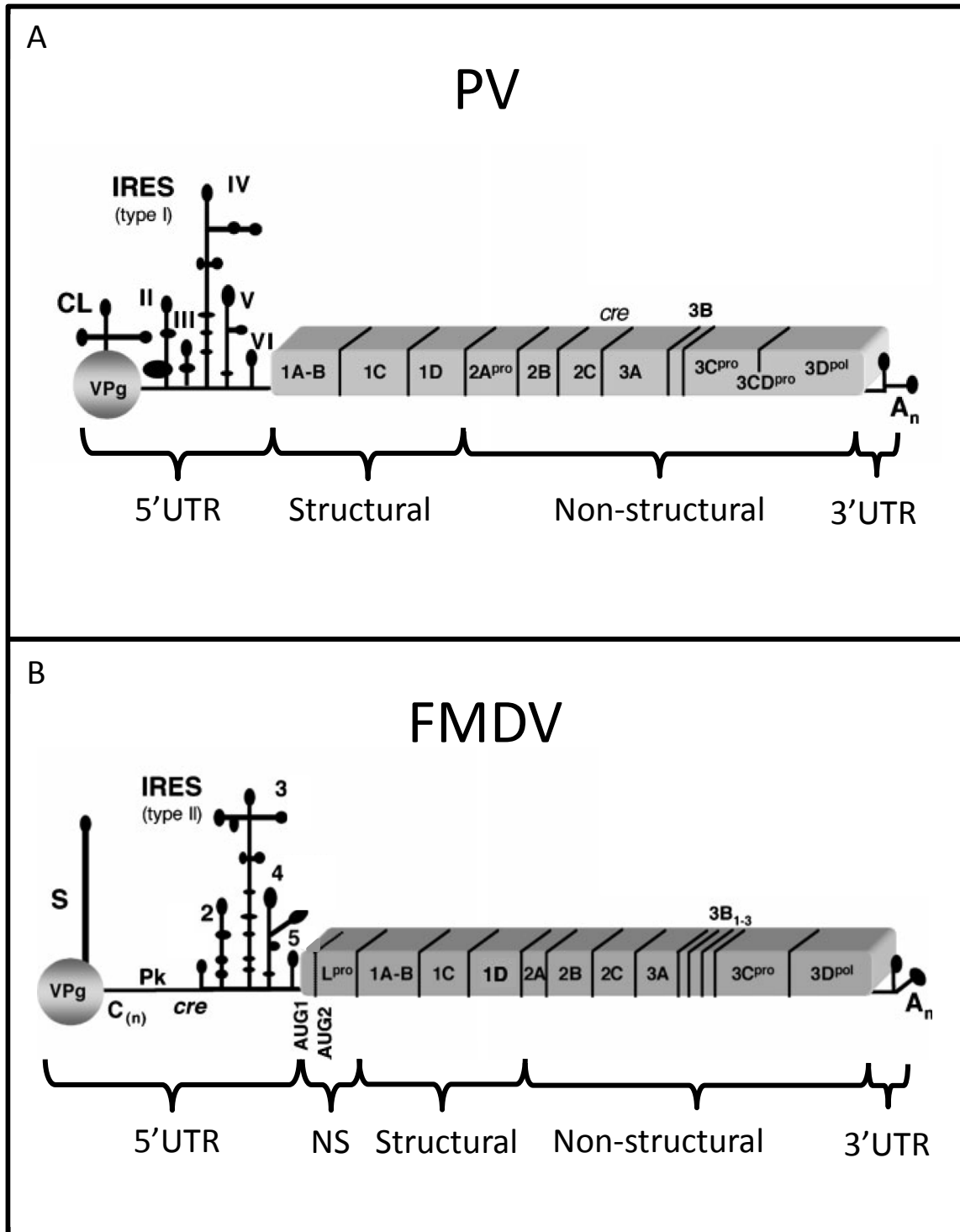


Figure 1.3. The genomic structures and encoded proteins of foot-and-mouth disease virus (FMDV) and poliovirus (PV). Diagrammatic representations of the RNA structural elements (black lines and ellipses) present in the 5'UTR (untranslated region), 3'UTR and the order and structure of mature protein products produced by the translated polyproteins (grey boxes) of PV (A) and FMDV (B).

Key: VPg; Viral-protein, genome linked, CL; Clover-leaf, numbers next to stem loops are the loops that make up the Internal ribosome entry site (IRES), *cre*; cis-acting replication element (loop not represented diagrammatically in the 2C protein of PV), A_n; poly(A) tail, NS; non-structural. Adapted from Martinez-Salas et al. (2008).

1.5.1 RNA structural elements

The picornaviral genome (especially the 5'UTR and 3'UTR) contains RNA structural elements for a host of functions including the translation and replication of the genome. The 5'UTR of FMDV is longer than in other members of the family (approximately 1300 bases in FMDV compared to 740 bases in PV (Mason et al., 2004)) partly due to structures known as the S-fragment and pseudoknots.

1.5.1.1 S-fragment (*Aphthovirus*) and clover leaf (*Enterovirus*)

The S-fragment is uniquely found in the aphthoviruses and is a large stem loop at the 5' most end of the genome but its function is unclear (Newton et al., 1985). It has been shown to bind to cellular RNA Helicase A which also interacts with other viral and host-proteins required for replication (Lawrence and Rieder, 2009). In support of a function for the S-fragment in replication, a structure at this position in all *Enterovirus* species known as the cloverleaf is required for circularisation of the genome that allows its replication (section 1.5.3) (Barton et al., 2001, Herold and Andino, 2001).

1.5.1.2 Poly-cytosine(C) tract and pseudoknots (PK)

Downstream of the S-fragment in aphthoviruses (or the clover leaf in enteroviruses) are two further features, known as the poly(C) tract and PK which are a run of cytosine residues, or two to five repeats of knot-like structures, in the RNA respectively and are found in cardioviruses, aphthoviruses and several other genera (reviewed in Palmenberg et al., 2010). The poly(C) tract can be hugely variable in length (Costa Giomi et al., 1984). Enteroviruses lack the PK structures and have much shorter C-rich regions but it is unknown whether they are analogous to the poly(C) tract. Truncation of the poly(C) tract in the *Cardiovirus*, EMCV, attenuates its pathogenicity (Duke et al., 1990). In PV, the cellular protein poly(C) binding protein 2 (PCBP2) interacts with the C-rich region and the cloverleaf (via interaction with a further viral protein) and mutation of a few C-residues affects its viability (Toyoda et al., 2007). These interactions are thought to be important for genome replication (section 1.5.3).

1.5.1.3 *Cis-acting replication element (cre)* and 3B-uridylylation site (bus)

The *cre* is an RNA hairpin loop, structural element essential for genome replication, which is found in the structural coding region of HRV14 (McKnight and Lemon, 1998) and cardioviruses (Lobert et al., 1999), and in the non-structural coding region of poliovirus (Rieder et al., 2000, Paul et al., 2000, Goodfellow et al., 2000) and HAV (Yang et al., 2008). This element acts in *cis* as a template for the uridylylation of the protein primer, VPg, for RNA synthesis. The analogous loop in FMDV is found in the 5'UTR (Mason et al., 2002), and can also function in *trans* (Tiley et al., 2003, Nayak et al., 2006).

1.5.1.4 *Internal ribosome entry site (IRES)*

All picornavirus genomes contain an RNA structure known as the IRES. The IRES functions as an internal site on the RNA for the initiation of protein synthesis (Jang et al., 1988, Pelletier and Sonenberg, 1988). The IRES structure consists of branched and un-branched stem loops (figure 1.3) which depend upon the RNA sequence for their structure (Skinner et al., 1989, Pilipenko et al., 1989). As such, the structure of the IRES elements in different picornavirus genera is variable and there are also differences between species. They are broadly characterised into four categories with PV, FMDV and HAV having type I, type II and type III respectively. The fourth class of IRES is thought to originate from a recombination event between picornaviruses and flaviviruses and is found in tescho-, sapelo-, seneca- and tremoviruses (reviewed in Palmenberg et al., 2010 and Belsham, 2009). IRES elements have also been recognised on mRNAs in mammalian cells (Komar and Hatzoglou, 2005).

1.5.1.5 3'UTR

Hairpin loops and a poly-adenine (poly(A)) tract are found in the 3'UTR at the end of the genome and are at least partially encoded by the genome rather than being added post-transcriptionally as seen in cellular mRNA (Baxt et al., 1979). The poly(A) tract is also thought to be required for genome circularisation in RNA replication and translation in concert with a cellular poly(A) binding protein (PABP) (reviewed in Palmenberg et al., 2010).

1.5.2 Encoded proteins

All picornaviruses encode the structural proteins upstream of the non-structural proteins, except for viruses that encode the non-structural L protein immediately upstream of the structural proteins (figure 1.3). The proteins are translated as a single large polyprotein before subsequent proteolytic processing. The protein naming system employed here is that of Rueckert and Wimmer (1984), whereby the translation products are ordered in the scheme L-P1-P2-P3 based upon the primary polyprotein cleavages observed within infected cells. In viruses which encode “L”, its sequence encodes for an L protein or protease, and the P1 sequence encodes the structural proteins 1A, 1B, 1C and 1D also known as VP4, VP2, VP3 and VP1 respectively. The P2 sequence encodes the three non-structural proteins 2A, 2B and the 2C adenosine triphosphatase ($2C^{ATPase}$), and the P3 sequence encodes non-structural proteins 3A, 3B, the 3C protease ($3C^{pro}$) and the 3D polymerase ($3D^{pol}$).

1.5.2.1 Structural proteins

Most picornaviruses encode four structural proteins; VP- 4,2,3,1, which are the mature proteins of the P1 translation product. In aphthovirus, cardioviruses and hepatoviruses, the P1 region is separated from the growing polyprotein chain still attached to the P2 protein 2A. P1-2A is later cleaved in all viruses apart from the hepatoviruses where it can still be attached and is required for virion assembly (Cohen et al., 2002). A maturation cleavage event separates the VP4 and VP2 proteins in most picornaviruses which occurs later in the processing cascade than the other cleavages. Before the maturation cleavage the VP4-VP2 protein is known as VP0. Viruses of the *Kobu*-, *Parecho*- and *Avihepatovirus* genera do not undergo the maturation cleavage and the sequence at the N-termini of VP0 is not similar to VP4 sequences. Hepatoviruses have a very small (approximately 20 amino acids compared with 60 and 80 in PV and FMDV respectively) N-terminal VP4 extension of VP2 that can undergo maturation (Feng et al., 2013) and has been shown to perform a function similar to that of the VP4 in other viruses (Shukla et al., 2014).

The structural proteins are the basis for much of the investigation presented in this thesis and, as such, their functions will be described in much greater detail in later sections.

1.5.2.2 Non-structural proteins

Leader protein and 2A

The Leader and 2A proteins have some analogous functions in different genera but can also perform functions that are unique to each genus. The Leader of FMDV is a protease (L^{pro}) and is expressed as two nested forms; Lab^{pro} and Lb^{pro} which is as a result of two start codons present in the sequence at the most 3' loop (figure 1.3; loop 5) of the IRES. While functionally similar, it is thought the Lb^{pro} is the more important protease as mutation of its start codon prevents viral replication whereas mutation of the first does not (Cao et al., 1995). The N-terminal extension of Lab^{pro} is thought to provide a nuclear localisation signal for its translocation to the nucleus (de los Santos et al., 2009).

A protein is named L if it is a mature protein found between the start codon for the polyprotein and P1 regardless of function. Such proteins are found in the *Aphtho-*, *Erbo-*, *Cardio-*, *Kobu-*, *Sapelo-* and *Seneca Valley virus* genera. The function of the *Aphthovirus* L^{pro} appears analogous to the function of *Enterovirus* and *Cardiovirus* 2A, which is that of a protease that cleaves the eukaryotic initiation factor-4GI (eIF4GI) shutting down host-translation and promoting viral translation (Krausslich et al., 1987, Devaney et al., 1988, Svitkin et al., 1999, Groppo and Palmenberg, 2007). The L^{pro} of aphtho- and erboviruses performs an autocatalytic cleavage releasing it from the N-terminus of P1 but *Erbovirus* L^{pro} does not cleave eIF4G (Hinton et al., 2002). The L protein in cardioviruses is not a protease, but instead regulates virus translation (Dvorak et al., 2001) and inhibits interferon signalling (Hato et al., 2007). The biological function of L proteins from the other genera listed above is unknown.

As well as cleaving eIF4GI in enteroviruses and cardioviruses, and performing a critical function in the proteolytic processing cascade, the 2A proteins of these virus genera have other important

functions. These include impairing host innate immune functions and the redistribution of nuclear proteins required for virus RNA replication and translation to the cytoplasm, possibly in conjunction with the L protein (Petty et al., 2014, Feng et al., 2014, de los Santos et al., 2009) and (reviewed in Castello et al., 2011). The role that 2A plays in separating the structural from non-structural proteins will be discussed in section 1.5.5.

2B, 2C and 2BC

The proteins 2B and 2C^{ATPase} are P2 products derived from the stable precursor protein 2BC^{ATPase}. Picornaviruses cause a rearrangement of host cell membranes into replication complexes and the ATPase activities of 2BC^{ATPase} and 2C^{ATPase} are highly active in this rearrangement (reviewed in van Kuppeveld et al., 2010). In PV and CV-B3 these proteins have been shown to reorganise membranes of the early secretory pathway (Hsu et al., 2010, Rust et al., 2001) and the finding that FMDV requires endoplasmic reticulum (ER) exit sites for replication suggest a similar mechanism (Midgley et al., 2013a). The ATPase activity of 2C is inhibited by guanidine hydrochloride (GuHCL) which specifically inhibits RNA synthesis (Pfister and Wimmer, 1999).

Protein 2B of enteroviruses has been shown to interact with membranes and reduces Ca²⁺ levels in the ER and golgi which is suggested to provide optimum conditions for RNA replication (Martinez-Gil et al., 2011a, Martinez-Gil et al., 2011b). This does not appear to be the case in the aphtho-, cardio- and hepatoviruses due to a 2B protein that lacks the membrane binding alpha-helix domain (de Jong et al., 2008).

The 2C^{ATPase} protein is among the most conserved among picornavirus species. It has been shown to directly interact with the capsid structural proteins during virion morphogenesis in several enteroviruses (Liu et al., 2010, Wang et al., 2012a, Wang et al., 2014a) (section 1.7.3). Evidence also

suggests that FMDV 2C^{ATPase} can stimulate a specific type I interferon response in infected cells (Zheng et al., 2014).

3A, 3B, 3AB and 3ABB

The precursor 3ABB (FMDV only), 3AB and the mature proteins 3A and 3B all appear to have functions in the picornavirus lifecycle. Mutations in protein 3A have been shown to affect culture adaptation in HAV (Graff et al., 1994) and host tropism in FMDV (Pacheco et al., 2003, Nunez et al., 2001). Protein 3B is VPg and is linked to the 5' terminus of the genome. FMDV is the only member of the *Picornaviridae* to encode three copies of the 3B (VPg) protein, (the other viruses only encode one) (Knowles, 2014). The reasons for this are not understood although deletion of one or two 3B proteins makes virus replication less efficient (Mason et al., 2003). The 3B protein is covalently attached to the 5' terminus of the genome in mature virions (Flanegan et al., 1977, Lee et al., 1977). The function of 3B is to act as a primer for RNA synthesis in concert with 3D^{pol} (Paul et al., 1998). In its uncleaved form, 3A has been suggested to act as a membrane anchor for 3B to perform its functions in replication although the 3A domains required for membrane attachment are not universally present in picornaviruses (reviewed in Palmenberg et al., 2010).

3C^{pro}, 3D^{pol} and 3CD

The mature proteins 3C^{pro} and 3D^{pol} are derived from the 3CD precursor. The 3C^{pro} enzymes of most picornaviruses are responsible for the majority of the proteolytic processing cascade performed on the viral polyprotein (described in section 1.5.5) but also have other functions. For example, the 3C^{pro} of FMDV is also responsible for cleaving an array of host factors required for; innate immune signalling (Du et al., 2014, Wang et al., 2012b) (also performed by the 3C^{pro} of CV-B (Mukherjee et al., 2011) and HAV (Wang et al., 2014b)), cap-dependent translation (Belsham et al., 2000), the cytoskeleton (Armer et al., 2008) and Sam68, which is a factor required for RNA binding in the nucleus

subverted for viral translation (Lawrence et al., 2012) (analogous to a function of 2A in enteroviruses (Fitzgerald et al., 2012)).

The precursor protein 3CD is more efficient at cleaving some P1 and P3 substrates than 3C^{pro} in enteroviruses (Ypma-Wong et al., 1988, Jore et al., 1988). 3C^{pro} has RNA binding activity and part of the 3CD precursor has been shown to bind to the *Enterovirus* cloverleaf (in concert with 3AB or PCBP) (Walter et al., 2002) and *cre* to facilitate RNA synthesis (reviewed in Pathak et al., 2007). FMDV 3C^{pro} has also been shown to possess RNA binding activity in the uridylylation of VPg (Nayak et al., 2006). The 3CD of HAV can also impair cellular innate immune signalling (Qu et al., 2011).

3D^{pol} is the picornavirus RNA-dependent RNA polymerase that synthesises both negative strands from positive strand templates, and positive strands from these newly formed negative strands in the generation of new genomes (Paul et al., 1998). RNA replication will be discussed in more detail in the following section.

1.5.3 Genome replication

1.5.3.1 Membrane rearrangement

The replication of the picornavirus genome produces both RNA for translation of viral proteins and RNA for inclusion into new virus particles. Infection with many picornaviruses causes the rearrangement of host cell membranes (figure 1.4), which function as a platform from which viral replication complexes can form. These are discreet factories where high concentrations of replication proteins can interact (reviewed in van Kuppeveld et al., 2010, Chase and Semler, 2012).

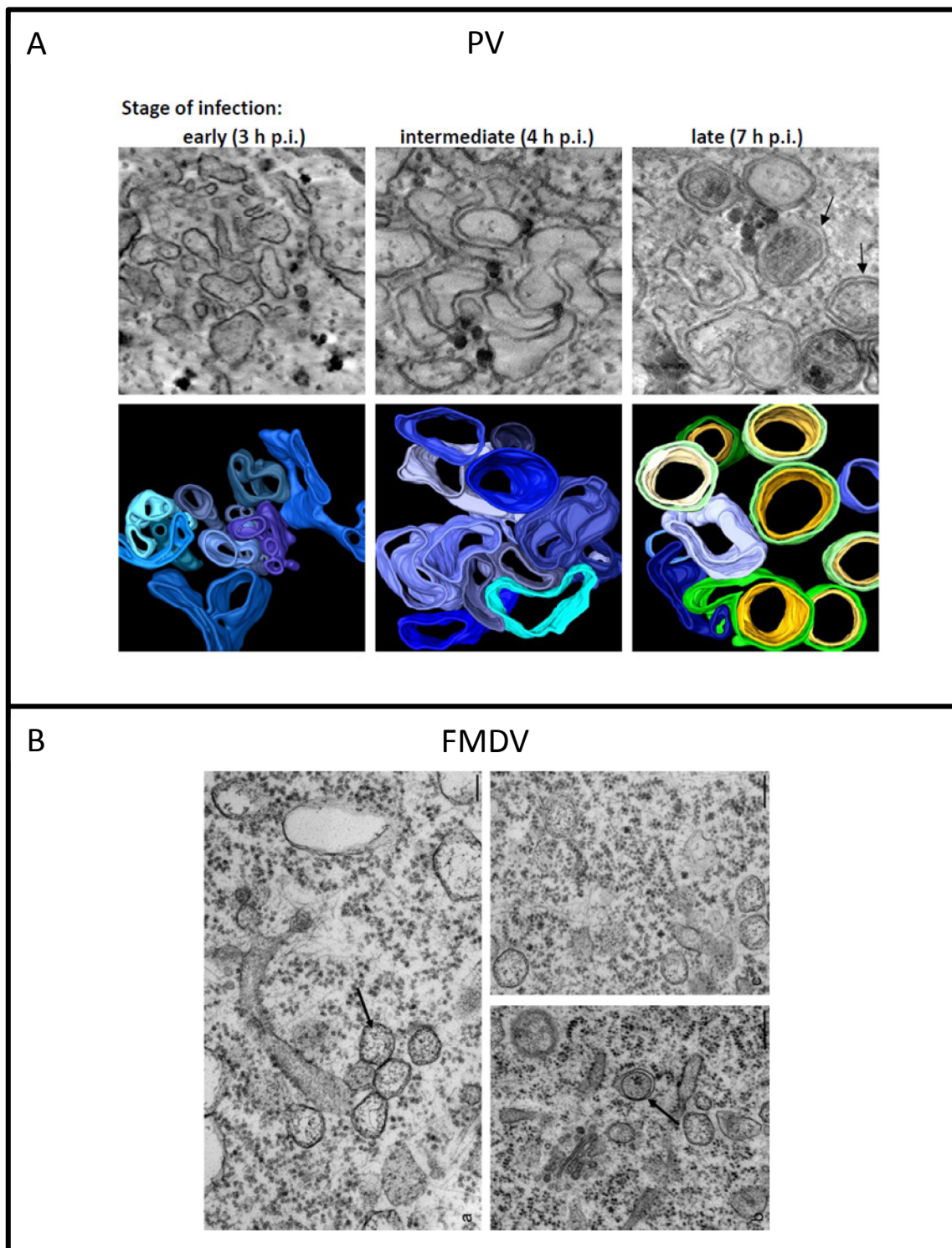


Figure 1.4 Membrane rearrangements during poliovirus infection of HeLa cells and FMDV infection of BHK-38 cells. Electron microscopy (top set) and 3-dimensional tomographic reconstructions (bottom set) of membrane rearrangements induced by infection of HeLa cells by PV at various time-points (A) (image taken from Belov and Sztul, 2014). Electron microscopy images of membrane rearrangements in FMDV infected BHK-38 cells at 2 hours post infection (image taken from Monaghan et al., 2004)

Picornaviruses use different methods to generate these membranes for replication. While the drug, GuHCl, inhibits RNA synthesis by disabling the 2C proteins of many picornaviruses (Belsham and Normann, 2008 and Pfister and Wimmer, 1999), drugs such as brefeldin A (BFA) have different effects depending on the virus genus. BFA is an inhibitor of the guanine-nucleotide exchange factor GBF1 which is required for the trafficking of transport vesicles between the ER, ER-golgi intermediate compartment (ERGIC) and the golgi apparatus (Kawamoto et al., 2002). PV 3A protein binds to GBF-1 and regulates its function to enrich membranes for phosphatidylinositol 4-kinase (PI4K) which are bound by 3D^{pol} in replication complexes and therefore the virus is sensitive to BFA (Hsu et al., 2010). FMDV is insensitive to BFA because it does not bind to GBF1 to reorganise membranes, and instead uses proteins 2B, 2C^{ATPase} and 2BC^{ATPase} (Moffat et al., 2007) to affect the formation of transport vesicles at the ER exit sites (Midgley et al., 2013a). This suggests that the ER exit site membranes are the source of membranes for the FMDV replication complex.

1.5.3.2 Molecular mechanism

The synthesis of RNA is performed within replication complexes and requires many of the non-structural proteins described in section 1.5.2. Negative sense templates for further positive strand synthesis are generated from viral RNA deposited into the cells during the infection process. Both positive and negative strand synthesis are performed by 3D^{pol}, but the process cannot occur simultaneously with translation (section 1.5.4) (Gamarnik and Andino, 1998), and several regulatory factors have been identified that mediate the switch between translation and replication in PV (Chase et al., 2014, Chase and Semler, 2014 and reviewed in Daijogo and Semler, 2011). In FMDV the 3'UTR may switch from binding to the IRES to binding to the S-fragment to achieve the switch (Martinez-Salas et al., 2008).

RNA synthesis begins with the addition of two phospho-uridines (pU) to VPg by 3D^{pol} using the *cre* as a template (Paul et al., 1998, Paul et al., 2000). VPg-pUpU acts as the primer for RNA synthesis from

the poly(a) tail by 3D^{pol} in a complex with PABP and PCBP that circularises the genome (Herold and Andino, 2001).

1.5.4 Translation from picornavirus IRES elements

The picornaviral RNA genome is translated upon its translocation into the cytoplasm. The method that picornaviruses use to translate their genome is not the canonical method of ribosome recruitment to a 5' methyl guanosine cap structure and Kozak or Shine-Dalgarno sequence. Instead, picornavirus RNA, like in many other viruses (and some host mRNAs) utilises IRES elements (Komar and Hatzoglou, 2005). In cap-dependent translation, the 43S ribosomal subunit complex (40S ribosome, eIF2, Guanosine triphosphate (GTP) and an initiating methionine residue carried by a transfer RNA (tRNA)) is recruited to the mRNA by PABP, eIF4B, eIF3 and the eIF4F cap-binding complex consisting of eIF4E which binds to the cap, eIF4G which acts as a bridge connecting the ribosome complex to eIF4E and the eIF4A helicase that removes secondary structure from the RNA. This complex scans along the RNA until a start codon is reached. At this point, eIF1 is released and eIF5B and eIF1A recruit the 60S ribosomal subunit to assemble the 80S ribosome and initiate protein synthesis (reviewed in Aitken and Lorsch, 2012) (figure 1.5).

As explained in the descriptions of the non-structural proteins (section 1.5.2.2) picornavirus infection results in the cleavage of eIF4G. The type I and II IRES elements of aphtho- entero- and cardioviruses only require the N-terminal of eIF4G which remains intact after cleavage, and this cleavage shuts-off cap-dependent protein synthesis in the host. The remaining eIF4G central domain, the 43S subunit, eIF4A and eIF3 can now bind to the IRES (Jang et al., 1990). Several other eIFs (4B, 1, 1A, 5 and 5B), IRES *trans*-acting factors (ITAFs), pyrimidine tract binding protein (PTB) and PCBP2 can influence the initiation and regulation of translation (reviewed in Chase and Semler, 2012) (figure 1.5).

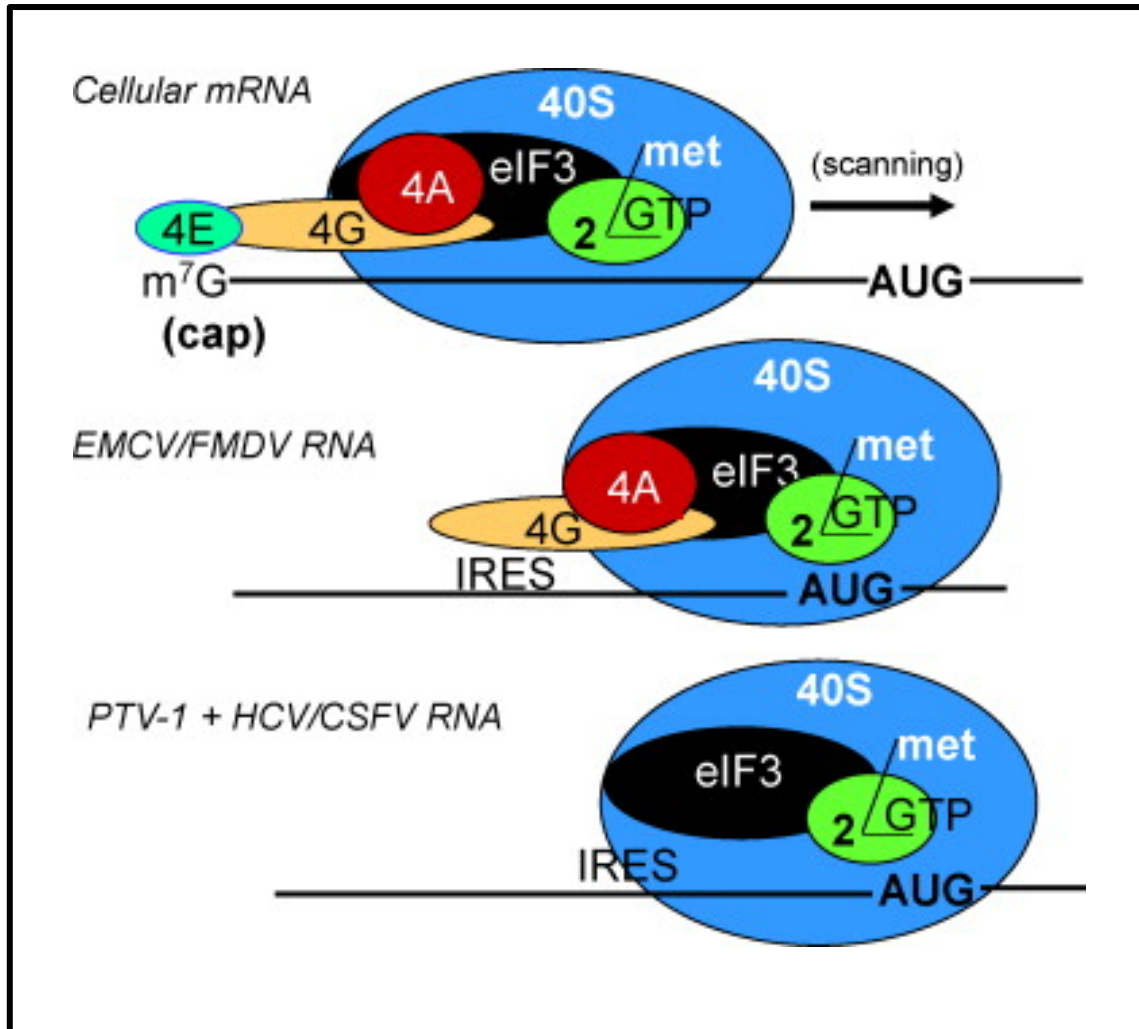


Figure 1.5 Requirements for translation initiation factors in selected picornavirus IRES elements and the 7-methylguanosine cap structure. Diagrammatic representation of the required eukaryotic initiation factors (eIFs) in the recruitment of ribosome initiation complexes to initiate translation on capped cellular mRNA (top), type II IRES RNA (e.g. Encephalomyocarditis virus (EMCV)/foot-and-mouth disease virus (FMDV)) (middle) and HCV-like type IV IRES RNA (e.g. porcine teschovirus-1 (PTV-1), hepatitis C virus (HCV) and classical swine fever virus (CSFV)) (bottom). Figure taken from Belsham (2009).

Key; 4G; eIF4G, 4E; eIF4E, 4A; eIF4A, 2; eIF2, GTP; Guanosine triphosphate, met; initiating methionine, 40S; 40S ribosomal subunit.

The type III IRES elements of HAV and the type IV (HCV-like) IRES elements found in viruses such as porcine teschovirus-1 (PTV-1) appear to operate in a slightly different manner, which require different initiation sequences, eIFs and ITAFs for initiation and regulation (reviewed in Belsham, 2009; figure 1.5).

1.5.5 Polyprotein processing

Picornavirus genomes code for a single large open reading frame (ORF) of approximately 6kb depending upon the virus. The single polyprotein that is produced can only be visualised in infected cells in the presence of reversible inhibitors such as amino acid analogues or zinc, or non-reversible inhibitors of the proteases due to the occurrence of rapid co-translational processing events (reviewed in Putnak and Phillips, 1981b). While there are many similarities in the processing of the translated polyprotein to produce mature proteins among the different picornavirus genera there are some key differences (Castello et al., 2011) (figure 1.6A). The mechanisms by which the structural proteins become separated from the growing polyprotein can be achieved in a number of ways. In enteroviruses, 2A is an auto-protease that cleaves at its N-terminus (Toyoda et al., 1986, Sommergruber et al., 1989). *Aphtho*-, *Cardio*-, *Erbo*-, *Tescho*-, and *Sencavirus* genera encode a sequence (DxExNPG/P – using single letter amino acid code, “x” is any amino acid and “/” is the junction between 2A/2B) in the C-terminal region of 2A that causes a ribosomal slippage at the 2A/2B junction (Donnelly et al., 1997, Donnelly et al., 2001, Ryan et al., 1991). In these viruses, 2A is removed from P1 by 3C protease or 3C^{pro} and is not required for viral assembly (Goodwin et al., 2009), however assembly is not affected when 2A is artificially fused to P1 (Gullberg et al., 2013b). Hepatoviruses have an enveloped particle with 2A still attached to P1 (known as P1-pX), and a non-enveloped particle where 2A is removed by an unknown host-cell protease (Graff et al., 1999, Cohen et al., 2002, Feng et al., 2013).

This co-translational separation yields the P1-2A in cardio-, erbo- and aphthoviruses, P1 in enteroviruses, and P1-2A in hepatoviruses (2A/B cleavage performed by 3C^{pro}) and is known as the

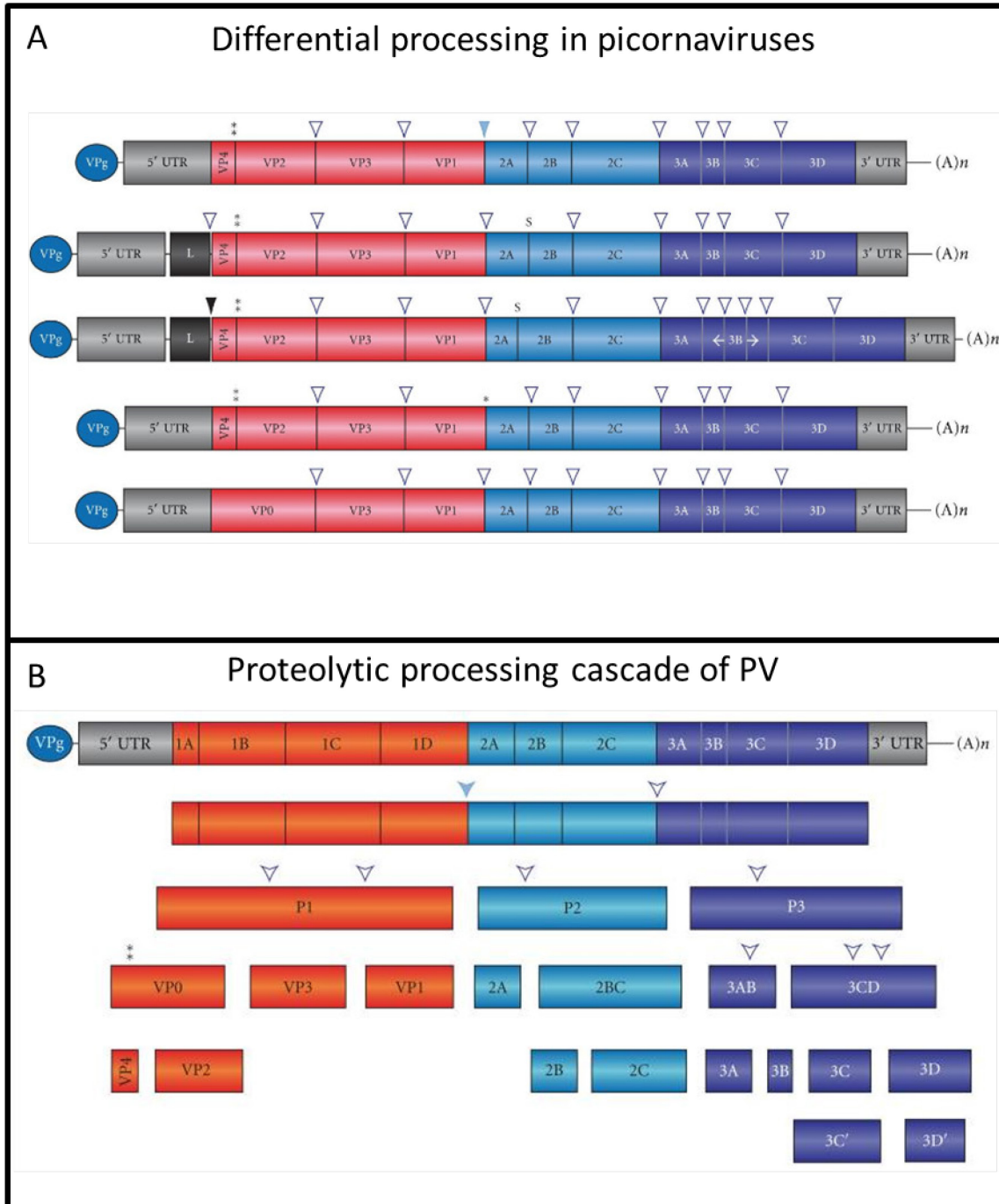


Figure 1.6 The use of different viral proteases in picornaviruses to perform the proteolytic processing cascade to achieve mature proteins. Diagrammatic representation of important picornavirus genera to show the differences between the encoded proteins and how mature proteins are generated. From top to bottom; *Enterovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Parechovirus* (A). Diagrammatic representation to show how the proteolytic processing cascade by the virally encoded proteins sequentially cleaves the PV polyprotein from top to bottom to show the order of processing (B). Figures adapted from (Castello, et al. 2011)

Key: ▽; L^{pro} cleavage, double asterisk; maturation cleavage, ▽; 3C^{pro} or 3CD cleavage site, ▽; 2A^{pro} cleavage site, S; polyprotein separation by ribosome slippage at DxExNPG/P sequence, *; cleavage by an unknown protease.

primary protein cleavage (along with the autocatalytic cleavage of L^{pro} in viruses that code for it) (reviewed in Palmenberg et al., 2010). The P1 N-terminus of most picornaviruses (apart from hepato- and parechoviruses) is co-translationally myristoylated by host enzymes (Chow et al., 1987) which indicates that the L protein must also be removed co-translationally to reveal the myristoylation signal sequence (section 1.5.6).

Secondary protein cleavages of the processing cascade are almost uniformly performed by 3C^{pro} or 3CD, (apart from the L^{pro} autocatalytic cleavage in aphtho- and erboviruses that releases L from P1-2A (Hinton et al., 2002)) (figure 1.6A). The cleavages occur at different rates (e.g. in PV (figure 1.6B)) and the sequence of cleavage is determined by the amino acids that make up the cleavage junction in the polyprotein (Zunszain et al., 2010). Structural studies of the 3C^{pro} enzymes of several picornaviruses have determined that their overall fold resembles trypsin-like serine proteases (Allaire et al., 1994, Matthews et al., 1994, Birtley et al., 2005, Cui et al., 2011). The active site of 3C^{pro} is enclosed in a peptide binding cleft flanked by two β -barrels configured similarly to the active site of serine proteases, but has a catalytic triad of cysteine-histidine-aspartate/glutamic acid instead of the serine-histidine-aspartate triad found in most serine proteases (Birtley et al., 2005, Cui et al., 2011). The nomenclature that will be used to discuss cleavage site specificity is NH₂-...P_n...-P4-P3-P2-P1/P1'-P2'-P3'-P4'-...P_n'...-COOH, where P and P' represent amino acids on the N- and C-terminal sides of the scissile bond respectively. The residues at the P1 and P1' sites of the cleavage junction are most highly conserved and considered most important however, the whole P4-P4' sequence although slightly less conserved is also critical for the overall cleavage specificity. For example PV and HRV 3C^{pro} cleaves between glutamine-glycine amino acids at the P1-P1' sites, but not all glutamine-glycine amino acid pairs are cleaved in the polyprotein (Blom et al., 1996). The requirement for a cleavage recognition sequence that can span up to 8 residues is thought to be related to the ability of the enzyme binding cleft to accommodate the amino acid chains of the substrate (Zunszain et al., 2010). Whereas the 3C^{pro} of HRV and PV has stringent requirements for

glutamine in the P1 position and has strongly selected against glutamic acid in this position, FMDV 3C^{pro} is more promiscuous in this respect and glutamic acid is present at several sites in the polyprotein of FMDV strain A₁₀61 (e.g. Blom et al., 1996). Differences are also present between the 3C^{pro} enzymes of different FMDV serotypes as seen by the wide range of P1-P1' cleavage pairs seen in serotype C viruses compare to other serotypes (Carrillo et al., 2005). This apparent promiscuity of FMDV 3C^{pro} appears to arise from the lack of a prominent β -ribbon structure seen in several enterovirus 3C^{pro} structures that confers increased substrate specificity to the protease (Birtley et al., 2005, Cui et al., 2011). The specificity for the cleavage sites is important because less efficiently cleaved sequences such as the 2B/C, 3A/B and 3C/D junctions allow the proteins to perform multiple functions both as precursors and mature proteins.

While the cleavage recognition site in the immediate vicinity appears to be important for 3C^{pro} processing at the scissile bond, evidence also suggests that the gross overall structure of the protein is important for its cleavage. This is most obvious in the cleavage of the P1 structural proteins into the mature capsid products VP0, VP3 and VP1 (figure 1.6B). Truncations at the N-, or C-termini of PV P1 (Ypma-Wong and Semler, 1987) prevent its processing, and truncations at the C-terminus of FMDV P1 (Ryan et al., 1989) effect the efficiency of its processing. Mutations some distance from the scissile bond both in the protomer structure and in the amino acid sequence can also affect processing (Gullberg et al., 2014, Escarmis et al., 2009). Additionally, in assays where intact P1-2A is presented to 3C^{pro} (Ryan et al., 1989, Gullberg, 2013a, Gullberg et al., 2013b) processing of the FMDV VP1/2A junction is the slowest of the processing events in the capsid protein. Whereas, in an assay where VP1/2A is presented to 3C^{pro} as a short synthetic peptide (assumed to lack any specific structure), cleavage is rapid (Birtley et al., 2005). HAV protomers lacking VP4 can still be processed normally although the VP4 protein in HAV is very small and so may not have a large impact on the structure of the protomer in different positions (Probst et al., 1999).

The presence of 3D^{pol} may be required for efficient 3C^{pro} processing of the VP3/VP1 and VP1/2A junctions in FMDV (Ryan et al., 1989) but processing can occur efficiently in systems lacking 3D^{pol} (e.g. Abrams et al., 1995). In PV, the precursor protein 3CD is more efficient at cleaving P1 than 3C alone (Ypma-Wong et al., 1988, Jore et al., 1988). Indeed, PV 3C^{pro} has been shown to only cleave the VP0/VP3 junction in the presence of 3D or when at very high, non-physiologically concentrations (Krausslich et al., 1990, Nicklin et al., 1988). In the PV sequence at the VP0/VP3 junction, when an unfavourable proline residue at the P4 position relative to the cleavage site is substituted for a favourable alanine residue, the site is cleaved much more efficiently by 3C^{pro}. However, when this sequence is mutated into the RNA genome, it is lethal for the virus (Blair et al., 1993). Protein cleavages are also staggered in FMDV (Grubman et al., 1995). This sequential processing in the capsid proteins is likely to provide a functional benefit to the virus such as a structural rearrangement conferred by the VP3/VP1 cleavage occurring first that is essential for particle assembly. This has also been investigated in chapter 4.

After secondary processing of P1 by 3C^{pro} into VP0, VP3 and VP1, the proteins undergo significant rearrangement (Hogle et al., 1985), but remain together as a 5S protomer (Bruneau et al., 1983) to become the basic unit of the virus particle.

The tertiary processing event called the maturation cleavage, is an intramolecular event whereby VP0 is cleaved into VP4 and VP2 and is required for the stabilisation of virus particles (section 1.7.3.5) and is the basis for investigation in chapter 6.

1.5.6 Myristoylation

The N-terminus of VP4 is co-translationally myristoylated by host N-myristoyl transferase in many picornaviruses (Chow et al., 1987) which occurs after the removal of the N-terminal methionine of P1 by host aminopeptidase. Myristoylation is a protein modification whereby a myristic acid fatty

acid is covalently bound to the Gly¹ of a consensus sequence Gly¹-x²-x³-x⁴-Ser/Thr/Cys⁵ either co- or post- translationally on nascent polypeptide chains (reviewed in Martin et al., 2011).

Myristoylation of proteins often functions as an important targeting signal which locates them to membranes and stabilises membrane association by anchoring the hydrophobic myristate into the hydrocarbon portion of the lipid bilayer. Myristoylation, however, is insufficient for membrane targeting without additional factors such as basic amino acid residues at the N-terminus of the protein (Peitzsch and McLaughlin, 1993, McLaughlin and Aderem, 1995, Yalovsky et al., 1999). The function of myristoylation in several viral proteins has been also elucidated e.g. myristoylation of HIV Nef protein facilitates its co-localization to interacting partners on membranes (Maurer-Stroh and Eisenhaber, 2004). The structural (gag) protein of HIV is also myristoylated and this has been shown to aid in the targeting of this protein to lipid rafts for multimerization and capsid assembly (section 1.7.3) (Li et al., 2007, Lindwasser and Resh, 2001).

Co-translational myristoylation of the N-terminus of most picornavirus P1 molecules occurs and is purported to have several functions. The lack of myristoylation has been shown to inhibit, but not block the proteolytic processing of P1 in FMDV (Abrams et al., 1995) and PV (Krausslich et al., 1990, Marc et al., 1989), as well as reduce the affinity for receptor binding in FMDV (Goodwin et al., 2009). This suggests that myristate molecules can influence the structure of P1 at sites which are distant to it in both sequence and in 3-dimensional space. The lack of myristoylation also prevents the entry of poliovirus (Marc et al., 1990), which may be a result of the inability of myristoylated VP4 molecules to penetrate membranes as effectively in the translocation of the RNA from the capsid to the cytoplasm (section (1.6.3) (Panjwani et al., 2014).

Several studies have demonstrated a role for myristoylation in the capsid structure and its assembly and these will be discussed further in sections 1.6 and 1.7 respectively and investigated in chapter 4.

1.6 The Picornavirus Particle

1.6.1 Structure overview

The structures of many picornavirus virions have been solved by x-ray crystallography e.g. PV (Hogle et al., 1985), and FMDV (Acharya et al., 1989). The structural architecture of these viruses is largely similar, but there are also important differences between them. Sixty copies of each of the structural proteins VP4, -2, -3 and -1 are assembled via twelve pentameric structures into pseudo T=3 icosahedral capsid structures that have 2-, 3- and 5-fold symmetry axis (figure 1.7A). In viruses that encode a VP4 protein, it is found on the inside of the capsid structure at the 5-fold axis (along with its N-terminal myristate group) with the processed N-termini of the other capsid proteins. VP1 is located around the 5-fold axis of symmetry where five copies of the P1 structural precursor come together to form a pentamer, and VP2 and VP3 are found around the two- and three-fold symmetry axes. The C-termini of these proteins are found on the exterior of the capsid (reviewed in Fry and Stuart, 2010). While the core features of the different structural precursors found within the family are relatively similar, their capsid structures have differences, such as surface topology (figure 1.7B) which are important for a number of processes.

Each capsid subunit is derived from one copy of P1 that has been processed into VP4, -2, -3 and 1 (section 1.5.5). The structures of VP2, -3 and -1 all resemble a “wedge-shape” and are composed of eight β -strands arranged in an anti-parallel conformation that form a β -barrel. Each β -strand is named alphabetically from the N-terminus of the protein from B to H. The shape of the β -barrel is conferred by two sets of β -sheets composed of four β -strands each (strands CHEF and BIDG) (Hogle et al., 1985) (figure 1.8). The loop structures that connect the strands are named by the β -strands that they connect. They are generally located on the outside of the virus particle and contain the major antigenic sites in the virus for example the G-H loop antigenic loop of FMDV.

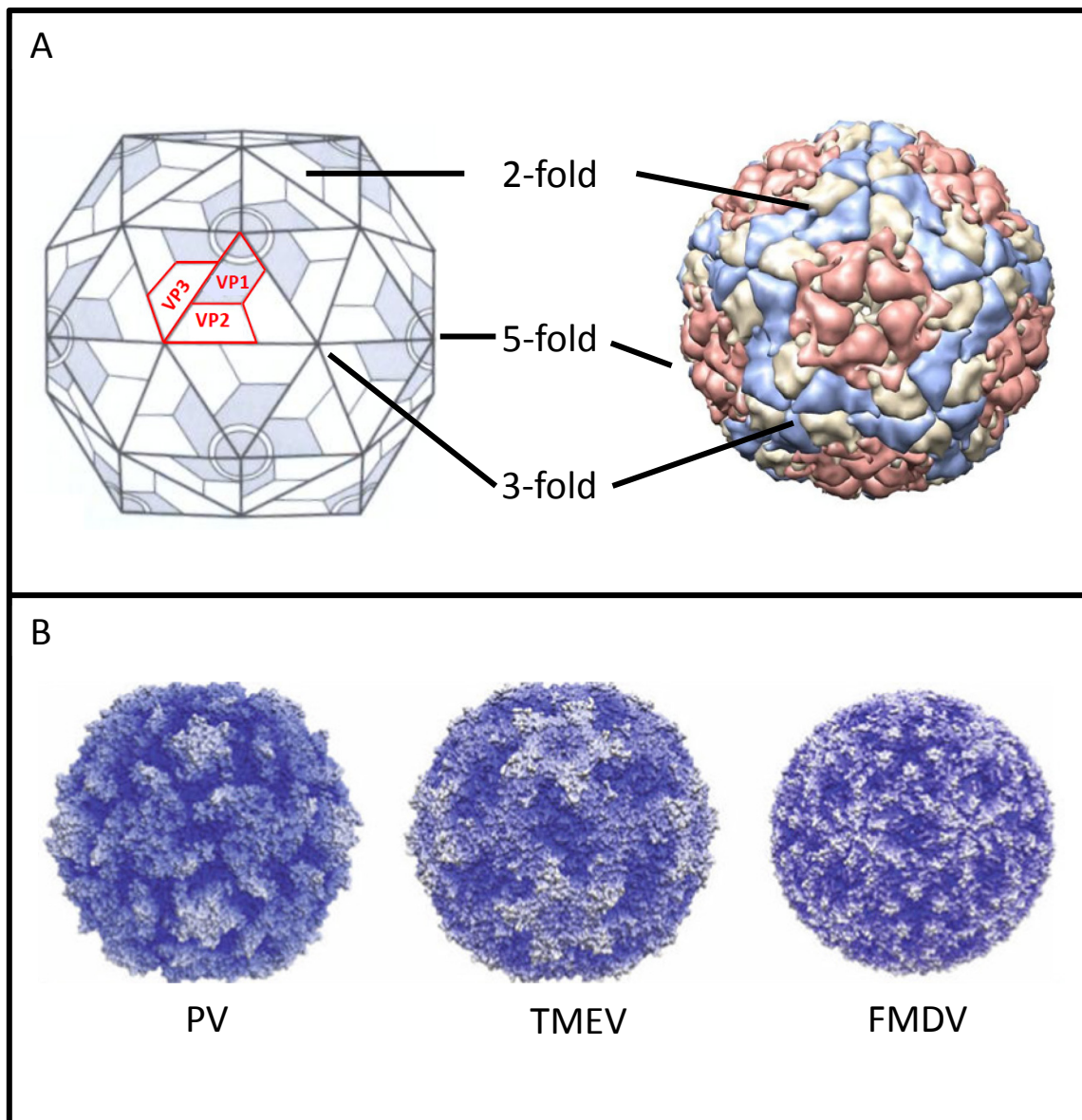


Figure 1.7 Structure of the picornavirus particle.

A; Diagrammatic representation of the picornavirus capsid structure showing the locations of the capsid proteins VP1, VP2 and VP3 and the symmetry axis (left). This view is of a capsid tilted slightly upwards in comparison to the computer generated space filling model generated from the capsid crystal structure of FMDV strain A22 Iraq which is orientated to look straight into the five-fold axis (right). In the crystal structure model, VP1 is coloured pink, VP2 in blue and VP3 in yellow. Space filling model obtained from the protein data bank ID: 4GH4 (Curry et al., 1996).

B; Radial depth cued images to compare the picornavirus capsid topology in:
 Poliovirus (PV) - 32nm in diameter with a 5-pointed star shape at the 5-fold axis, surrounded by a "canyon" with a three-bladed "propeller" at the three-fold axis.
 Theiler's murine encephalitis virus (TMEV) – 32nm in diameter with an extended star shape at the fivefold axis and surface depressions spanning the two-fold axis
 Foot-and-mouth disease virus (FMDV) – 30nm in diameter with a smooth surface relative to the other viruses. Figure taken from Tuthill et al. (2010).

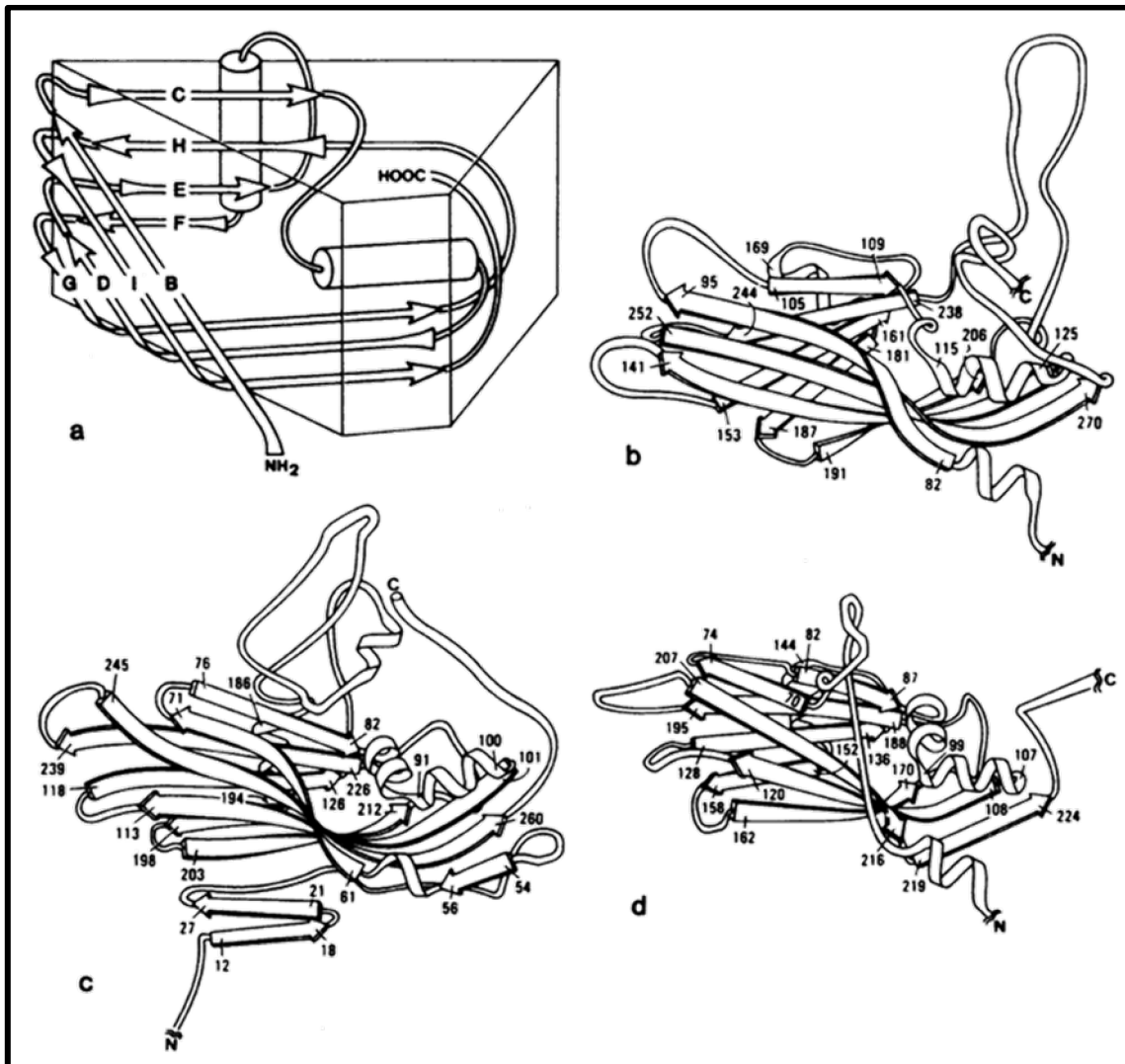


Figure 1.8 Schematic to show the structure of the individual PV capsid proteins in the virion. Simplified diagram of the “wedge structure” conferred by the eight antiparallel β -strands that make up two sets of β -sheets in the wedge with strands labelled alphabetically from B to H (A). Ribbon diagrams show the individual VP1 (B), VP2 (C) and VP3 (D) structures with numbers representing amino acid positions starting from the N-termini of the proteins. N.B. the N- and C-termini of VP1 and the N-terminus of VP3 has been truncated for clarity. Figure taken from Hogle et al. (1985).

The FMDV capsid proteins VP1, -2 and -3 average 24 kilodaltons (kDa) in molecular weight whereas in other picornaviruses they are approximately 30kDa. This is thought to be due to variations in the length of the surface exposed loops which consequently make the FMDV capsid structure thinner than many other picornaviruses at about 33 angstroms (Å) (Acharya et al., 1989) compared with 42.5Å in HRV (Rossmann et al., 1985). A conserved feature in picornavirus structures is that the N-termini of five VP3 molecules in a pentamer form β -sheets in a “twisted tube” resulting in a β -annulus at the five-fold axis of symmetry with a gap in the middle. The N-termini of five myristyl-VP4 molecules are also in close proximity. In enteroviruses the myristate molecules are thought to provide structural support to the “twisted tube” formed by the VP3 N-termini by hydrogen bonding with other VP4 and VP3 molecules in the same pentamer stabilising the formation of viruses and empty capsids (Moscufo and Chow, 1992). However, in FMDV, the N-terminus of VP4 and myristic acid are disordered which suggests that this region may be performing a different function (Acharya et al., 1989).

The central and N-terminal domains of VP4 (including the myristate group) are largely disordered in most picornavirus crystal structures along with the N-terminal domain of VP2 in FMDV and the N-terminal domain of VP1 in PV and HRV. This indicates that these regions have some flexibility in the capsid and they have been implicated in the cell entry process. The RNA is generally not seen in picornavirus crystal structures because it does not conform to the symmetry of the capsid, but some structures have shown what appears to be RNA bases stacked up against VP2 (Arnold and Rossmann, 1990, Filman et al., 1989) and VP4 (Lentz et al., 1997) inside the capsid.

The capsid has an important role in protecting the RNA from the external environment, but its structure is also important for several processes within the virus lifecycle.

1.6.2 Capsid structure and its relationship to receptor binding

Picornaviruses have evolved to use a diverse range of cell surface receptors to target host-cells and the capsid is key to receptor recognition. As shown in figure 1.7(B), the PV five-fold axis has a prominent five-pointed star shape surrounded by a deep depression known as the “canyon”, and it is a feature present in many *Enterovirus* species. The surface depressions in cardioviruses are less well pronounced than the canyon but they appear to have an analogous function, which is that of receptor binding. Major receptor group HRVs which bind to the cellular receptor ICAM-1 have been shown to use the canyon in this way (Colonno et al., 1988, Olson, 1993) as have polioviruses which uses the poliovirus receptor (Pvr) (Belnap et al., 2000b) and coxsackie viruses which use the coxsackie-adenovirus receptor (CAR) on HeLa cells (He et al., 2001). In contrast, FMDV field strains bind to integrin receptors (Monaghan et al., 2005) and this is mediated by an arginine-glycine-aspartate (RGD) sequence on the prominent G-H loop of VP1 (Acharya et al., 1989, Logan et al., 1993).

1.6.3 Capsid alterations during genome delivery

The attachment of the viral capsid to a cellular receptor is the first step in the entry of picornaviruses into cells and this is followed by endocytosis. Field isolates of FMDV that bind integrins use a clathrin-dependent pathway for endocytosis (Berryman et al., 2005, O'Donnell et al., 2005b), and genome delivery to the cytoplasm takes place in early endosomes (Johns et al., 2009). In contrast, PV is thought to enter cells by a non-canonical endocytic pathway because it is insensitive to inhibitors of clathrin, caveoli, macropinocytosis and microtubule polymerisation. PV may rapidly travel around the cell while inside an endocytic vesicle by using actin filaments before translocating its RNA into the cytoplasm (Brandenburg and Zhuang, 2007, Vaughan et al., 2009).

During the process of RNA release into the cytoplasm, changes occur to the capsid. The capsid proteins become separated from the RNA in PV infection remain inside endocytic vesicles which traffic to the perinuclear region (Vaughan et al., 2009). During RNA translocation VP4 is also lost and

the remaining capsid proteins remain associated as a slightly expanded empty particle in enteroviruses (that sediments in sucrose at 80S) (Belnap et al., 2000a, De Sena and Mandel, 1977, Levy et al., 2010) but dissociate into pentamers that sediment at 12S in aphtho- and cardioviruses (Mak et al., 1970, Dubra et al., 1982). This dissociation occurs in acidic conditions, and as such, it is thought that RNA translocation in aphtho- and cardioviruses is linked to endosomal acidification (Berryman et al., 2005, Johns et al., 2009).

Several capsid alterations are observed to occur which facilitate the process of RNA translocation. In enteroviruses, a process called “breathing” occurs at physiological temperatures, which involves the transient externalisation and re-internalisation of VP4 and the N-terminus of VP1. The trigger for the loss of VP4 appears to be receptor binding to the canyon in PV and major receptor group rhinoviruses, whereas the trigger in minor group viruses is an acidic pH (reviewed in Tuthill et al., 2010). The loss of VP4 in enteroviruses results in the appearance of an altered or “A” which sediments at 135S in sucrose compared with 160S sedimentation for the corresponding viruses. A particles have irreversibly externalised the N-terminus of VP1 and are slightly expanded (Fricks and Hogle, 1990, Lewis et al., 1998). The conversion to RNA containing “A” particles precedes the formation of 80S particles that have lost RNA suggesting that the “A” particles are involved in genome delivery. Indeed, cryo-EM reconstructions using “A” particles and model membranes have revealed an “umbilical connector” which connects the capsid to the membrane (Strauss et al., 2013) and myristoylated VP4 has been shown to make pores in membranes (Panjwani et al., 2014) indicating a route for RNA egress from the capsid to the cytoplasm across the endosomal membrane.

Due to the high sensitivity of *Aphthovirus* capsids to dissociation in acidic conditions, the RNA translocation mechanism was presumed to be different. However, the discovery of expanded dissociation intermediates in ERAV (Tuthill et al., 2009, Bakker et al., 2014) and preliminary evidence

of the same in FMDV (Gold et al., unpublished data), may indicate a conserved mechanism similar to that seen in enteroviruses.

1.6.4 Structure and antigenicity

Major antigenic sites on picornaviruses are found on the loops connecting the β -strands between the capsid proteins. The immunodominant epitope in many FMDV strains is found on the large protruding G-H loop of VP1, although there are several other important immunogenic epitopes (Mateu et al., 1995) and on O-serotype viruses antigenic site 2 in the B-C loop has been shown to be immunodominant (Mahapatra et al., 2012). Counterintuitively, the G-H loop is also required by the virus in cell entry and neutralising escape mutations occur continuously but map to sequences away from the RGD binding motif (Mateu et al., 1994). The G-H loop can also adopt several conformations, for example, when disulphide bonds at the base of the loop are reduced, the loop lies flat on the capsid surface (Logan et al., 1993). Neutralising escape mutations have been found at positions distant from the G-H loop that affect recognition of the loop and it is thought that these mutations have the effect of flattening the loop so that the neutralising epitope is no longer accessible to antibodies (Parry et al., 1990) (figure 1.9A).

The capsids of many enteroviruses such as PV bind what are thought to be lipids in the canyon (Filman et al., 1989) that are thought to confer the native N-antigenicity to virus capsids (reviewed in Ansardi et al., 1996). However, these “pocket factors” are not found in HRV14, HRV3, EMCV or mengovirus, and their function is poorly understood (reviewed in Jiang, 2014). Polioviruses express major (N-AgIA, -IIA, -IIIA, and -IIIB) and minor neutralisation sites (N-AgIB and -IIB) as three sets (figure 1.9B), which results in three PV serotypes (Jiang et al., 2014). Unlike FMDV, where constant divergence within serotypes hinders vaccine efficacy, no new serotypes of PV have emerged since the three serotypes were first discovered. HRV, which has a similar capsid structure to PV exists as many serotypes and it appears that for a new serotype of PV to evolve changes would be required to

occur to all of the N-Ag sites on the capsid simultaneously (reviewed in Jiang et al., 2014). Picornavirus capsids which sediment in sucrose at 160S (PV) and 146S (FMDV) have been shown to have different antigenic characteristics than particles on the disassembly pathway such as the 135S “A” particle with RNA and 80S capsids that lack RNA in enteroviruses (Hummeler et al., 1962 and reviewed in Hogle, 2002), and the 12S dissociated pentamers in FMDV (Doel and Chong, 1982, Cartwright, 1962, Cartwright et al., 1980, Rowlands et al., 1975). All of the particles on the disassembly pathway lack VP4 whereas VP4 is present internally in the intact virion and this is the subject of investigation in chapter 6 and further introduction in section 1.7.3.5.

1.7 Capsid assembly

1.7.1 Assembly overview

Nucleic acids are highly labile molecules and are rapidly digested by intra- and extracellular nucleases. Viruses overcome this problem by generating a capsid protein shell which is essential for the protection and carriage of the viral genome cargo from one host cell to another. As shown by the examples in section 1.6, the structure of the capsid is fundamentally important for many viral processes to occur. The mechanisms of capsid assembly have been well characterised in several bacteriophages and other viruses and these will be discussed before picornavirus assembly for comparison.

1.7.2 Model systems for virus capsid assembly

1.7.2.1 Mathematical models for virus assembly

To conserve the size of their genome, viruses encode only a limited amount of information for their capsid, that can be repeatedly expressed to form identical subunits that fit together to form the closed capsid structure. The repetitive nature of the subunits has allowed the fitting of mathematical models to the capsid assembly process. Modelling of capsid assembly originates from the work of Casper and Klug (Casper and Klug, 1962) who hypothesised that spherical capsids have icosahedral,

quasi-equivalent symmetry. They hypothesised that icosahedral virus capsids must conform to similar principles in their assembly in which triangular subunits make up pentameric and/or hexameric subunits which then make up the complete capsid structure. This theory was subsequently demonstrated to be accurate for the structures of many small animal virus capsids in which 180 chemically identical (T=3) or chemically similar (pseudo T=3 e.g. picornaviruses) subunits form pentameric subunits in the capsid surface structure (reviewed in Rossmann and Johnson, 1989). This theory also holds true for the capsids of large viruses such as the T=7 bacteriophages P22 (Jiang et al., 2003) and HK97 (Wikoff et al., 2000) which have 420 capsid proteins arranged into 60 hexamers and 12 pentamers. There are some notable exceptions to the quasi-equivalence theory including members of the adeno-, papilloma-, polyoma- and reoviruses that encode flexible arms on the inside surface of the capsid that allow the bonding of capsid subunits together in shapes that do not conform to an icosahedral lattice (Prasad and Schmid, 2012).

A key question in protein folding is that of Levinthal's paradox in which proteins can adopt a vast number of conformations based upon their sequence but appear to rapidly fold into the correct conformation (Zwanzig et al., 1992). In the case of capsids, this applies to the folding of the capsid subunits in such a way as to permit their rapid assembly into capsids. Capsids have been modelled to assemble by a process in which a couple of subunits come together slowly (nucleation) followed by a faster process (elongation) in which capsid subunits assemble more rapidly once a critical nucleus has been reached (Endres and Zlotnick, 2002). Using an *in silico* modelling approach to small single stranded RNA viruses, Dykeman and colleagues (2014), showed that as the concentration of capsid subunits increases in virus infection, there is a concurrent increase in the efficiency of capsid assembly and genome packaging specificity (section 1.7.3.4) which is achieved by a decrease in the number of nucleation events.

1.7.2.2 Large DNA viruses

The assembly mechanisms in a number of DNA bacteriophages have been well characterised and are similar to the assembly mechanisms used by herpesviruses and adenoviruses demonstrating that they have potential as models for the assembly of animal viruses (Prevelige and King, 1993). The T=7 capsid of the tailed DNA phage P22 (*Podoviridae*) requires multiple virus encoded proteins including a scaffold protein (gp8) for its assembly and packaging. Approximately 300 gp8 proteins ultimately form an inner shell onto which 420 coat proteins (gp5) sit, and this is achieved through the addition of sequential gp8 subunits which accommodate a gp5 subunit at their terminus in the creation of a protein shell called the procapsid (Prevelige and King, 1993). During assembly of the gp5 coat, several injection proteins are assembled onto one of the 12 capsid vertices creating a portal through which the DNA genome is inserted, the scaffold proteins exit (Earnshaw and Casjens, 1980) and the tail-spike is attached (Goldenberg and King, 1981). The maturation of the capsid is complete after large scale structural changes increase the size of the procapsid making it bigger, angular and more stable (figure 1.10; reviewed in Aksyuk and Rossmann, 2011). Similarly, the T=16 capsid of herpesvirus (composed of 150 hexamer and 12 pentamer subunits) assembles via an empty procapsid lacking DNA on a scaffold protein which is removed from the mature virus via a portal (Brown and Newcomb, 2011).

1.7.2.3 Human immunodeficiency virus-1 (HIV-1)

Enveloped viruses also contain capsids inside their membranous envelope. The capsid structure of the retrovirus HIV-1 is composed of approximately 1500-3000 Gag polyproteins which assemble into spherical capsids on membranes (sedimenting at approximately 750S) in a process much more complex than has been described for the non-enveloped viruses so far (reviewed in Lingappa et al., 2014). This is in part due to the presence of many domains in the approximately 500 amino acid Gag polyprotein that can influence different parts of the assembly pathway (figure 1.11). The assembly of immature HIV-1 particles occurs inside cells, whereas the maturation of the capsid in which Gag is cleaved into its four constituent domains occurs after the viruses have become enveloped by host

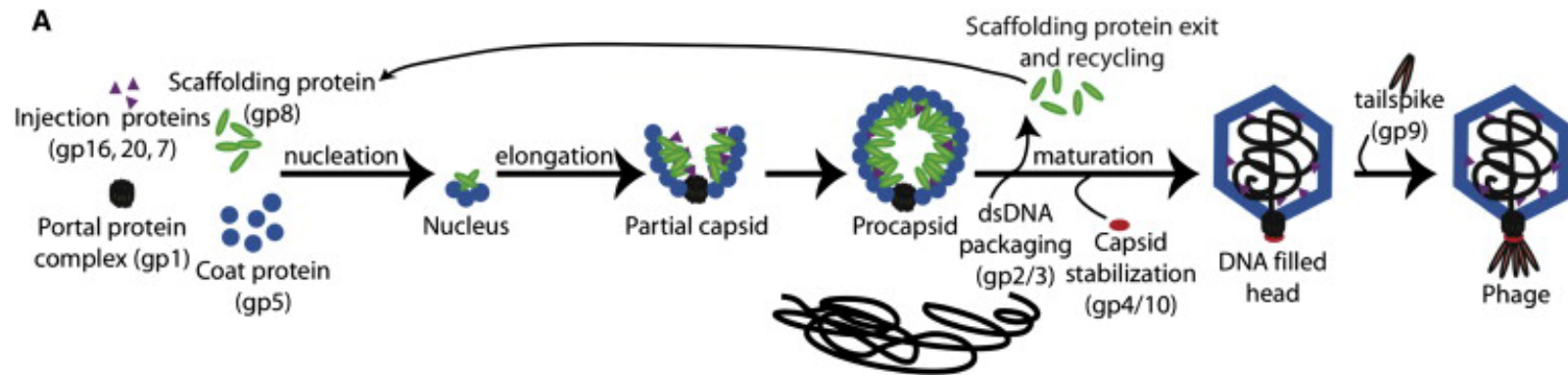


Figure 1.10 Assembly pathway in bacteriophage P22. The coat, portal, infection and scaffold proteins nucleate the assembly process. The coat protein assembles around the scaffold proteins using the injection proteins for support. Procapsids are formed that contain a portal into which the DNA is packaged, scaffold proteins are removed and the capsid is stabilised before the attachment of the tail-spikes. Image taken from Rizzo et al. (2014).

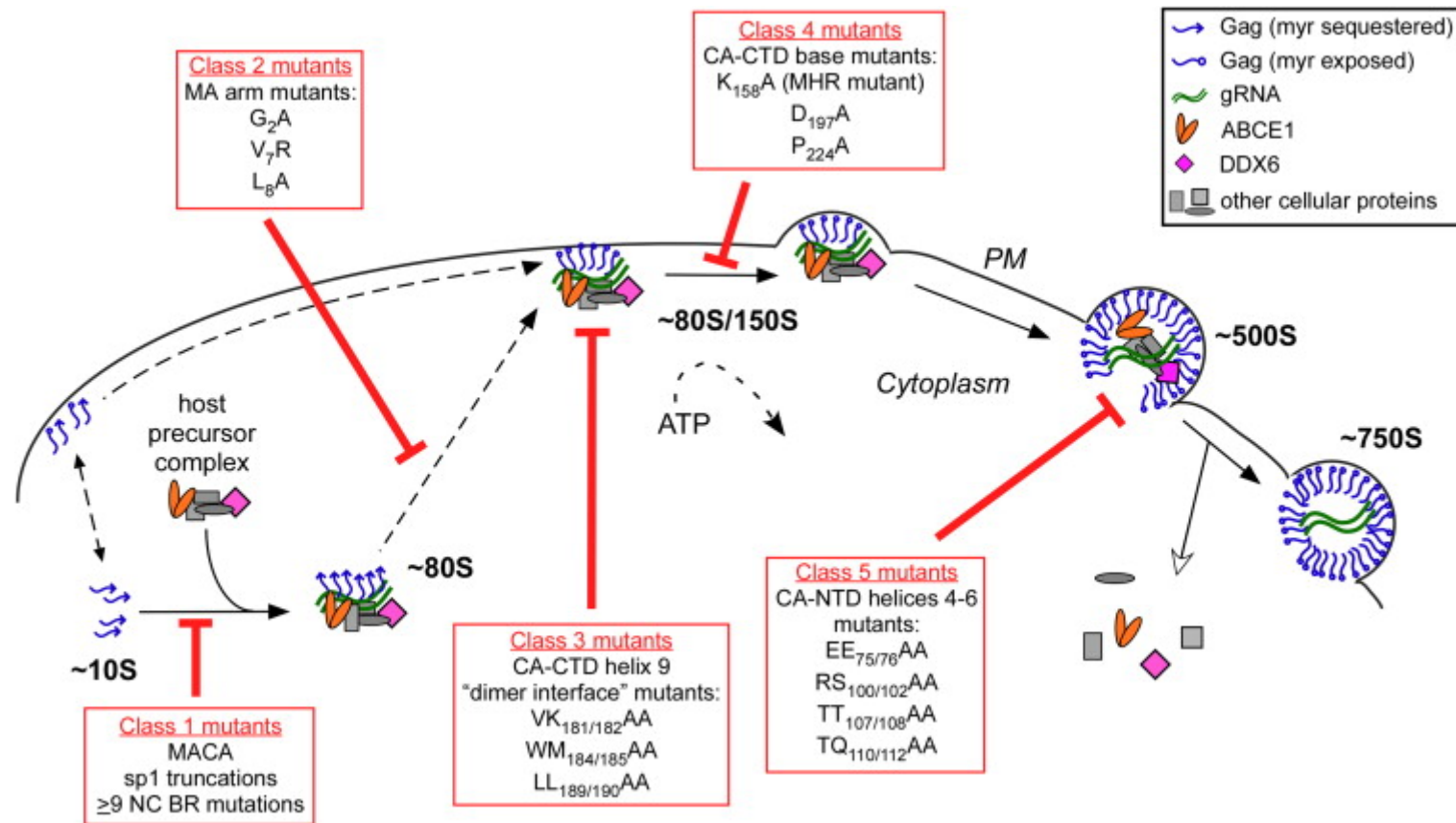


Figure 1.11 Assembly of HIV capsids and mutations that can prevent assembly. The Gag protein transitions through several multimeric states with the help of host proteins such as ABCE1 and DDX6. Mutations in the matrix (MA) and capsid C-terminal (CA-CTD) domains and sub-domains that inhibit the assembly pathway at different points are shown in pink boxes and are organized into classes depending on the stage of assembly that they prevent. The mutations are presented as single letter amino acid codes with the numbers representing the position of each amino acid from the N-terminal start point of that domain. E.g. EE_{75/76}AA in CA-CTD means that the two glutamic acid residues present at positions 75 and 76 from the start of the CA-CTD have been mutated to alanine. Figure taken from Lingappa et al. (2014).

membranes and the virions have budded from the cells. The Gag polyprotein is co-translationally myristoylated (like the N-terminus of picornavirus P1 (section 1.5.6)) at the N-terminal matrix (MA) domain, which, along with MA contributes to the recruitment of Gag to the plasma membrane (reviewed in Chukkapalli and Ono, 2011). Gag multimerisation which starts in the cytoplasm and continues at the plasma membrane is a poorly understood process, progresses through $\approx 10S$, $\approx 80S$, $\approx 150S$ and $\approx 500S$ sedimentation intermediates as more Gag molecules are recruited (figure 1.11). The multimerization process sequesters several host proteins to facilitate assembly (ABCE-1 and DDX6) (Robinson et al., 2014) and package RNA (Staufen) into the multimerising complex (reviewed in Cochrane et al., 2006) which are removed from the particles before they bud from the membrane and acquire an envelope (figure 1.11). HIV-1 maturation occurs during, or immediately following budding and involves the cleavage of Gag into its four domains; MA, capsid (CA), nucleocapsid (NC) and P6 by the viral protease. Maturation results in a significant rearrangement of Gag domains resulting in the characteristic conical capsid formed from approximately 1200 copies of the CA domain (reviewed in Sundquist and Krausslich, 2012)

1.7.2.4 Hepatitis B virus (HBV)

A member of the *Hepadnaviridae* family, HBV is also an enveloped virus but encodes a DNA genome of approximately only 3kb. The capsid is made up of a single protein known as the core protein. In a similar fashion to the assembly of RNA phages, the core protein assembles via dimer formation (Zhou and Standring, 1992), but in contrast, these dimers have the same conformation as each other and are linked by a disulphide bridge (Nassal et al., 1992). The assembly of dimers into the complete core is not well understood, but the cellular chaperone nucleophosmin binds to HBV core dimers and promotes their assembly (Jeong et al., 2014). Interestingly chaperones have been implicated in picornavirus assembly and are the subject of investigation in chapter 5. The C-terminal 34 amino acids of the approximately 180 amino acid core protein (C) form a domain which is dispensable for capsid assembly, but critical for binding to and encapsidating RNA (Gallina et al., 1989). HBV cores

assemble aggressively into icosahedral capsids with either T=3 (90 C protein dimers) or T=4 (120 C protein dimers) symmetry (Crowther et al., 1994), and both are found in the livers of infected patients (Kenney et al., 1995) but the significance of this is not known. HBV C protein non-specifically packages RNA *in vitro* but packaging of RNA *in vivo* requires phosphorylation of the C-terminal domain (Gazina et al., 2000, Chen et al., 2011). A host protein kinase (SRPK) has been shown to bind to the C-terminal domain and stall capsid assembly until, upon phosphorylation, the capsid is released, demonstrating the role for a second chaperone in the HBV capsid assembly and packaging process (Chen et al., 2011). The maturation of the capsid is said to occur when a partial DNA copy is synthesised from the RNA template by the viral reverse transcriptase enzyme. This process occurs inside the formed capsid and is facilitated by naturally occurring 15Å pores in the capsid dimer structure (Zlotnick et al., 1999) that allow nucleotides into the capsid lumen for the reverse transcription reaction.

1.7.2.5 RNA viruses

The capsid assembly process of RNA bacteriophages of the *Leviviridae* family such as MS2, phiCb5 and Qβ are also well characterised. They are some of the most simple viruses with a 3.5kb genome and a T=3 icosahedral capsid measuring approximately 30nm diameter. Like picornaviruses the capsid surface is composed of 180 proteins, but in contrast, these proteins have the same amino acid sequence and the way they interact with each other changes their structure into three “conformers” (A, B and C). The virions also contain one copy of a maturation protein known as the A protein. Ninety dimer pairs are formed in total between AB and CC conformers of the capsid. These dimers have a highly extensive network of interactions and the two types are governed by the way a loop connecting the F and G β-strands is folded (Valegard et al., 1990). The conformation of the capsid protein can be switched (CC to AB) by the binding of an RNA stem loop (Stockley et al., 2007) and the RNA can interact with neighbouring dimers that bring the capsid together (Plevka et al., 2009).

1.7.3 Picornavirus assembly

1.7.3.1 Overview

The picornavirus assembly process starts with the P1-2A subunit, proceeds through a pentameric intermediate and is completed, when, upon the encapsidation of RNA, the virus matures and is competent for the infection of new cells. The processes of myristoylation and proteolytic processing modify the P1-2A subunit to facilitate its assembly into pentamers. However, the only structural information available for processed P1 and pentamers has been inferred by the nature of these structures in the completed capsid. The same applies for the process of RNA encapsidation. The next few sections summarise what is known about the picornavirus assembly process (figure 1.12), much of which has been investigated in PV and FMDV but where differences are known between other viruses, such as HAV, they are highlighted.

1.7.3.2 5S protomer to 14S pentamer

The P1 (PV) or P1-2A (FMDV) capsid subunit is generated upon its translation (section 1.5.4), and separation from the non-structural proteins. In PV, 2A^{pro} is a protease that cleaves itself from P1 during or immediately after translation (Toyoda et al., 1986) and although 2A remains attached to FMDV P1 after primary processing it has been shown not to be required for the assembly of pentamers *in vitro* (Goodwin et al., 2009). The 2A protein remains attached to VP1 after 3C^{pro} processing in HAV and is required for its assembly (Cohen et al., 2002). It is removed from P1 at a late stage of infection by an unknown cellular enzyme and its removal confers infectivity to the virus (Cohen et al., 2002). Neither P1, VP0 nor VP4 are myristoylated in HAV and a recent crystallographic study has suggested that 2A may perform the role of myristate in helping to stabilise the virus at the 5-fold interface (Stuart et al., 2014).

Co-translational myristoylation of P1 is conserved in all picornaviruses apart from HAV and parechoviruses. It appears to stabilise the β -annulus structure at the 5-fold axis of symmetry (section 1.6.1) and the lack of it prevents the assembly of pentamers (Ansardi et al., 1992) or assembles

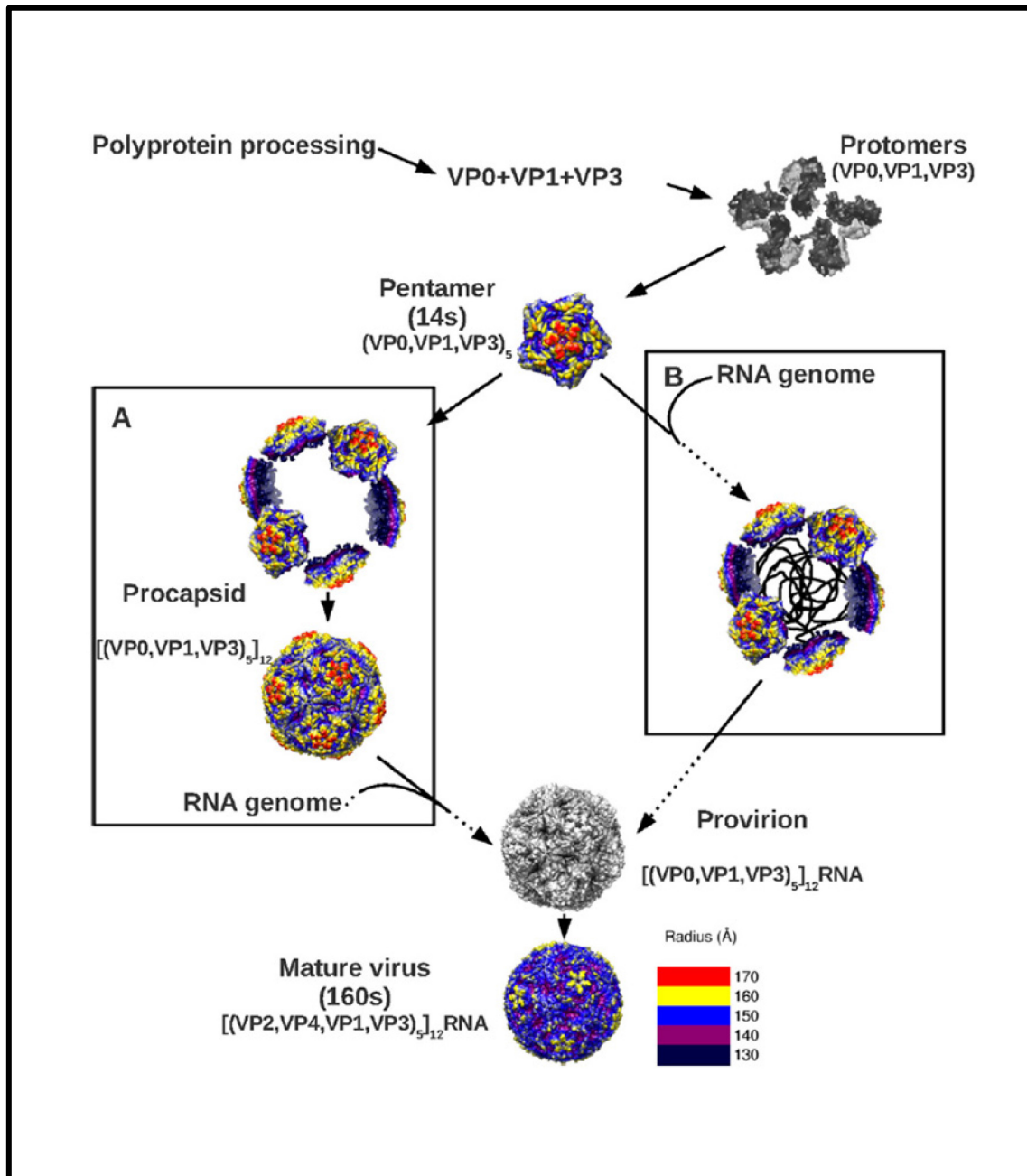


Figure 1.12. A schematic of picornavirus assembly. After polyprotein processing, the capsid protomer remains intact and five copies assemble into pentamers. Encapsidation models suggest that either RNA is inserted into pre-formed empty capsids (left fork) or that pentamers condense around the RNA to form the procapsid structure (right fork). The maturation cleavage of VP0 into VP4 and VP2 is the final step of the assembly pathway and locks capsids into a “meta”-stable state for the infection of new cells. The capsid depicted is EV71 and the topology of the capsid is shown using the radial heat map described before whereby the radius is the distance from the centre of the particle in angstroms. Figure taken from Cifuentes et al. (2013).

pentamers and capsid components with abnormal sedimentation characteristics (Goodwin et al., 2009, Moscufo and Chow, 1992). If the 2A protein performs the role of myristate in HAV, then quite how members of the parechovirus stabilise their capsids is unknown. The contribution of myristoylation to pentamer formation in FMDV is one of the subjects of investigation in chapter 4. The capsid monomer or protomer is produced by 3C^{pro} processing and before and after its processing has a sedimentation in sucrose of 5S (Goodwin et al., 2009). Processing of P1 by 3C^{pro} yields the capsid proteins VP0, VP3 and VP1 that remain associated as the protomer (Bruneau et al., 1983). It is general accepted that processing of P1 or P1-2A by 3C^{pro} facilitates its assembly into higher order structures because the protein termini are not attached to each other in crystal structures e.g. FMDV (Acharya et al., 1989) and PV (Hogle et al., 1985). These termini are thought to contribute to inter-protomer interactions required for pentamer stabilisation (reviewed in Ansardi et al., 1996).

There is a growing pool of evidence to suggest that many viruses use the cellular chaperone machinery for various parts of their life cycle including assembly (reviewed in Geller et al., 2012). For example, rabies virus requires the same host protein (ABCE-1) (Lingappa et al., 2013) for its assembly as HIV-1 (section 1.7.2.3) and adenoviruses interact with the same host protein (nucleophosmin) in capsid formation as HBV (section 1.7.2.4) (Ugai et al., 2012). Assembly of protomers into higher order structures in several enteroviruses (PV, HRV, CV and EV71) also appears to be dependent on the action of cellular molecular chaperones, in this case, the heat shock protein 90 (Hsp90) and its co-chaperone, (p23) (Geller et al., 2007). Pharmacological inhibition of this cellular chaperone prevents processing of P1 by 3C^{pro} and targets it for degradation by the proteasome (Geller et al., 2007, Tsou et al., 2013) suggesting that pentamer formation will be prevented (figure 1.13). Escape mutants do not appear in cells or animals infected with PV and treated with Hsp90 inhibitors suggesting that it has an obligate requirement for Hsp90 where there is a high fitness cost paid by not using the chaperone (Geller et al., 2007). HAV is the only picornavirus to behave differently in

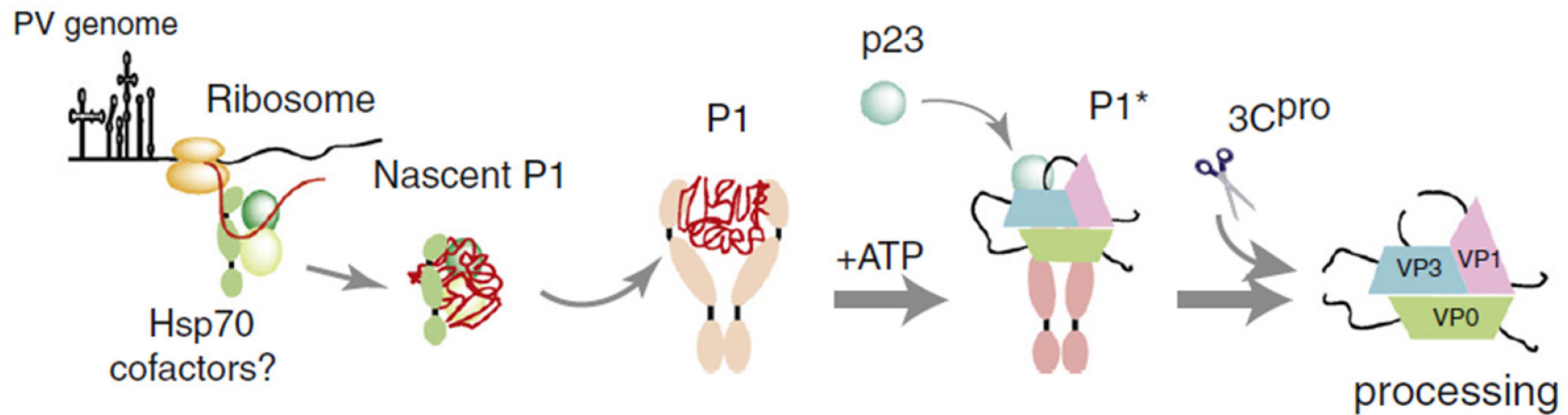


Figure 1.13 Model for the interaction of Hsp90 with poliovirus P1. The poliovirus genome is translated by the ribosome and the nascent P1 polyprotein produced is bound by the cellular chaperone Hsp70. Hsp70 performs initial folding on the P1 molecule before it is cycled to Hsp90 for more extensive folding, in concert with the Hsp90 co-chaperone p23 and requiring the hydrolysis of ATP. P1 is folded into a conformation that allows access to the scissile bonds by the 3C^{pro}. Image taken from Geller et al. (2012).

this respect. It is relatively insensitive to Hsp90 inhibitors when compared with poliovirus (with an IC_{50} to geldanamycin (GA) almost 20 times greater) and it has been suggested that HAV P1 may use an alternative pathway (Aragonès et al., 2010). The requirement for Hsp90 in the FMDV lifecycle is the subject for investigation in chapter 5. The cellular tripeptide, glutathione, has also been shown to be required for the assembly of pentamers from protomers in two recent studies (Ma et al., 2014a, Thibaut et al., 2014). Both sets of authors found that enteroviruses were highly sensitive to glutathione depletion. The inhibitor was used to generate mutant viruses that could replicate in the absence of glutathione. These escape mutants displayed threonine to methionine mutations at interfaces between the protomers and methionine residues at this position were also found in viruses that were insensitive to the inhibitor. Glutathione was found to directly interact with the capsid protein VP1 suggesting that it is providing structural support to pentamers in the absence of methionine at this position.

Five copies of P1 assemble into pentameric structures which sediment at 14S in sucrose (Watanabe et al., 1962, Phillips, 1969, Phillips et al., 1968, Palmenberg, 1982).

1.7.3.3 14 S pentamer to 75S/80S empty capsids

Capsids lacking RNA, which are known as empty capsids or virus-like particles have been isolated from the infected cells of many picornaviruses including the entero- and aphthoviruses (Cowan and Graves, 1966, Su and Taylor, 1976, Korant et al., 1975, Maizel et al., 1967) but not cardiaviruses (Jiang et al., 2014). Empty capsid are formed by the assembly of twelve pentamers (Onodera and Phillips, 1987, Phillips, 1971, Rombaut et al., 1991) and are thought to spontaneously assemble in a concentration dependent manner (Rombaut et al., 1991, Li et al., 2012) when there is an absence of sufficient viral RNA to form virions (Verlinden et al., 2000, Li et al., 2012). Empty capsids of PV have been suggested to form as a by-product of insufficient RNA, and when PV infected cells are treated with GuHCL which inhibits RNA synthesis (section 1.5.3) large quantities of ECs are formed relative to

untreated cells (Jacobson and Baltimore, 1968b, Basavappa et al., 1994). These resemble empty capsids isolated from infected cells antigenically and in their ability to be dissociated into pentamers and reassembled into capsids (Icenogle et al., 1981, Marongiu, 1981 #8791, Rombaut et al., 1982).

Several alternative hypotheses have been suggested for the presence of empty capsids. One such hypothesis suggests that they are a precursor particle to viruses into which the RNA is inserted (Jacobson and Baltimore, 1968a) in a similar way to the provirions of P22 phage (section 1.7.2.2). A second theory suggests that they are a by-product of assembly and are formed due to an excess of pentamers as explained above (Koch and Koch, 1985) and a third theory suggests that empty capsids are merely an artefact of the methods used to solubilise infected cells (Pfister et al., 1992). While there is evidence for all of these theories (reviewed in Ansardi et al., 1996), the true role of empty capsid formation in infected cells is unknown. However, the ability to assemble empty capsids from pentamers at a high enough concentration has proved a useful tool in the development of novel vaccines. Expression cassettes encoding P1 or P1-2A and 3C^{pro} have been used in a number of systems to generate empty capsids for a number of viruses to examine their potential as novel vaccines (Lewis et al., 1991, Oem et al., 2007, Cao et al., 2009, Roosien et al., 1990, Porta, 2013, Li et al., 2013, Rombaut and Jore, 1997, Han et al., 2012, Pan et al., 2008, Abrams et al., 1995, Winokur et al., 1991, Ma et al., 2014b, Estes et al., 1987, Simons et al., 1993, Gullberg et al., 2013a, Polacek et al., 2013).

1.7.3.4 14S pentamers to 150S provirions – RNA encapsidation

According to the most widely accepted model for RNA encapsidation, pentamers condense around RNA in the formation of provirions. In support of this model (rather than a model where RNA is inserted into an empty capsid), and in parallel with the pathways of encapsidation in RNA phages (section 1.7.2.5) and small RNA plant viruses (Hogle et al., 1986), Ghendon and colleagues (1972) showed that in the presence of GuHCl, pentamers accumulate in PV infected cells. Upon the removal

of GuHCl from the medium, pentamers were converted into virions. While this is strong evidence that pentamers to virions is the pathway taken by viruses, the presence of a short lived empty capsid intermediate cannot be ruled out. Additional studies to support this model found that pentamers have RNA binding activity, whereas empty capsids do not, and the binding of RNA to pentamers changes their conformation (Nugent and Kirkegaard, 1995). RNA in replication complexes can also be cross-linked to protomers and pentamers which is suggested to be a first step in the RNA encapsidation pathway (Pfister et al., 1995). Only replicating RNA has been shown to be encapsidated into provirions (Nugent et al., 1999), and only pentamers are required for this interaction (Verlinden et al., 2000).

Recent evidence also suggests a role for the viral non-structural protein $2C^{ATPase}$ in facilitating and giving specificity to the RNA encapsidation process in enteroviruses. Viral chimeras in which the structural proteins are from coxsackie viruses in the *Enterovirus C* species and the non-structural proteins are from PV were shown to only encapsidate RNA upon mutations to the $2C^{ATPase}$ and VP3. These proteins were also shown to bind to each-other in immunoprecipitation assays (Liu et al., 2010). Alanine scanning mutagenesis of the $2C^{ATPase}$ showed that the C-terminal domain was required for encapsidation (Wang et al., 2012a, Wang et al., 2014a).

1.7.3.5 150S provirion to 146S/160S virion – the maturation cleavage

The tertiary protein cleavage is the maturation cleavage that occurs in VP0 and results in the production of the mature capsid proteins VP4 and VP2 following interaction with viral RNA (Hindiyeh et al., 1999). Provirions are relatively unstable in comparison to viruses because the maturation cleavage stabilises virus particles locking them into a meta-stable state for the infection of new cells. The maturation cleavage also imparts important entry and antigenic properties to the virus and the latter of these is the subject of investigation in chapter 6.

1.8 Aims

While it is known that capsids assemble from monomeric precursors through pentameric intermediates, little information is available as to the molecular mechanism for self-assembly of these protein subunits. The FMDV capsid precursor protein (P1-2A) is proteolytically cleaved by the viral 3C^{pro} at three protein junctions and is myristoylated during the translation of the polyprotein. P1-2A undergoes structural changes after processing for the assembly of viruses and in PV, Hsp90 has been shown to facilitate processing. Assembly pentamers are both competent for assembly and are antigenically similar to the virus, whereas pentamers formed when the virus dissociates upon re-entry into a cell are neither. A fundamental aim of this work is to improve our understanding of capsid assembly by characterizing the properties of 'assembly' and 'dissociated' pentamers, to understand if just the loss of VP4 is enough to cause the switch in antigenicity. An understanding of the contribution that myristoylation, 3C^{pro} processing and Hsp90 involvement has on the assembly process is a further fundamental aim.

1.8.1 Hypotheses

Hypothesis 1: The myristoylation and processing events that occur during the translation of the FMDV genome influence the ability of the structural precursor to assemble into pentamers.

Hypothesis 2: The cellular chaperone Hsp90, which is required for P1 processing in PV, is also necessary for FMDV P1-2A processing.

Hypothesis 3: The loss of VP4 in pentamers derived from the disassembly of FMDV virions is the reason for their altered antigenicity.

Chapter 2 Materials and Methods

2.1 General Reagents

2.1.1 Cell lines and media

An African Green Monkey kidney cell-line (Cos-1), a human osteomyeloma cell-line lacking the thymidine kinase gene (TK-) and a rabbit kidney cell-line (RK13) were used for the initial recombination, subsequent selection and propagation of vaccinia viruses respectively. The cells were obtained from the central services unit (CSU) at Pirbright and were propagated in Dulbecco's minimum essential medium (DMEM; Life Technologies) with 10% foetal calf serum (FCS; Autogen Bioclear), 2mM L-Glutamine, 100 units/L penicillin and 100µg/mL streptomycin (all Life Technologies).

The baby hamster kidney cell-line (BHK-21) and a pig kidney cell-line (IB-RS-2) were obtained from the CSU at Pirbright and used for the Hsp90 inhibition experiments (chapter 5). BHK-21 cells were grown in Glasgow's MEM (GMEM; Life Technologies), with 10% FCS, 2mM L-Glutamine, 100 units/mL penicillin, 100µg/mL streptomycin and 5% tryptose phosphate broth (CSU, Pirbright). The IB-RS-2 cells were grown in the same media as BHK-21 cells but with 10% adult bovine serum (ABS; Life technologies) instead of FBS. During luminescence counting experiments, cells were maintained in DMEM lacking phenol-red indicator (Life technologies).

2.1.2 Plasmids

Plasmid constructs encoding for the IRES-P1-2A-3B3-3C-3'UTR from the A22 Iraq strain of the FMDV in pBG200 vv transfer vectors, as well as the P1-2A region from O1 Manisa, inserted into this backbone, were obtained from Dr. Alison Burman at the Pirbright Institute.

The pCR-Blunt II-TOPO (pTOPO) vector was used as an intermediate cloning vector in which to perform mutagenesis of FMDV constructs.

Two pET28b plasmids encoding the $\Delta 3B1-3B2-3B3-3C$ -His⁶ sequence from FMDV A₁₀₆₁ were obtained from Stephen Curry (Imperial College London) (Sweeney et al. 2007). The constructs had

specific mutations to enable better solubility for expression and purification which were either C95K/C142A or C95K/C142T.

2.1.3 Viruses

Stocks of wild type Western Reserve (WR) vaccinia virus and a WR virus that expresses T7 RNA polymerase (vTF7-3) (Fuerst et al., 1986) were obtained from Dr. Alison Burman at Pirbright for the creation of recombinant viruses and for expression from these viruses respectively.

Stocks of the FMDV strains A22 Iraq and O1 Manisa were obtained from Dr. Sarah Gold at the Pirbright Institute for use in the virus inhibition experiments in chapter 5.

2.1.4 Antibodies

Guinea pig sera that had been raised against inactivated FMDV vaccine strains A22 Iraq and O1 Manisa was obtained from the World Reference Laboratory (WRL) at Pirbright for use in Western Blots and immunoprecipitation assays at a dilution of 1:1000. This antibody was detected with polyclonal rabbit antibodies raised against Guinea Pig immunoglobulins and conjugated to HRP (at a dilution of 1:1500).

A mouse monoclonal antibody specific for his⁶ tags (Abcam) was used to detect his-tagged proteins in western blots at a dilution of 1:4000. This antibody was detected with rabbit antibodies raised against mouse immunoglobulins and conjugated to HRP (at a dilution of 1:5000).

Two Llama single domain antibody fragments (VHHs) (Harmsen et al., 2011) were used to distinguish between intact particles (M170) and disassembled particles (M3) of FMDV in immuno-precipitation assays and were obtained from Dr. Eva Perez at the Pirbright Institute (at concentrations of 0.5µg/mL).

Two mouse monoclonal antibodies named B2 and D9 that are specific for the G-H loop of FMDV strain O1 Lausanne but are cross-reactive with O1 Manisa (Mahapatra et al., 2008) were obtained from Terry Jackson at Pirbright and used in immunoprecipitation assays at a dilution of 1:2000.

2.1.5 Bacterial strains

Chemically competent DH5 α *Escherichia coli* (*E. coli*) (Life technologies) (genotype F-, Φ 80/*lacZ* Δ M15, *endA1*, *recA*, *hsdA1*, (rk-, mk+), *phoA*, *supE44* λ -, *thi-1*, *deoR*, *gyrA96*, *relA1*, (Δ *lacZYA*-argF), U169, λ) were used for routine sub-cloning.

Chemically competent one shot Top 10 *E.coli* (life technologies) (genotype F- *mcrA*, Δ (*mrr*-*hsdRMS*-*mcrBC*), Φ 80/*lacZ* Δ M15, Δ *lacX74*, *recA1*, *araD139*, Δ (*ara-leu*)7697, *galU*, *galK*, *rpsL*, (StrR), *endA1*, *nupG*) were used for transforming pTOPO vectors after blunt end cloning of PCR products.

Chemically competent BL21(DE3) pLysS *E.coli* (Invitrogen) (genotype F- *ompT*, *hsdSB*, (rB-mB-), *gaI*, *dcm*, (DE3), pLysS, (CamR)) were used for expression of 3C^{pro}.

Chemically competent XL-10 Gold *E.coli* (Agilent) (geneotype TetrD(*mcrA*)183, D(*mcrCB*-*hsdSMR*-*mrr*)173, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *lac*, The, [F' *proAB lacI*^fZDM15, Tn10 (Tet^r), Amy, Cam^r) were used for Quikchange mutagenesis transformations.

2.2 DNA manipulation

2.2.1 Primers

Sequences required for cloning were amplified by using oligodeoxynucleotide primers (Sigma) that were designed to introduce restriction sites and tags at the ends of the amplicon (chapter 3). The sequence of the primers is given in appendix 1. The design of primer sequences was performed by analysing sequences of the P1-2A proteins from A22 Iraq and O1 Manisa obtained from the

Nucleotide database on the NCBI website (Accession numbers AY593764.1 and AY593823.1 respectively) and by using the Sigma DNA calculator (<http://www.sigmaaldrich.com/life-science/custom-oligos/custom-dna/learning-center/calculator.html>).

All primers were purchased from Sigma-Aldrich as lyophilised powders and were resuspended in deionised water (dH₂O) to a final concentration of 100µM.

2.2.2 Polymerase chain reaction (PCR) for generating blunt ended cloning products

To generate blunt ended inserts, PCR reactions were assembled using hot-start KOD polymerase (Merck, NJ, USA) according to the manufacturers instruction; 100ng template DNA, 0.3µM primers, 0.2mM dNTPs, 5µl 10x reaction buffer, 3µl of 25mM MgSO₄, 1µl KOD polymerase and nuclease free water to 50µl. Typical amplification reactions were performed in a thermo cycler (Eppendorf), with the following cycling conditions typical for the ~2800bp IRES-P1 region: 95°C for 2 minutes, 35 cycles of (95°C for 30 seconds, 63°C for 30 seconds and 70°C for 1 minute 15 seconds) and a final elongation step of 70°C for 2 minutes. Amplified DNA was separated from the reaction components by agarose gel electrophoresis (section 2.2.6).

2.2.3 Quikchange mutagenesis PCR

Mutagenesis was performed by using the Quikchange mutagenesis kit (Agilent) for single or triple point mutations. Primers for the Quikchange procedure were designed according to the instructions given in the Quikchange Lightning Site-Directed Mutagenesis manual. The primer sequences are given in appendix 2. Reaction mixtures were assembled with 100ng of plasmid template, 5µl of 10X reaction buffer, 125ng of each mutagenic primer, 1µl of supplied dNTP mix, 1.5µl of QuikSolution Reagent and nuclease free water was added to make a 50µl total reaction volume. Once mixed, 1µl of QuikChange Lightning enzyme was added and the reactions were placed in a thermo cycler (Eppendorf). Amplification reactions to generate a methylated, mutated amplicon from the non-methylated template were performed in a thermo cycler (Eppendorf) with the following cycling conditions typical for the mutagenesis of capsid sequences in pTOPO vectors: 95°C for 2 minutes,

followed by 18 cycles of 95°C for 20 seconds, 60°C for 10 seconds and 68°C for 30 seconds/kb of plasmids and a final extension of 68°C for 5 minutes. Following temperature cycling, the reactions were placed on ice for 2 minutes and the non-methylated template was digested by the addition of 1µl *Dpn* I (10U/µl) restriction enzyme to the reactions which were incubated at 37°C for one hour. Mutated plasmids were then transformed into XL-10 Gold competent cells (method 2.2.12) in the presence of 1µl 2-mercaptoethanol.

2.2.4 Overlap PCR mutagenesis

Overlap PCR was used to mutate sections of DNA that were too large to be performed by Quikchange mutagenesis (24bp in the case of the mutation to a PreScission Protease Cleavage site; chapter 3). Initially reactions were assembled to amplify two products from one template that overlapped at the mutation site. Two primer pairs were required for this purpose; one set containing a 5' flanking primer and a 3' mutagenic primer, and the second which contained a 5' mutagenic primer that overlapped the mutagenic sequence in the first reaction, paired with a 3'flanking primer (appendix 2). Both amplicons were prepared using the KOD PCR set up described in section 2.2.2. Products from this initial round of synthesis were analysed by gel electrophoresis before extraction and purification from the gel. The two sets of purified products were then added in equi-molar ratios, into a second PCR reaction identical to the first with the exception that only flanking primers were added to the reaction and this was done after five cycles so that full length copies of the construct could be obtained. These products were gel extracted as above and cloned back into the parental vectors to introduce the mutation.

2.2.5 Sequencing PCR

Plasmid DNA was sequenced using a standard fluorescent dideoxy chain termination method provided by the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Plasmid DNA was first denatured by heating for 1 minute at 96°C before being placed into an assembled

sequencing reaction containing 1.88µl of 5x sequencing buffer, 0.25µl BigDye Terminator v3.1, 1.6pmol sequencing primers, plasmid template (50-300ng) and nuclease free water to a final volume of 10µl. The reactions were added to 96-well 200µl PCR plates (Applied Biosystems) and run on a thermo cycler (Eppendorf) at 96°C for one minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Upon completion, the DNA was precipitated with 60µl of 100% ethanol and 5µl of 125mM EDTA, the samples were mixed, incubated at room temperature for 15 minutes and then centrifuged at 2000 x *g* for 45 minutes at 4°C. The precipitation solution was removed from the precipitated DNA, which was then washed with 60µl of 70% ethanol and centrifuged at 1650 x *g* for 15 minutes. The wash was removed from the DNA which was then resuspended in 20µl Hi-Di Formamide (Applied Biosystems). The samples were run on an in-house ABI 3730 DNA analyser (Applied Biosystems).

2.2.6 Agarose gel electrophoresis

Agarose gels (typically 0.8-1.2% w/v agarose (Sigma) in Tris-acetate-EDTA (TAE) buffer (40mM Tris-base, 40mM glacial acetic acid, 1mM EDTA)) were used to resolve DNA for analysis or gel extraction. DNA samples were prepared in 6X loading buffer (Promega), loaded onto agarose gels and resolved by the application of an electric current. DNA markers were loaded alongside the samples for size comparison and the samples were visualized and photographed using a UV transilluminator (Biorad).

2.2.7 Gel Extraction

A sterile scalpel was used to excise DNA in gel fragments. DNA fragments were extracted from gels using the QIAquick gel extraction micro centrifuge kit (QIAGEN). Briefly, gels slices were dissolved in a high salt acidic buffer before binding to a silica membrane contained in a micro centrifuge spin column. Impurities were removed from the DNA by washing with 70% alcohol solution before the DNA was eluted in 10mM Tris buffer pH8.5.

2.2.8 Restriction endonuclease digestion

Analysis of purified DNA was performed using restriction endonucleases (Promega or NEB). When performing a diagnostic digest, a typical quantity of 250ng of DNA, was digested for 2 hours (usually at 37°C depending on the enzyme) with 2 units of the appropriate restriction enzyme, 2µl of the appropriate 10 x reaction buffer, 10 µg/ml bovine serum albumin (BSA) and made up to 20µl in nuclease free water. Preparative digests for use in ligation reactions contained up to 10µg of DNA in a final reaction volume of 50µl.

2.2.9 DNA dephosphorylations

Linearised vectors from restriction digests were dephosphorylated to improve the efficiency of ligation. Dephosphorylation reactions were performed with rAPid alkaline phosphatase (Roche). Typically, 1µg of digested DNA was dephosphorylated in a 20µl reaction with 1U of rAPid and 2µl of 10X reaction buffer for 15 minutes at 37°C before rAPid was inactivated by heating at 75°C for 2 minutes.

2.2.10 DNA ligation

2.2.10.1 *pTOPO* vectors

Blunt ended PCR products were ligated into pTOPO cloning vectors (Invitrogen) as intermediate vectors for the mutagenesis of inserts (sections 2.2.3 and 2.2.4). Briefly, 2µl gel purified PCR products were mixed with 0.5µl salt solution and 0.5µl pCR-Blunt II TOPO vector (Invitrogen). The reactions were left for 30 minutes at room temperature before transformation into Top 10 *E.coli*.

2.2.10.2 *non-pTOPO* vectors

Ligation reactions were performed between insert DNA and dephosphorylated restriction digested vectors. Typically, vector DNA and inserts were ligated together using a 3:1 insert to vector molar ratio. DNA was ligated in a reaction mixture containing 1µl T4 DNA ligase (400U/µl; NEB) with 1µl of 10X T4 ligation buffer (NEB) made up to 10µl with nuclease free water. The ligation reactions were

typically incubated at room temperature before the ligase was deactivated by incubation at 65°C for 10 minutes.

2.2.12 Transformation of DNA into *E.coli*

Plasmid DNA was propagated in chemically competent *E.coli* strains by heat shock. Briefly, frozen tubes of *E.coli* were thawed on ice before typically 250ng was gently mixed into the bacteria suspension and the reactions incubated on ice for 30 minutes. The bacteria were then heat shocked for 45 seconds at 42°C and placed back on ice for 2 minutes. SOC medium (0.5mL, CSU) was added to the transformation mixtures and the cells were left to recover for one hour at 37°C. After this time the bacteria were pelleted in a micro centrifuge at 1500rpm for 3 minutes, the SOC medium decanted, the pellet resuspended in 200µl of SOC medium and the mixture spread on the appropriate antibiotic selective agar plate (Agar and antibiotics (Kanamycin at 50µg/mL and ampicillin at 100µg/mL) from Sigma) and incubated overnight at 37°C. The following day, colonies from these plates were picked into 5mL Luria-Bertani medium (LB) (CSU) containing the appropriate antibiotic selection and incubated with shaking either overnight at 37°C or if large scale plasmid preparation was required, they were incubated for 8 hours at 37°C before transfer to a larger volume of culture before overnight incubation with shaking at 37°C .

2.2.13 Plasmid prep and quantification

Plasmid DNA from bacterial cultures was extracted using the method of alkaline lysis provided by the QIAprep Spin Miniprep Kit or the QIAGEN Plasmid Maxiprep Kit (both Qiagen). Briefly, overnight cultures were centrifuged into a pellet and the bacterial pellets were resuspended in a cell resuspension buffer before lysis in an alkaline buffer containing SDS. This buffer was neutralised with a high salt sodium acetate solution which precipitated the bacterial genomic DNA away from the plasmid of interest. Plasmid DNA was purified by the use of silica spin columns which operate in the

same way as those described for gel extraction (method 2.2.7). Purified DNA was quantified on a Nanodrop spectrophotometer (Thermo Scientific) at a wavelength of 260nm.

2.3 Generating recombinant vaccinia viruses

2.3.1 Recombination

Recombinant vaccinia viruses expressing FMDV P1-2AΔ were generated in COS-1 cells. The cells were prepared in 6-well plates with virus growth medium (VGM, DMEM with 2.5% FCS) so that the cells were 75% confluent at the start of the experiment. The WR strain of vaccinia virus was added at a multiplicity of infection (MOI) of 10 to each dish, which were mixed and incubated at 37°C for one hour. During the incubation, 8μl of lipofectamine 2000 was mixed with 192μl of Opti-MEM (both Invitrogen) for each well to be transfected, and incubated at room temperature for 45 minutes. 3μg of plasmid DNA was diluted in 200μl of dH₂O, and 15 minutes before the virus and cell incubation was finished, added to the Lipofectamine mixture. During the last 5 minutes of the Lipofectamine incubation, the dishes were washed with FCS free DMEM and replaced with the 400μl of the DNA/Lipofectamine 2000/Optimem. The dishes were incubated at 37°C for five hours which were tilted every 30 minutes to ensure they did not dry out, before 2mL VGM was added to each well and they were incubated overnight at 37°C. The cells were then removed with a cell scraper into the growth medium and frozen and thawed three times to release the recombinant vaccinia viruses.

2.3.2 Plaque purification

Cells lacking the thymidine kinase gene (TK-) were used to select for and plaque purify recombinant vaccinia viruses (described in chapter 3). Six-well plates of TK- cells were grown in DMEM until the day of infection which was then replaced with 1mL VGM per well. One, 5, 10, or 25μl of thawed vaccinia cell lysate was mixed into different wells and left at 37°C for 2 hours. At this point, a further 1mL VGM containing 25μg/mL 5-bromo-2'-deoxyuridine (BUdR) (Cambridge Bioscience) was added and the plate was incubated for 48 hours at 37°C. Single plaques were identified and picked into

200µl VGM from which the DNA was extracted using a QIAmp Viral RNA kit (similar methodology to 2.2.13, Qiagen). Extracted DNA was tested for the presence of P1-2AΔ insert by PCR (method section 2.2.2) and agarose gel electrophoresis (method section 2.2.6). Plaques that were positive for vaccinia virus with inserts were used in two further plaque purifications before being propagated in RK13 cells. When extensive CPE was observed in the RK13 cell sheet, the cells and medium were removed and pelleted by centrifugation, and the final pellet was resuspended in 5mL Tris-EDTA pH9 (TE9) and homogenised in an IKA homogeniser (Sigma). The homogenised lysate was separated into aliquots and frozen at -20°C.

2.4 Protein Expression

2.4.1 FMDV 3C

To express FMDV 3C^{pro}, bacterial expression plasmids encoding for the protein were transformed into BL21(DE3) pLysS *E.coli* and the bacteria were grown overnight at 37°C. The following day, this culture was diluted 1/100 in Luria-Bertani (LB) broth, and grown to an optical density at 600nm of 0.4-0.7 absorbance units. To induce expression, 1mM Isopropyl-β-D-thio-galactosidase (IPTG; Sigma) was then added to the remaining culture. The culture was induced for four hours at 37°C and then pelleted by centrifugation for purification of recombinant protein.

2.4.2 FMDV Capsid Precursor

2.4.2.1 Recombinant Vaccinia Virus

To express FMDV capsid precursor, RK13 cells were prepared in 10mL VGM and infected with vTF7-3 and the recombinant vaccinia virus encoding the capsid precursor in its genome under the control of a T7 promoter, both at an MOI determined to be optimal for protein expression (chapter 3). The virus was adsorbed to the cells by incubation at 37°C for 2 hours after which an additional 40mL VGM was added and the flasks were incubated at 37°C overnight. The following day, the cells were removed and pelleted by centrifugation for purification of recombinant protein.

2.4.2.2 Cell-Free Transcription and Translation

Wild-type and mutant versions of the capsid precursor under the control of a T7 RNA polymerase promoter were translated in a rabbit reticulate lysate transcription and translation system (TnT quick; Promega). Transcription and translation reactions were typically performed by mixing 40µl TnT quick master mix (Promega) with 1µg template DNA, 2µl ³⁵S methionine (EasyTag; Perkin Elmer), and nuclease free water was added to a final volume of 50µl. The reactions were incubated at 30°C for 1.5 hours, before further treatment or analysis.

2.5 Solubilisation of recombinant proteins from expression cultures

2.5.1 FMDV 3C

To generate a soluble lysate from 3C^{pro} bacterial expression cultures, they were resuspended in a lysis buffer containing 50mM HEPES (Sigma) pH 7.1, 200mM NaCl (Sigma), 1X HALT protease inhibitor cocktail (PIC; Thermo Scientific), 1% Igepal CA-630 (Sigma), 100µg/mL lysozyme (Sigma), 100µg/mL DNase (Invitrogen) left on ice to lyse for one hour and then subjected to 12, 30 second sonication cycles at amplitude of 10 microns with 30 seconds on ice between each sonication. A total lysate sample was taken, before the lysate was pelleted by centrifugation at 4000 x g in a bench-top centrifuge for 1 hour at 4°C. The supernatant was decanted, the pellet was resuspended in lysis buffer and samples were taken from each as soluble and insoluble lysate fractions respectively.

2.5.2 FMDV capsid precursor

The conditions for the lysis and solubilisation of FMDV P1-2AΔ from mammalian cells had to be highly optimized experimentally and is described in chapter 3, but typically, mammalian cell pellets were resuspended in lysis buffer (20mM Na₂HPO₄ pH7.4, 200mM NaCl, 1X PIC) with up to 1% detergent for upwards of one hour on ice. In some cases this was followed by the addition of up to 8M urea (Sigma) and in others, the detergent treated lysates were pelleted by centrifugation and treated with a 1:1 ratio of PBS to chloroform (Sigma). After lysis, a total lysate sample was taken, before the lysate was pelleted by centrifugation at 4000 x g in a bench-top centrifuge for 1 hour at 4°C. The

supernatant was decanted, the pellet was resuspended in lysis buffer and samples were taken from each as soluble and insoluble lysate fractions respectively

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2.6 Protein purification

2.6.1 Nickel column

His-tagged FMDV 3C^{pro} and P1 were purified from the soluble lysates by nickel-ion affinity chromatography. This procedure also required high levels of optimisation (chapter 3) but typically, HisTrap columns (GE Healthcare) were wetted with dH₂O, and equilibrated in a high salt PBS solution (pH7.4) containing 5mM-30mM imidazole (sigma). Imidazole (5-30mM; pH7.4) was also added to the soluble protein to prevent non-specific binding before the lysate was pumped through the column by means of a peristaltic pump and tubing. The column was washed in equilibration buffer before the protein was eluted into fractions in a step-wise fashion using equilibration buffers with increasing concentrations of imidazole.

2.6.2 Cobalt Column

Purification of his-tagged recombinant protein was also attempted using a HisPur Cobalt Purification column (Pierce) with equilibration, binding, washing and elution steps as in 2.6.1.

2.6.3 Dialysis

2.6.3.1 FMDV 3C^{pro}

The 3C^{pro} fractions with the highest concentration of purified protein were determined by SDS-PAGE and pooled into pre-equilibrated Slide-A-Lyzer dialysis cassettes (Thermo Scientific) with a 10kDa molecular weight cut off. The pooled fractions were dialysed four times against a 500mL buffer consisting of 50mM HEPES (pH7.1), 0.2M NaCl, 1mM EDTA, 1mM β-mercaptoethanol and 5% glycerol. Purified dialysed proteins were then aliquoted and stored at -80°C.

2.6.3.2 RRLs

Following the RRL TnT reaction and/or 3C^{pro} processing of the TnT lysate (section 2.7), the lysates were pipetted into chilled Slide-A-Lyzer MINI Dialysis Cassettes (Pierce) that had been pre-blocked for at least one hour with 1% BSA in PBS and were dialysed twice against 500mL PBS (Severn Biotech; 137mM NaCl, 2.7mM KCl) for two hours at 4°C to remove free ³⁵S methionine radiolabel.

2.6.4 Spin columns

As an alternative to dialysis, Pierce Polyacrylamide desalting columns with a molecular weight cut off of 7kDa were trialled to remove radiolabel. Columns were centrifuged at 1500 x *g* for one minute to remove excess storage liquid before lysates were applied to the centre of the compacted polyacrylamide resin and centrifuged through the resin at 1500 x *g* for 2 minutes at 4°C into a collection tube

2.7 Processing and assembly of P1-2AΔ

The conditions with which to process P1-2AΔ with 3C^{pro} and assemble pentamers were determined experimentally in chapter 4. It was determined that 3C^{pro} be used in the assembly reactions at a concentration of approximately 1μM where it represented 1/20th of the final volume. The reactions were incubated for 1 hour at 37°C. Where the maturation cleavage event was mimicked (chapter 6), protease cleavages were performed using 0.2U/μl of Precision Protease (Novagen) and incubated for 1 hour at 37°C.

2.8 Sucrose density gradients (SDGs)

2.8.1 Gradient formation

SDGs were used to separate proteins by their sedimentation rate. Typically, large stock solutions of 5 and 30% w/v sucrose in PBS were prepared, filter sterilised and frozen in aliquots. Gradients were prepared in 5mL ultracentrifuge tubes (1/2" x 2" polyclear ultracentrifuge tubes; Biocomp) using the Biocomp gradient master system. 5% sucrose solution was pipetted into the tube to make up half

the total sucrose volume. The 30% sucrose solution was pipetted underneath the 5% solution using a syringe and cannula to make up the second half of the solution taking care not to mix the solutions. A short cap (Biocomp) was fitted into the top of the tube and excess 5% sucrose was removed with blotting tissue. Gradients were formed by rotation on the Gradient Master (Biocomp) using the rotation settings for 5-30% w/v sucrose solutions in 5mL tubes with short caps.

2.8.2 Ultracentrifugation

After the gradient was formed, a volume of 240µl sucrose solution was removed from the top of the gradient and the samples were diluted to this volume in PBS and layered on top of the sucrose. The tubes were placed into SW55Ti ultracentrifuge buckets (Beckman), weighed and slight differences in weight were corrected by the addition of minimal volumes of PBS. The buckets were sealed, hooked onto an SW55Ti ultracentrifuge rotor and subjected to ultracentrifugation for 6 hours at 367598 x *g* max, 286794 x *g* average at 10°C.

2.8.3 Fractionation

Following ultracentrifugation, gradients were fractionated using a Piston Gradient Fractionator (Biocomp). Initially the tubing was washed with PBS before the samples were fractionated into 23 equal fractions by the sequential movements of the piston 1.6mm through the gradient and the tubing was washed in between fractions with 125µl of cold PBS. The 24th fraction was obtained by removing a further 200µl from the ultracentrifuge tube with a pipette.

2.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples for analysis by SDS-PAGE were denatured and reduced in 1X SDS-PAGE loading buffer with 10mM dithiothreitol (DTT; both NEB) and heated in a water bath at 70°C for 10 minutes for rabbit reticulocyte lysates and 95°C for 3 minutes for all other samples.

Poly-acrylamide gels were prepared using a Mini-Protean 3 gel casting system (Biorad). Typically, 8mL of 12% resolving gel were made by mixing 3.4mL deionised water (dH₂O), 2.4mL (30% T, 2.67% C) acrylamide/Bis-acrylamide, 2mL 1.5M Tris (pH8.8) and 80μL 10% SDS (All Sigma). Gels were polymerised by the addition of 80μL of 10% ammonium persulphate (APS) and 8μL Tetramethylethylenediamine (TEMED) (both Sigma) and poured into the casting stand and the top was levelled with water-saturated 1-butanol (Sigma). Typically 5mL of 5% stacking gel were made by mixing 3.4mL dH₂O, 0.85mL 30% T acrylamide/Bis-acrylamide, 0.6mL 1M Tris (pH6.8), 50μL of 10% SDS and polymerised with 50μL 10% APS and 7.5μL TEMED. The stacking gels were poured on top of the resolving gels once they had set and all of the butanol had been removed.

Samples were resolved through SDS-PAGE gels in a mini-Protean Tetra cell (Biorad) for typically 45 minutes at 200 volts in a SDS-PAGE running buffer (25mM Tris, 192mM glycine, 0.1% SDS). The migration through the gels and molecular weight estimation was possible by using the Dual Color Precision Plus Protein Prestained Standard marker proteins (Biorad).

2.10 Protein analysis

2.10.1 Coomassie Staining

For coomassie staining of SDS-PAGE gels, the gels were washed three times for ten minutes in dH₂O and stained for one hour in a 0.3% coomassie brilliant blue r250 (Sigma) solution dissolved in destain (20% methanol, 10% glacial acetic acid in dH₂O). Gels were then soaked sequentially in destain solution until background staining was removed.

2.10.2 Silver Staining

For silver staining of SDS-PAGE gels, the gels were washed three times for ten minutes in dH₂O, fixed (30% ethanol and 10% acetic acid (sigma)), washed again, sensitised and stained by the impregnation of silver ions into the gel which were reduced to metallic silver when bound to protein

in the gels using the Silver Stain Kit (Pierce). The protein/silver complexes were then visualised and the reaction was stopped with 5% acetic acid in dH₂O.

2.10.3 Western Blotting

For the specific detection of proteins on membranes, SDS-PAGE gels were soaked in transfer buffer (12.5mM Tris-Base, 100mM Glycine and 10-20% methanol in distilled H₂O (all Sigma)) and proteins were transferred to nitrocellulose membranes (Pierce) by electro-blotting for 45 minutes at 95 volts. The membrane was placed into blocking buffer (PBS with 0.1% tween-20 (PBS-T; Sigma) with 5% non-fat dry milk (Premier foods)) for one hour at room temperature. The membranes were transferred into a solution containing primary antibody diluted in blocking buffer and incubated for one hour at room temperature and subsequently washed in PBS-T. Secondary antibody bound to horseradish peroxidase (HRP; Dako) was then applied to the membrane for one hour at room temperature before the membranes were washed again. The membranes were then soaked in ECL reagent (Thermo Scientific) for 1 minute, placed in a film cassette and exposed to x-ray film for various lengths of time (15 seconds to 30 minutes) before the bands were developed in film development solution, washed in dH₂O and fixed in film fixative solution (all Kodak).

2.10.4 Fluorography

For the detection of radioactive proteins, SDS-PAGE gels were soaked in 1M sodium salicylate (VWR) for 30 minutes and then dried on a Slab gel dryer (DrygelSR) for up to 1 hour at 80°C before being exposed to film as described for Western blots, but in contrast, the exposure was performed at -80°C and for longer periods of time (4 hours to 2 days) and developed as described previously.

2.10.5 Gel and film images

Images of gels and films were generated by an image scanner (Epson). The images that are presented in this thesis were de-coloured, and the contrast and brightness settings adjusted minimally to improve the clarity of the image.

2.10.6 Scintillation counting

Radioactive SDG fractions for scintillation counting were diluted 1:5 in Optiphase Supermix (Perkin Elmer) and counted in scintillation vials for three minutes on an LS6500 Multi-purpose scintillation counter (Beckman). The counts were read in the wide setting to account for all ^{35}S signal.

2.10.7 ELISA

SDG fractions for ELISA were fractionated into high bind ELISA plates (Greiner Bio-one) and left to coat overnight at 4°C. The samples were then washed 3X in PBS-T with 0.2% BSA (Sigma) and blocked with PBS-T with 2% BSA for 2 hours at room temperature. Primary antibodies diluted in block buffer were then added to the wells for 1 hour before washing 3X with wash buffer. Secondary antibody bound to horseradish peroxidase was then applied to the membrane for one hour at room temperature before being washed again. SigmaFast OPD tablets (Sigma) were dissolved in dH_2O and added to the wells, and when colour development was deemed sufficient the reaction was stopped with 2.5M sulphuric acid (Fischer Scientific). The absorbance of the samples at a wavelength of 492nm was read on a Synergy 2 spectrophotometer (Biotek).

2.10.8 Bradford Assay

A Bradford assay was employed to calculate the concentration of 3C^{pro} . Dilution series of BSA and 3C^{pro} in 3C^{pro} storage buffer and triplicate 5µl dilutions were pipetted into a 96 well microplate (Greiner Bio-one). 250µl of Coomassie reagent was added to each well, mixed and the plate was

incubated for 10 minutes at room temperature. The absorbance values at a wavelength of 595nm were read on a spectrophotometer. Concentration calculations are shown in chapter 3.

2.10.9 Plate based immunoprecipitation assay

To compare the binding of antibodies to different assembly components, a plate based immunoprecipitation assay was designed. High bind ELISA plates (Greiner Bio-one) were coated with 100µl of antibody diluted in 0.5M carbonate/bicarbonate buffer pH9.8 (Pirbright CSU) and incubated at 4°C overnight. The plates were then washed three times with blocking buffer (PBS-T, 1% BSA) and then blocked in the same buffer for 1 hour at room temperature. The blocking buffer was washed three times and 100µl of gradient fractions for analysis were added in triplicate to the plates and they were incubated for one hour at room temperature. The plates were washed three times and the samples were removed with 2% SDS in PBS and counted on a scintillation counter.

2.11 Virus titration

2.11.1 Plaque assay

To quantify the recombinant vaccinia viruses, serial dilutions were made of one 50µl aliquot of the frozen lysate in VGM. Six-well plates containing 80% confluent RK13 cells were infected in duplicate with 100µl of dilutions between 10^{-4} and 10^{-9} and the virus adsorbed to cells for 1 hour at 37°C. At this point, the infection medium was removed; the cells were washed with PBS and then covered with a molten plaquing overlay (74% Eagles overlay, 5% Tryptose phosphate buffer (Pirbright CSU), 2.5mL FCS, 1mL penicillin/streptomycin, 0.6g indubiose (Pall Europe) and 25mL sterile dH₂O (Pirbright CSU) and incubated at 37°C for 48 hours. The cells were then fixed and stained (0.1% methylene blue, 4% formaldehyde solution (both Sigma) in PBS (Pirbright CSU)) for up to 4 hours before the plaque overlay was removed by agitation.

To quantify FMDV by plaque assay the procedure above was performed on BHK-21 cells rather than RK13 and the plaques were left to develop for 72 hours instead of 48 hours

2.11.2 Tissue culture infectious dose 50 (TCID₅₀)

Virus titrations were also performed by TCID₅₀ on BHK-21 cells. Log₁₀ dilutions were made of FMDV lysates in the range of 10⁻¹ to 10⁻¹⁰ in serum free DMEM (SFM). The cells were washed in the SFM and 100µl of SFM was added to each well. Eight replicates of 100µl of each virus dilution were added to each well in a row across the plates before the plates were incubated at 37°C for 72 hours. Media was then removed from the plates and they were fixed and stained with the same stain as for plaque assay. Titres are calculated using the Reed-Muench method (Reed and Muench, 1938).

2.12 Toxglo assay

To calculate cellular toxicity caused by Hsp90 inhibition, the Viral Tox-Glo assay (Promega) was used. Cells were grown in µclear 96-well plates (Greiner Bioone) and maintained in phenol red-free DMEM (Life Technologies). A dilution series of drug concentrations were made in phenol red-free media and 100µl replaced the media in each well. At the end of the toxicity period required, room temperature ATP detection reagent was added to ATP detection substrate, mixed thoroughly and 100µl was added to each well. Luminescence counts were obtained after 10 minutes by measuring absorbance values using a Plate Chameleon spectrophotometer (Hidex).

2.13 FMDV replicon assay

An FMDV replicon was used to calculate the effect of Hsp90 inhibition on FMDV translation and replication (chapter 5). The replicon was obtained from Stephen Berryman at Pirbright who had taken the FMDV GFP replicon described by Tulloch and colleagues (2014) and had replaced the GFP gene with a Renilla-luciferase gene. IB-RS-2 cells were grown in µclear 96-well plates maintained in phenol red-free DMEM. Media was removed from the wells and replaced with triplicate conditions

of fresh media or different drug treatments. 1µl of Lipofectamine 2000 was mixed with 25µl of Optimem for 5 minutes and at the same time 90ng of FMDV O1K replicon RNA was mixed with 25µl of Optimem before the two mixtures were combined. The media and drug treatments were removed from the cells and replaced with the RNA-Optimem-Lipofectamine mixtures. EnduRen Live Cell Substrate (Promega) was prepared in fresh media with drug treatments as previously described which were then added to cells on top of the transfection mixtures. Luminescence was read periodically on the Plate Chameleon spectrophotometer.

Chapter 3 Generation of reagents to analyse FMDV assembly in cell free systems

3.1 Introduction

This chapter describes the generation of recombinant reagents used in experiments for the study of FMDV assembly in subsequent chapters. This chapter also documents preliminary work towards a system for the large scale production of recombinant FMDV capsid precursor protein. The terms P1, P1-2A and P1-2A Δ are used throughout the results chapters in this thesis. For clarity, P1 is the structural precursor found in several picornaviruses such as PV. The FMDV structural precursor is P1-2A. To generate the constructs used here, recombinant versions of P1-2A have been made to assist with purification and detection. These are P1-only constructs (P1) or constructs where only 4 amino acids of 2A are coded for in the sequence (P1-2A Δ).

3.1.1 Rationale for using recombinant proteins to study picornavirus assembly

A reductionist approach can aid the understanding of complex biological processes, where a small part of a larger system can be investigated in isolation. This kind of approach can be applied to the process of picornavirus assembly by using recombinant proteins. Fundamental information from these recombinant systems can then be compared with and used to inform more complicated models to improve the understanding of the whole system.

3.1.2 Rationale for separating the generation of 3C and P1-2A

Picornavirus assembly is initiated when the capsid precursor P1-2A is proteolytically processed by the viral protease 3C^{pro}, triggering the assembly of five processed precursors into pentamers. Proteolytic processing by picornavirus proteases is rapid and occurs both during and immediately following translation. As such, in natural infection the single ORF encoded polyprotein is rarely visible (except in one account of an *in vitro* CVB1 infection (Kiehn and Holland, 1970), or when amino acid analogues are used to prevent proteolytic processing e.g. in poliovirus (Jacobson et al., 1970)). Processing of the VP2/3 and VP3/1 junctions of P1-2A, in the case of EMCV, occurs more slowly than the primary protein cleavages with the half-lives of the uncleaved intermediates being in the order of minutes (Butterworth and Rueckert, 1972).

The ability to study the processing of P1-2A by 3C^{pro}, and the subsequent assembly into pentamers is hampered by an inability to control the initiation of assembly in virally infected cells. This has led researchers to use recombinant proteins to separate the translation and processing steps. By producing P1 and 3C^{pro} in separate systems before re-combining them, capsid assembly can be controlled with precision. Additionally, due to the ability of 3C^{pro} to induce shut-off of host cell protein synthesis by cleavage of eIF4A and eIF4G (Belsham et al., 2000, Li et al., 2001), 3C^{pro} can be toxic to host cells and as such the yield of P1-2A when produced in the same system is variable (Polacek et al., 2013, Porta et al., 2013b).

3.1.3 Rationale for the choice of systems to express recombinant P1-2A

Due to the high error rate of picornavirus polymerases (Hicks and Duffy, 2011), a mutant sequence inserted into the viral genome is likely to revert back to the wild-type sequence if it produces a deleterious effect on the virus. If the mutation cannot be easily compensated for by extra mutation then mutations affecting capsid protein can have a dominant inhibitory effect and are likely to be lethal to the virus (Crowder and Kirkegaard, 2005). Therefore the production of proteins in recombinant systems is beneficial to study the phenotypic changes that mutations can have on the function of viral proteins.

Recombinant P1 or P1-2A expressed on its own has been demonstrated to retain important properties that it possesses when translated from a complete genome or in tandem with 3C^{pro} i.e. in a system where it is rapidly processed. In systems where P1 or P1-2A are produced separately, they can still be processed normally by 3C^{pro} resulting in assembly into higher order structures (Goodwin et al., 2009) and possess the same antigenic characteristics as P1-2A expressed in tandem with 3C^{pro} (Grace et al., 1991, Balamurugan et al., 2005, Saiz et al., 1994, Sanz-Parra et al., 1998, Lewis et al., 1991, Han et al., 2012).

Recombinant protein expression in *E.coli* and yeast are relatively cheap and fast methods of producing recombinant protein, however, there are a lack of the correct enzymes required for post-translational modifications of mammalian proteins. For example, to obtain myristoylated P1-2A in *E.coli* an additional plasmid encoding NMT has to be transformed into the bacteria (Duronio et al., 1990). Although the reconstitution of an infectious virus from purified components has not been achieved, Goodwin et al. (2009) showed that purified P1 expressed in this system with NMT could assemble into pentamers after the addition of 3C^{pro}. Further assembly and characterisation of this material proved difficult because the amount of purified material from this system was limiting. More recently, an approach where the individual capsid proteins (VP0, VP3 and VP1) were produced in *E.coli* as SUMO fusion proteins has been used to produce vast amounts of material (Cao et al., 2009, Guo et al., 2013). After expression and purification, the SUMO moieties were removed by proteolytic cleavage resulting in the assembly of immunogenic empty capsids; however, this system does not allow the analysis of early P1 interactions or processing. Recombinant baculoviruses allow high levels of protein expression in insect cells but the purity of recombinant protein obtained from these systems is a drawback if the material is required for analyses requiring high purity (Roosien et al., 1990, Oem et al., 2007, Cao et al., 2011, Li et al., 2011, Bhat et al., 2013, Ruiz et al., 2014). Although one study was able to produce about 50% soluble P1-2A from a P1-2A-Δ2B precursor in insect cells (Lewis et al., 1991), other groups obtained completely insoluble material when P1 was expressed alone (Porta, 2011 - personal communication).

Functional recombinant protein production in heterologous hosts can be impaired by codon usage biases when expressing mammalian proteins resulting in reduced expression levels, frame shifting and incorrect amino-acid incorporation (Norkiene and Gedvilaite, 2012). The choice of expression system for producing P1 was based upon the findings of previous published studies and optimisation experiments performed as part of this study.

3.1.4 Mammalian cell-free expression

Lysates of mammalian cells allow cheap and efficient production of protein from an environment containing many, if not all of the necessary factors required by mammalian viruses. It has been shown that HeLa lysates programmed with PV or EMCV RNAs are able to produce infectious virus and empty capsids with native structures and antigenic states, demonstrating that they provide an authentic environment for the production of recombinant protein (Molla et al., 1991, Kobayashi et al., 2007, Verlinden et al., 2000). The type II IRES elements found in aphthoviruses and cardioviruses have different requirements than the *Enterovirus* type I IRES (Ngoi et al., 2004), and as such, rabbit reticulocyte lysates (RRL) have proven useful for the production of FMDV and EMCV proteins that are competent for processing and assembly (Boege et al., 1986, Grubman and Baxt, 1982, Grubman et al., 1985, Shih et al., 1979, Palmenberg, 1982). Human cells are non-, or less-permissive to the growth of FMDV compared with animal cells, even when stably transfected with the av β 6 entry receptor (Jackson et al., 2000, O'Donnell et al., 2005a) and FMDV 3C^{pro} cleaves human eIF4G but not rabbit eIF4G (Strong and Belsham, 2004). For these reasons, the RRL was used for the production of the FMDV capsid precursor and the analysis of its assembly.

3.1.5 Large scale P1-2A expression in vaccinia virus infected mammalian cells

Much of the analysis of FMDV processing and assembly that we wanted to perform was possible on a small scale for which the RRL system provided an ideal environment. A further goal of this work was to develop a system in which large amounts of P1-2A could be expressed and purified. It was hoped that sufficient material could be produced to use in future studies to image assembly intermediates and to analyse the kinetics of assembly. The vaccinia virus expression system also allows high levels of protein expression in mammalian cells and has been used extensively for the production of recombinant picornaviral proteins (Abrams et al., 1995, Ansardi et al., 1991, Porter et al., 1993, Sanz-Parra et al., 1998, Winokur et al., 1991, Zhu et al., 1994). The system is well established for the production and purification of FMDV empty capsids in the lab using an expression

cassette expressing P1-2A and 3C (Porta et al., 2013a, Porta et al., 2013b). This system was adopted to express and purify large quantities of recombinant FMDV P1-2AΔ.

3.1.6 Rationale for exploring differences between strains from serotypes A and O

A- and O-serotype viruses are present on all continents where FMDV is found (reviewed in Jamal and Belsham, 2013) and these two strains have known differences in their assembly. For example in cell culture, A serotype viruses produce significantly more empty capsids than O's (Rweyemamu et al., 1979). Normally, the VP0 protein of empty capsids does not undergo the maturation cleavage but A serotype empty capsids can be found to have a large proportion of VP4 and VP2 (Curry et al., 1995), and this phenomenon has recently been reported for O-serotype empty capsids as well (Gullberg et al. 2013a). A-serotype empty capsids are also more stable than O's (Doel and Baccarini, 1981).

3.2 Results

3.2.1 Generating and quantifying 3C^{pro}

3.2.1.1 *Expression and purification of recombinant FMDV 3C^{pro}*

FMDV 3C^{pro} was expressed in *E. coli* from plasmid constructs supplied by Prof. Stephen Curry (as described in methods section 2.1.2). The proteases expressed from these constructs comprised the amino acid sequence of FMDV strain A₁₀61 modified to contain the amino acid mutation C95K as well as either C142A or C142T which are required in order to improve the stability and solubility of the protease when expressed to high levels in *E. coli* (Sweeney et al., 2007). These will be referred to as 3CKA or 3CKT respectively from here on. The plasmids code for the sequence of a T7 promoter and Shine-Dalgarno sequence upstream of the coding sequence to initiate transcription and translation respectively. The protease is expressed downstream of FMDV 3B proteins to give 3C^{pro} the authentic FMDV N-terminus and with a C-terminal 6-his tag to facilitate its purification using immobilized metal ion affinity chromatography (IMAC). Hence the proteins encoded are Δ3B1-3B2-3B3-3C-6xHis (35kDa) in the bacteria and 3C^{pro} (23kDa) cleaves itself from this chain at its N-terminus. It has been demonstrated that 3C^{pro} can be purified from this system in high

concentrations and retain high proteolytic activity for its use in cleaving P1 or P1-2A in assembly assays (Goodwin et al., 2009).

Competent bacteria (*E. coli* strain BL21 (DE3) pLysS) were transformed with the plasmids described above and expression was induced by the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) (method in 2.5.1). This *E. coli* strain will express T7 RNA polymerase from a λ DE3 lysogen using the *lacUV5* promoter where IPTG is present and bound to the *lac* repressor (Studier and Moffatt, 1986), and background expression is reduced by the pLysS plasmid. Following expression, bacterial lysates of 3C^{pro} expression cultures were sonicated and the supernatant was cleared of insoluble material by centrifugation (figure 3.1 first three lanes). Soluble material was applied to a Ni^{2+} IMAC column, and retained proteins were eluted in step-wise increases in imadazole concentration (figure 3.1 remaining lanes). Both forms of 3C^{pro}, 3CKA (figure 3.1A) and 3CKT (figure 3.1B) eluted from the column with high purity as shown by silver stain (top panels) and were shown to be his⁶-tagged by western blots stained with an α -his tag antibody (bottom panels). 3C^{pro} migrated with the expected apparent molecular weight of 23kDa indicating that active protein had been produced by self-cleavage from the 35kDa precursor as described above. While 3C^{pro} was detectable at every stage of the purification, peak amounts of pure 3C^{pro} were seen in the 200 and 300mM eluted fractions for each mutant. These fractions were pooled, dialysed into a storage buffer and frozen at -80°C in small aliquots.

3.2.1.2 Measuring the concentration of purified 3C^{pro}

The concentrations of 3C^{pro} were measured by Bradford assay (Bradford, 1976) (method in 2.10.8).

The Bradford assay was chosen instead of other common protein quantification methods such as the bicinchoninic acid (BCA) assay or Lowry assay due to the presence of the reducing agent β -mercaptoethanol in the 3C^{pro} storage buffer which is incompatible with these methods. The principle of the Bradford assay is that the absorbance of an acidic solution of Coomassie brilliant blue (CBB) (G-250 form) dye will shift its absorbance maximum from 465nm to 595nm upon binding to protein

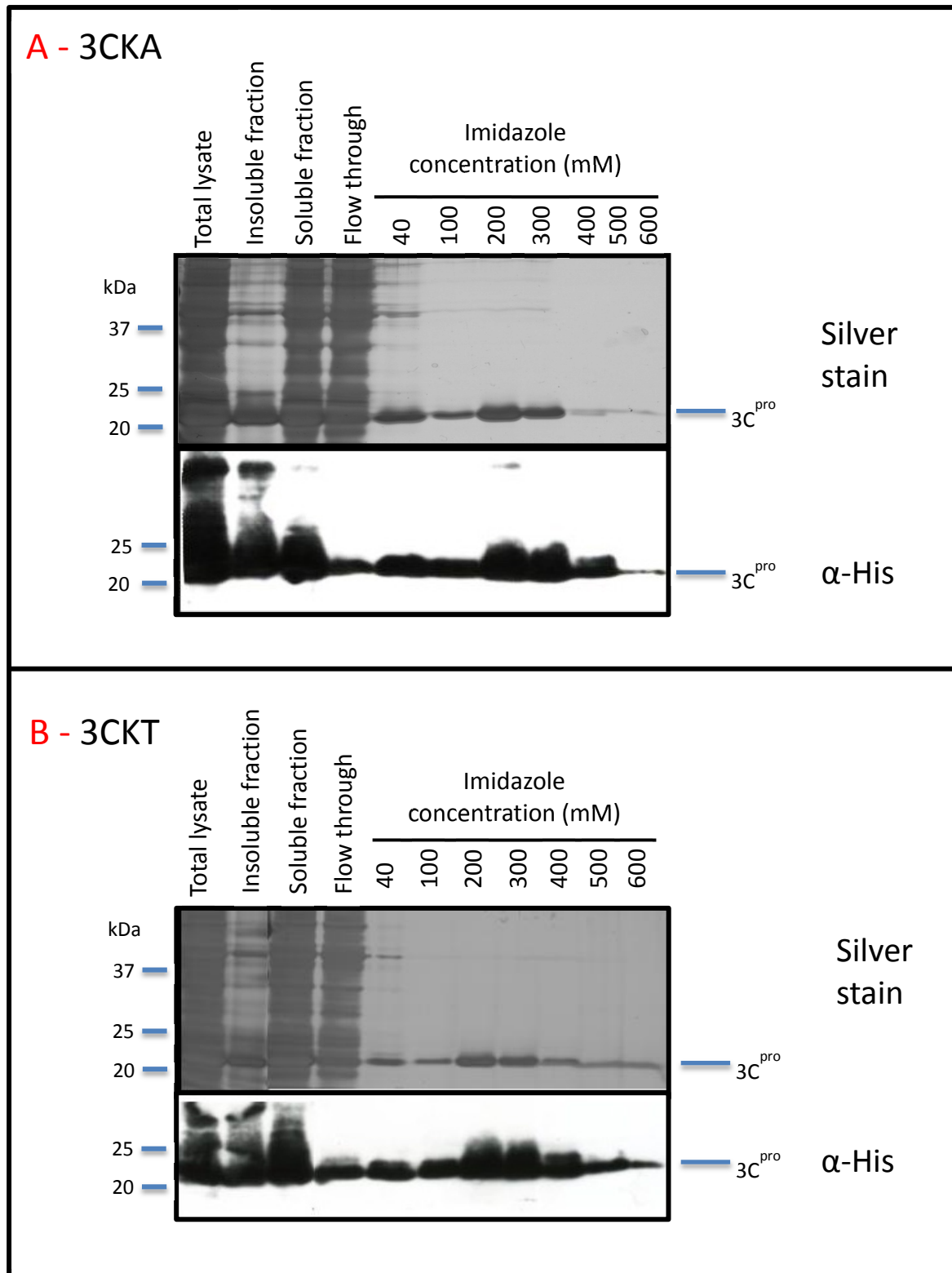


Figure 3.1 Purification of FMDV 3C^{pro} from an *E. coli* expression system. The mutant FMDV 3C^{pro} enzymes 3CKA (A) and 3CKT (B) were expressed in *E. coli*, lysates were prepared and then separated based upon solubility. The proteases were bound via their C-terminal His-tag to nickel columns and eluted by stepwise increases in imidazole concentration. Samples were collected at every stage, normalized for loading and separated on 10% SDS-PAGE gels. 3C^{pro} was visualised by Silver Stain (top panels in A and B) or WB (bottom panels in A and B) using an anti His-tag antibody.

due to a stabilisation of the neutral and anionic forms of the dye. CBB binds to amino acids via electrostatic and hydrophobic interactions with arginine residues providing the strongest interactions and other positive and aromatic amino acids also important (De Moreno et al., 1986, Fountoulakis et al., 1992, Georgiou et al., 2008). To calculate the concentration of 3C^{pro} in the samples, a dilution series of BSA, in 3C^{pro} storage buffer, and a four-fold dilution of both 3C^{pro} mutants were prepared and triplicate samples were analysed spectrophotometrically following Bradford assay. The OD⁵⁹⁵ of the samples were read and a second order polynomial model was applied to the BSA standards to generate a standard curve (figure 3.2A) using Prism software (Graphpad) that had an R-square of 0.9931. A second order polynomial model was used based upon Pierce Protein Assay Data Analysis (<http://www.piercenet.com/method/protein-assay-data-analysis>) suggesting that this type of model best describes the relationship between the data points of the BSA standard curve. The OD⁵⁹⁵ of the 3C^{pro} samples was used as an input in the quadratic equation to obtain their concentrations and the molarity of enzyme in each solution (figure 3.2B). Both enzymes were obtained at a high concentration with the 3CKA mutant at a higher concentration (48.5µM) than the 3CKT (23.8µM).

3.2.2 Generation of plasmids for capsid precursor expression

3.2.2.1 *Wild-type plasmids*

Expression plasmids under the control of a T7 promoter were required so that they could be used in both the cell-free and cell-based mammalian expression systems. In the cell-free system T7 RNA polymerase, ribonucleotide triphosphates (rNTPs) and an appropriate buffer are supplied in a RRL to generate a transcription and translation (TnT) system for plasmids encoding the sequence for the protein of interest under the control of a T7 promoter. In the cell-based expression system, a recombinant vaccinia virus provides T7 RNA polymerase. The genes for cloning were obtained from existing expression plasmids which have been successfully used to generate recombinant vaccinia virus for the expression of empty capsids in mammalian cells (Figure 3.3 (1)). These constructs lack

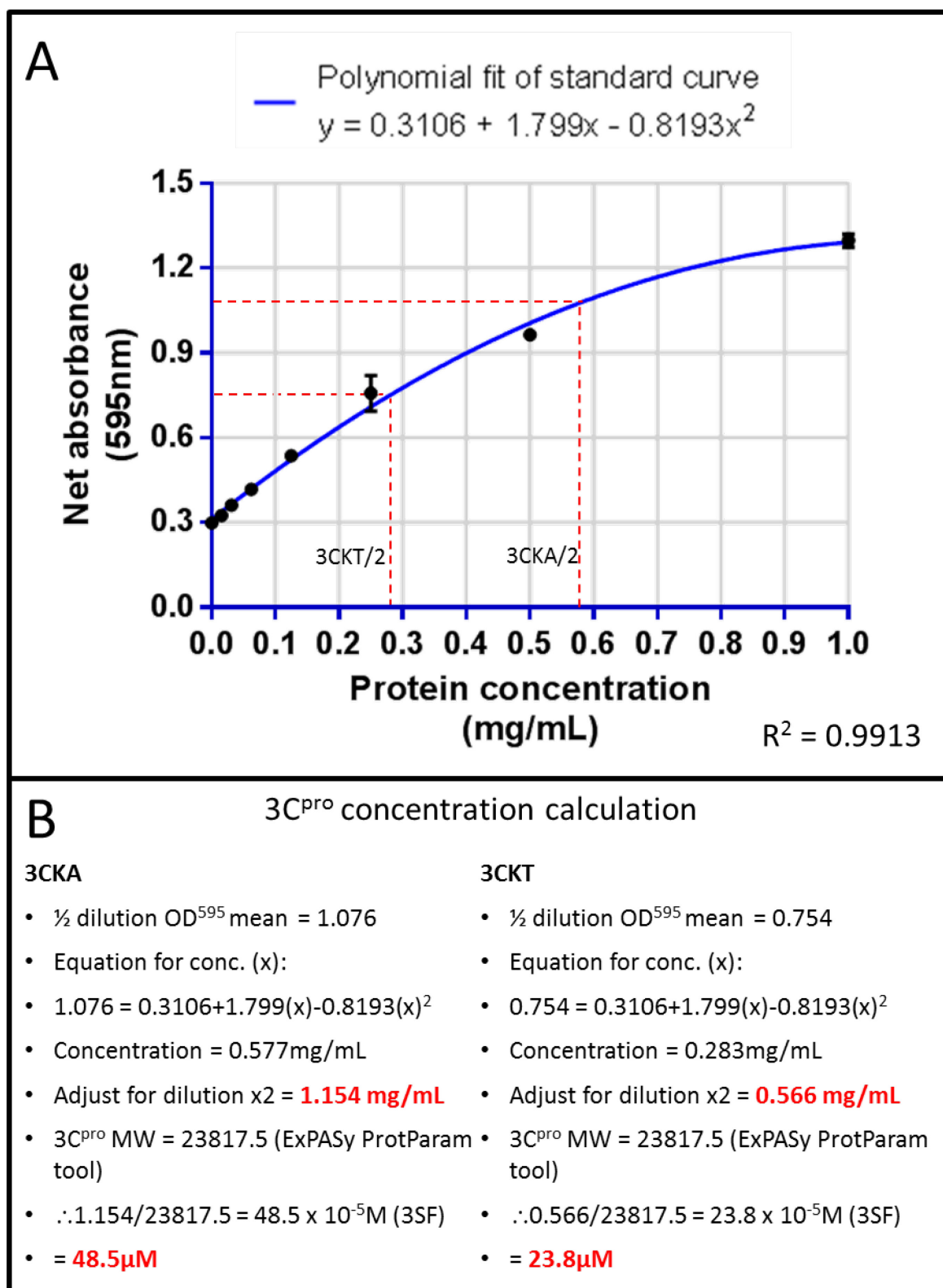


Figure 3.2 $3C^{pro}$ quantification by Bradford assay. A standard curve was generated by measuring the optical densities of a two-fold dilution series of triplicate BSA samples spectrophotometrically at 595nm after Bradford assay and applying a polynomial regression model (A). Triplicate dilutions of 3CKA and 3CKT were assayed by the same method and their concentrations were calculated by inputting their A^{595} into the equation of the standard curve (B). (Red hashed lines on graph A show where the protease absorbance values fell in relation to the standard curve - - -)

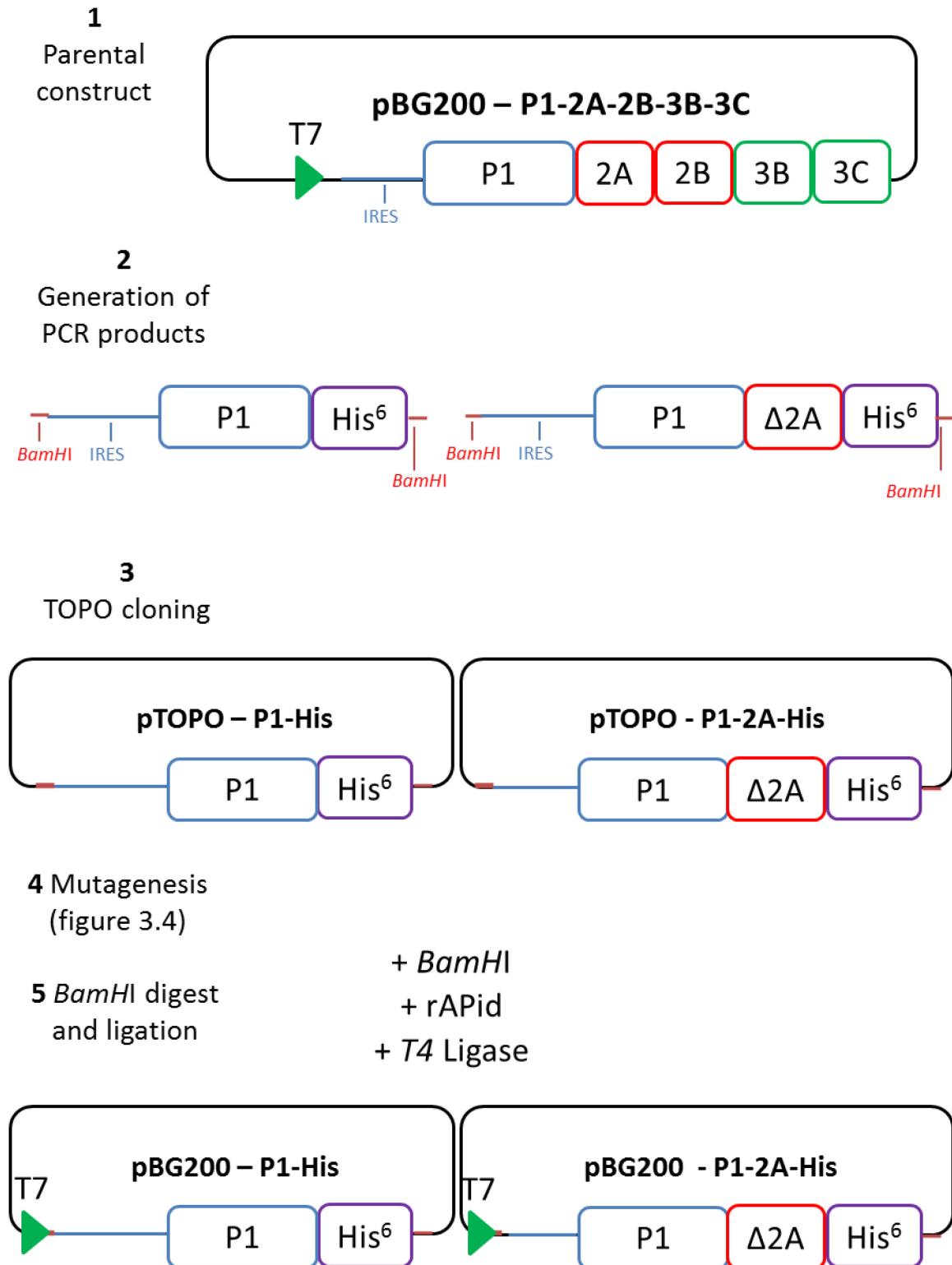


Figure 3.3 Cloning strategy used to design P1 and P1-2A Δ expression constructs. P1 and P1-2A Δ amplicons were made from a parental construct encoding for the FMDV IRES-P1-2A-2B-3B-3C (1) introducing a sequence for a His⁶ tag and flanking both ends of the amplicon with *Bam*HI restriction sites (2). PCR products were blunt-end cloned into pTOPO vectors (3), before mutagenesis was performed (4; displayed in figure 3.4). The wild-type and mutant P1 and P1-2A Δ sequences were then digested with *Bam*HI, along with the destination pBG200 vector. The vector was dephosphorylated and the inserts were ligated into it (Methods 2.2)

L^{pro} and as such, have a novel start codon at the start of P1 and encode the sequence for 2A and several non-structural proteins including 3C^{pro}. P1 and P1-2AΔ versions of the constructs were designed, whereby the sequence coding for P1 was either followed by a double alanine spacer and then the sequence for a his⁶ tag or the sequence of 12 nucleotides of 2A (2AΔ) followed by the his⁶ tag. This was designed so that the P1-only constructs would retain the his⁶ tag for detection and purification in the subsequent assembly steps and the P1-2AΔ construct would yield a more authentic assembly product whereby the tag should be cleaved away by 3C^{pro} at the native P1/2A junction during processing. The native FMDV IRES element was amplified with all constructs to provide a site for ribosome entry and translation and sets of amplicons were made for the two sets of sequences from the viruses A22 Iraq and O1 Manisa. Unique *Bam*HI restriction enzyme sites were introduced at both ends of the amplicon to facilitate future cloning steps. PCR was performed using the high fidelity proof-reading KOD polymerase to avoid unwanted mutations entering the sequence through PCR error (figure 3.3 (2) (Primer sequences in appendix 1).

The PCR products were ligated into pCR-Blunt II- TOPO vectors (figure 3.3 (3)) to perform mutagenesis (Figure 3.3 (4)).

The vaccinia virus transfer vector, pBG200 (Abrams et al., 1995) was chosen as the destination vector for the insert of capsid precursor sequences. This vector is a derivative of pH3IV (Belsham et al., 1990), in which the P7.5 vaccinia virus promoter has been replaced with a BglII linker into which the T7 promoter and terminator BglII- BglII fragment from pAR2529 (Fuerst et al., 1986), has been cloned. This fragment is flanked by regions of the vaccinia virus thymidine kinase (TK) gene which allows for the recombination and selection of cloned products into recombinant vaccinia viruses. TOPO constructs containing P1 and P1-2AΔ inserts and the pBG200 destination vector were digested with BamHI and the linearised pBG200 vector was dephosphorylated before the inserts were cloned back into the pBG200 transfer vector using T4 DNA ligase (methods section 2.2.10).

3.2.2.2 Mutagenesis of plasmids for the generation of mutant forms of capsid precursor for studying myristoylation, processing and maturation

Myristoylation and proteolytic processing have been suggested to be responsible for the changes in capsid precursor that allow it to assemble into higher ordered structures such as pentamers and capsids (explained further in chapter 4). The maturation cleavage is a critical event that locks capsids into a stable state and controls the stability, antigenicity and infectivity of the virus (explained further in chapter 6). We designed plasmids (primer sequences in appendix 2) to generate a set of mutants to analyse the effect of myristoylation, proteolytic processing and maturation on the fate of recombinant P1 and P1-2AΔ.

The myristoylation signal at the N-terminus of the P1 sequence was altered to be non-functional by minimal mutation of the nucleotide sequence using Quikchange site-directed mutagenesis (section 2.2.3), resulting in the encoded sequence at the N-terminus of P1 mutated to encode alanine instead of glycine (figure 3.4A).

Mutations in one or other of the two (or three in P1-2A) 3C^{pro} cleavage recognition sites in the P1 and P1-2AΔ sequences were also introduced by site-directed mutagenesis. Mutations were designed at the cleavage junctions to prevent either the VP0/VP3 or VP3/1 cleavages while not grossly affecting the amino acid characteristics at these sites. Hence, for the A22 Iraq constructs, the nucleotide sequence was mutated to change the amino acid sequences (amino acids given in single letter code) PSKE/GIVP at the VP2/VP3 junction to PSKD/GIVP and PRSQ/TTTT at the VP3/VP1 junction to PRSN/TTTT (figures 3.4B and C). For O1 Manisa, the nucleotide sequence was mutated to change the amino acid sequence PSKE/GIFP at the VP2/VP3 junction to PSKD/GIFP and the ARTQ/TTSA at the VP3/VP1 junction to ARTN/TTSA (where '/' is the junction between the proteins in the wild type construct) (figures 3.4B and C). These mutations were also introduced using Quikchange mutagenesis by minimal mutation of the nucleotide sequence.

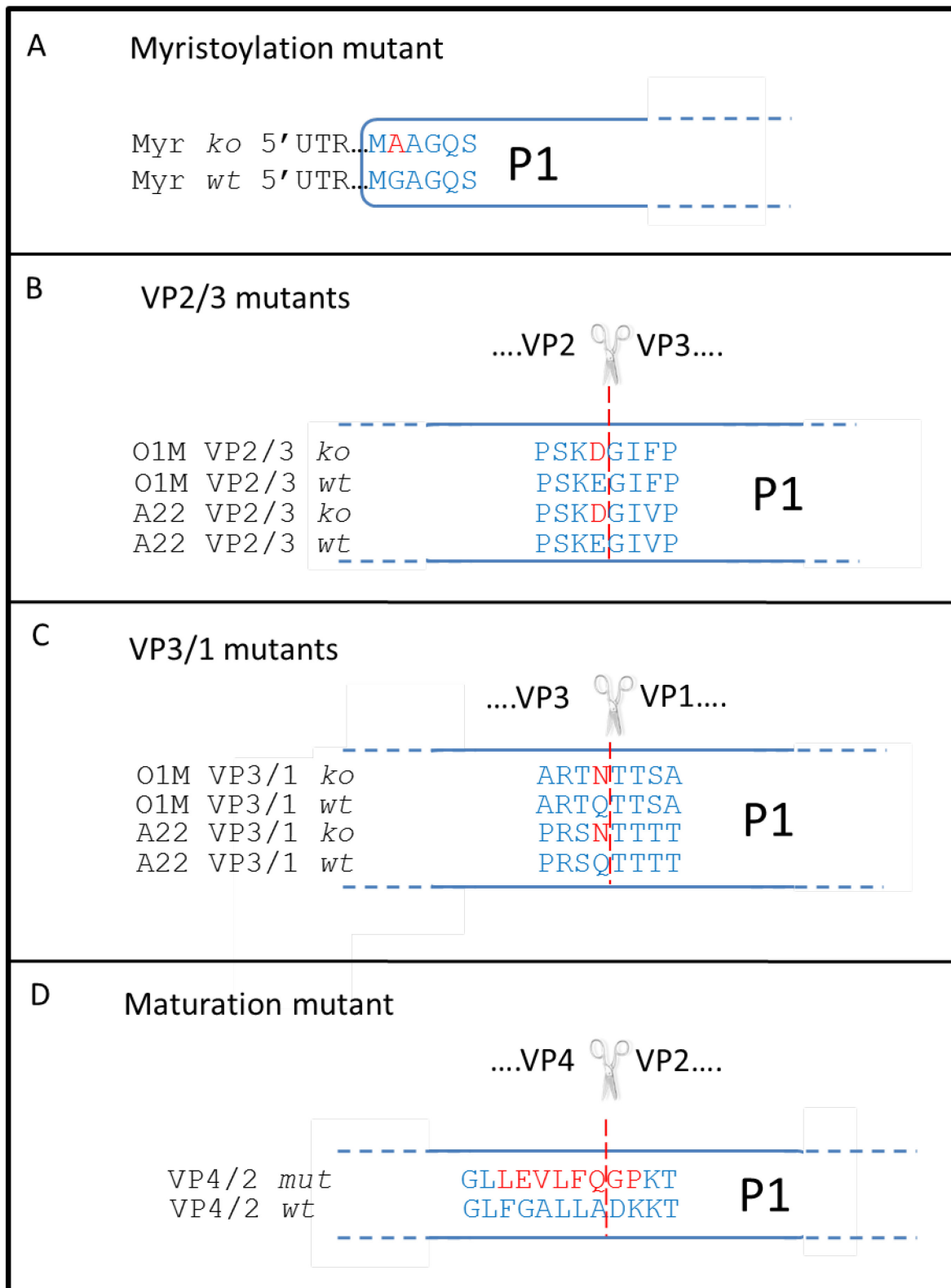


Figure 3.4 Mutagenesis strategies for P1 and P1-2AΔ constructs. pTOPO vectors containing the P1 and P1-2AΔ sequences were mutated by site directed mutagenesis and overlap PCR mutagenesis. The amino acid sequences are shown for the *wild-type* (*wt*), *knockout* (*ko*) and mutant (*mut*) sequences with the mutated amino acids in red in the position relative to the end of the genome for the myristoylation mutant (A), the VP2/3 cleavage junction (B), the VP3/1 cleavage junction (C) and the VP4/2 maturation junction (D). Scissors represent proteolytic cleavage and the red dashed line is the position of cleavage.

Lastly, a mutant was created in which the maturation cleavage could be mimicked in order to study the effect of the loss of VP4 on the formation and properties of recombinant pentamers (chapter 6). The maturation cleavage site for both FMDV serotypes A and O contains the amino acid sequence FGALLA/DKKT. The coding sequence at this site was mutated to code for LEVLFQ/GPKT which is predicted to permit a maturation-like event to be controlled in the capsid precursor and pentamers by a second recombinant protease called Precision Protease (figures 3.4D).

The mutagenesis for the Precision Protease site required a stretch of 24 nucleotides to be mutated which was above the limit that could be achieved using the Quikchange mutagenesis kit, and so, a strategy of overlap PCR mutagenesis was employed (method in section 2.2.4). Two sets of primers were designed for both A22 Iraq and O1 Manisa. The first set were a forward primer that bound at approximately 440bases upstream of the mutation site and a reverse primer that contained the 24 base pair mutation and a 23 base section that would anneal to the wild type sequence adjacent to the mutation site. The second set of primers were a forward primer containing the mutation and a run of adjacent annealing sequence, and a reverse primer that bound approximately 1370bp downstream of the site. Two PCR reactions were assembled using the wild-type sequences in pTOPO as a template and the primer sets described to amplify two overlapping mutated fragments which were separated by agarose gel electrophoresis (figure 3.5A). The two sets of fragments were excised and extracted from the gels before being combined in a second PCR reaction to generate a full-length mutated amplicon. The two mutated fragments were annealed together and flanking primers were added to extend the full length products. This second reaction was also separated on agarose gels and visualised by UV transillumination (figure 3.5B). The second round reaction had amplified a band of the expected size (~1770bp) in each case with a second non-specific band that migrated just below it. Gels were run further to separate the two bands and the full length product was excised and extracted as before. The full length amplicons with mutated maturation sites were cloned back into the pTOPO vectors containing wild-type sequence using the restriction enzymes BstEII and MluI

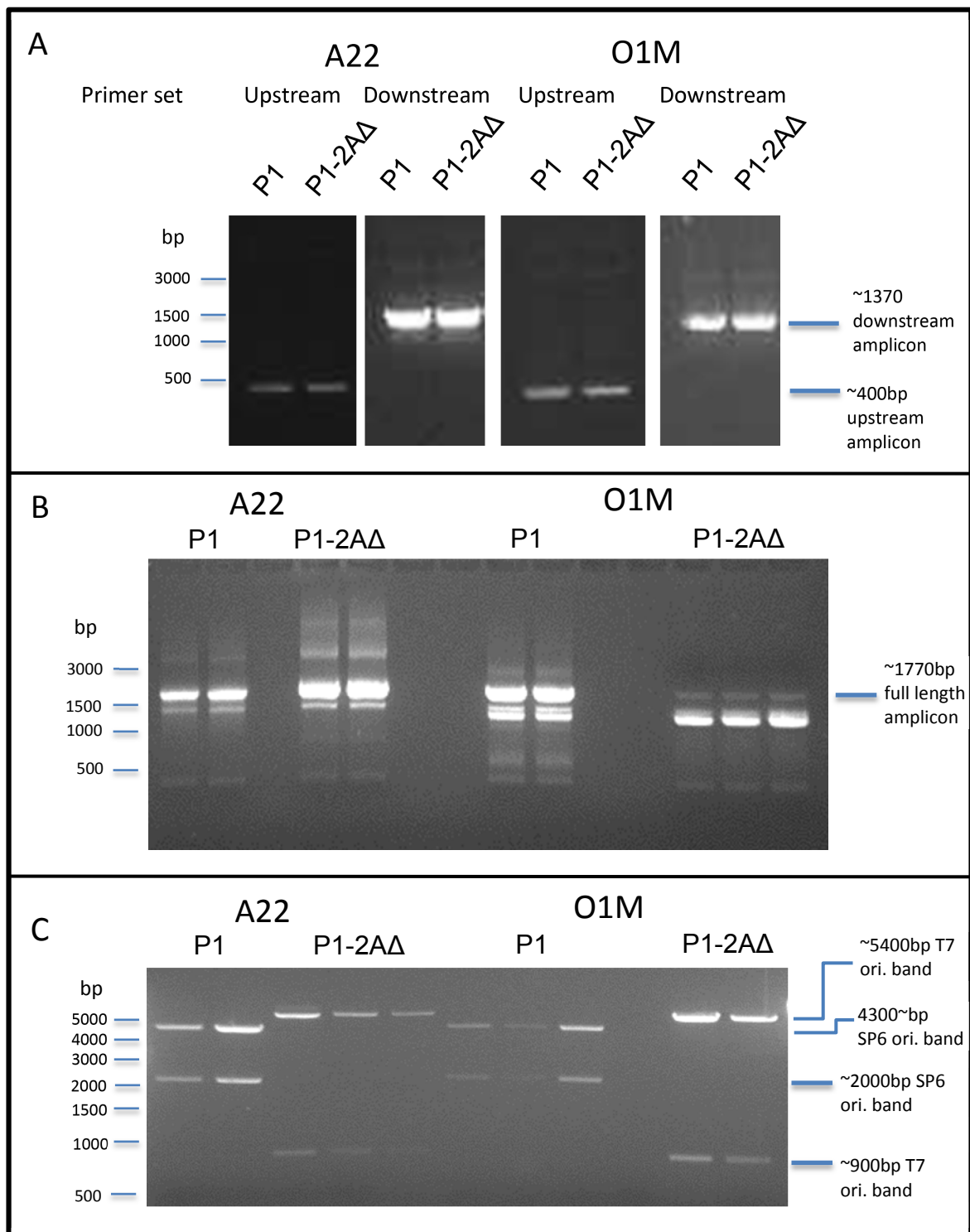


Figure 3.5 Agarose gels of maturation cleavage site overlap PCR mutagenesis. Upstream and downstream primer sets were used to generate two sets of mutant amplicons (~1370bp and ~400bp respectively) for A22 and O1M, P1 and P1-2AΔ sequences from the wild type sequence template (A). Matching pairs of amplicons were combined in a further PCR reaction where only flanking primers were used to generate the full length product (~1770bp) (B). Full length amplicons were excised from the gel, extracted and cloned back into pTOPO vectors. To differentiate mutant from wild-type sequence, pTOPO vectors were subjected to digestion with the enzyme *Apal* which cuts at the mutation site and at a site in the TOPO vector. Depending on the orientation of P1 in pTOPO (SP6 or T7 orientation (ori)) the sizes for correct pairs of digestion products were approximately 5400bp and 900bp (T7), 4300 and 2000bp (SP6) (C).

and transformed, propagated and extracted from *E.coli* (methods in section 2.2). To confirm that the vectors now contained the maturation mutant site in the wild-type P1 or P1-2AΔ background, a diagnostic *Apal* restriction digest was performed which would only digest a site in the mutant sequence and a site in the pTOPO vector. Depending upon the orientation of the inserts in the vector, the expected products from this digest in the presence of the mutation were sets of bands at approximately 5400bp and 900bp for inserts in T7 orientation, and 4300bp and 2000bp for inserts in SP6 orientation (figure 3.5C). This confirmed the success of the overlap PCR mutagenesis.

The maturation, myristoylation and processing mutant plasmids, along with the *wt* plasmids described in the previous sections resulted in twenty pTOPO constructs which were then cloned into the pBG200 transfer vector using the unique *BamHI* restriction site. The orientation of the capsid precursor inserts in the pBG200 transfer vectors was confirmed by digestion with the restriction enzymes *EcoRI* and *SpeI* that enabled the differentiation of inserts in the correct T7 orientation and the incorrect orientations. The orientation reactions were analysed by agarose gel electrophoresis (an example orientation digest is shown in figure 3.6). Inserts in the correct orientation yielded digestion products of approximately 1780bp and 1260bp and inserts in the wrong orientation yielded products of 1320bp and 1690bp. Once all of the inserts were inserted into the T7 orientation, the vectors were sequenced. The list of constructs are shown in table 3.1.

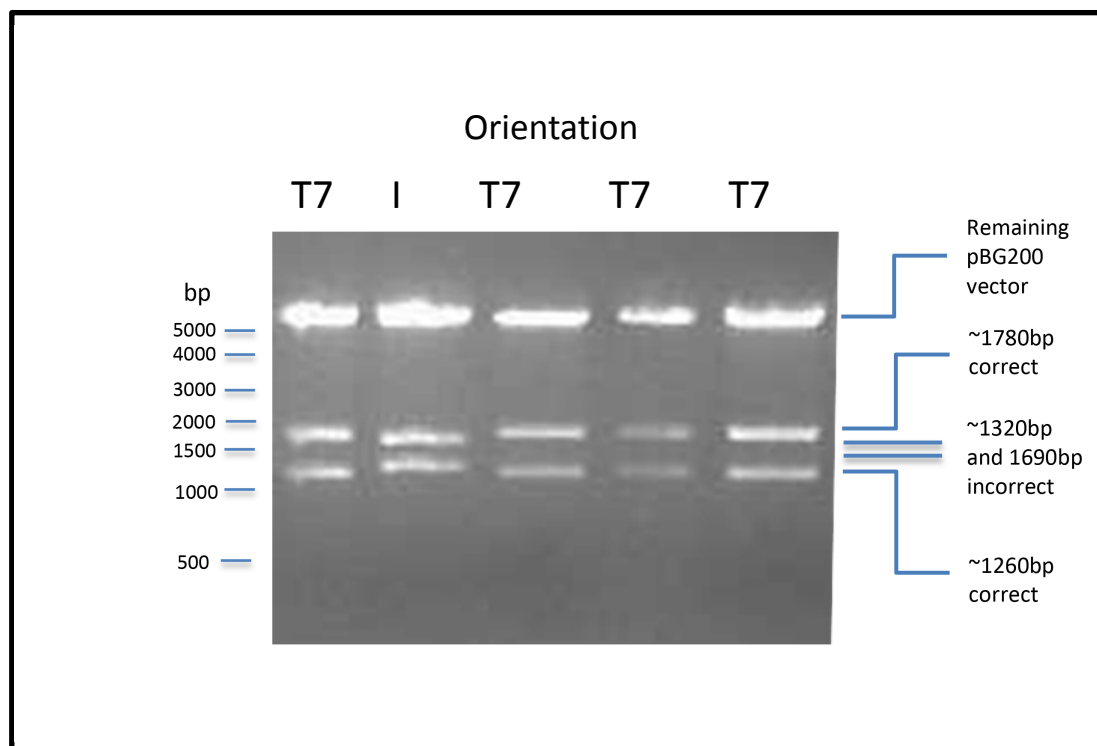


Figure 3.6 Example gel to show the orientation of capsid precursor sequences in pBG200 vectors. The orientation of capsid precursor inserts in pBG200 vectors was confirmed using a combination of the restriction enzymes *SpeI* and *EcoRI*. Inserts in the correct (T7) orientation yielded bands of ~1780bp and ~1260bp whereas inserts in the incorrect (I) orientation yielded bands of ~1320bp and ~1690bp.

Table 3.1: List of plasmid expression constructs. Plasmid constructs were generated to express *wt* and mutant FMDV capsids precursors under the control of a T7 promoter. The constructs were designed to express their encoded proteins in an *in vitro* expression system and also to be compatible for their recombination into vaccinia viruses.

FMDV Strain	Encoded regions	Mutant	Construct name
A22 Iraq	P1	Wildtype	pA22P1 <i>wt</i>
		Myristoylation	pA22P1G2A
		VP2/3 cleavage	pA22P1_2/3
		VP3/1 cleavage	pA22P1_3/1
		VP4/2 maturation	pA22P1 <i>mat</i>
	P1-2AΔ	Wildtype	pA22P12A <i>wt</i>
		Myristoylation	pA22P12AG2A
		VP2/3 cleavage	pA22P12A_2/3
		VP3/1 cleavage	pA22P12A_3/1
		VP4/2 maturation	pA22P12A <i>mat</i>
O1 Manisa	P1	Wildtype	pO1MP1 <i>wt</i>
		Myristoylation	pO1MP1G2A
		VP2/3 cleavage	pO1MP1_2/3
		VP3/1 cleavage	pO1MP1_3/1
		VP4/2 maturation	pO1MP1 <i>mat</i>
	P1-2AΔ	Wildtype	pO1MP12A <i>wt</i>
		Myristoylation	pO1MP12AG2A
		VP2/3 cleavage	pO1MP12A_2/3
		VP3/1 cleavage	pO1MP12A_3/1
		VP4/2 maturation	pO1MP12A <i>mat</i>

3.2.3 Development of a system for large scale production of purified recombinant P1-2AΔ

3.2.3.1 *Generating recombinant vaccinia viruses*

To analyse the suitability of the vaccinia virus expression system for the expression and purification of large quantities of capsid precursor, a recombinant vaccinia virus was generated using the pA22P12Awt expression construct (table 3.1). The P1 or P1-2AΔ inserts in the pBG200 plasmid are flanked by segments of the vaccinia virus thymidine kinase (*TK*) gene and can undergo homologous recombination with the infecting WR vaccinia virus, inserting the sequence of interest into the vaccinia. Infections are performed in the presence of the thymidine analogue 5-bromodeoxyuridine (BrdU). If BrdU is incorporated into replicating vaccinia virus genomes it is lethal. For BrdU to be incorporated, it has to be phosphorylated by thymidine kinase (*TK*). Vaccinia viruses that recombine with the *TK* flanked gene insert cannot produce *TK* and therefore survive whereas non-recombinant vaccinia viruses, with intact *TK*, phosphorylate BrdU and incorporate it into their genome making their replication non-viable.

The pA22P12Awt plasmid was transfected into cells which were pre-infected with Western Reserve (WR) and incubated overnight at 37°C. The cells were then homogenised and the virus was plaque purified in cells which lack thymidine kinase (*TK*- cells) in the presence of BrdU. Three rounds of plaque purification were performed with viruses tested by PCR for the presence of the recombined P1-2AΔ insert at each stage. The plaque purified virus was named vaccinia virus A22P12Awt and a large stock was prepared, titrated by plaque assay (methods section section 2.11.1) and frozen in aliquots.

3.2.3.2 *Optimisation of P1-2AΔ expression*

The source of T7 RNA polymerase in the vaccinia virus expression system is provided by a second recombinant virus called “vTF7-3”. The RNA polymerase in this virus is under the control of the vaccinia virus *P7.5* promoter (Fuerst et al., 1986) which allows continuous expression due to the presence of early and late vaccinia virus regulatory signal elements in the promoter region (Cochran

et al., 1985). Co-infection of vTF7-3 into cells with a second recombinant vaccinia virus encoding the protein of interest under the control of a T7 promoter results in the expression of the inserted protein. vTF7-3 was obtained from Alison Burman at Pirbright and used to generate a high titre stock. Initially, the length and MOI of the vaccinia virus co-infection of v-TF73 and vaccinia virus A22P12Awt was optimised. RK13 cells were infected with an array of MOIs of both viruses and expression performed for 8, 24, 32, 48 or 56 hours. After each time-point cell lysates were prepared and analysed by coomassie R-250 staining of SDS-PAGE gels (figure 3.7). After 24 hours a band of the expected size (88kDa) for P1-2AΔ was present and was seen in all subsequent time-points and MOI conditions suggesting that the recombinant vaccinia virus was expressing P1-2AΔ as expected and to a very high level of expression. The vaccinia virus MOI did not appear to have a noticeable effect on the levels of P1-2AΔ expression, but between 24 and 32 hours, the quantity of P1-2AΔ increased and was maintained in the cells at this level until the 56 hour time-point. At 32 hours the cell monolayer was beginning to detach and because the expression levels were deemed high enough, future expression experiments were stopped at 24hours.

While there was a significant quantity of P1-2AΔ being produced in our expression assays, attempts to obtain it in a soluble state were only partially successful as it appeared that a large proportion of the P1-2AΔ remained in the insoluble pellets following cell lysis. In attempts to improve this, several detergents and chaotropic denaturing agents were tested for their ability to increase P1-2AΔ solubility. The detergents Triton X-100 and Igepal were tested in combination with sonication and 6M urea was used as a positive control. A chloroform extraction on a cell pellet treated with Igepal was also performed due to this being a successful method of purifying ECs of FMDV. The cell pellets were re-suspended in each condition on ice for an hour before centrifugal separation into soluble and insoluble fractions. Analysis was performed by running the total (T), insoluble (I) and soluble (S) samples on SDS-PAGE gels and visualisation was achieved by WB (figure 3.8). Surprisingly extraction with 6M urea only resulted in a small amount of P1-2AΔ becoming soluble, however, in subsequent

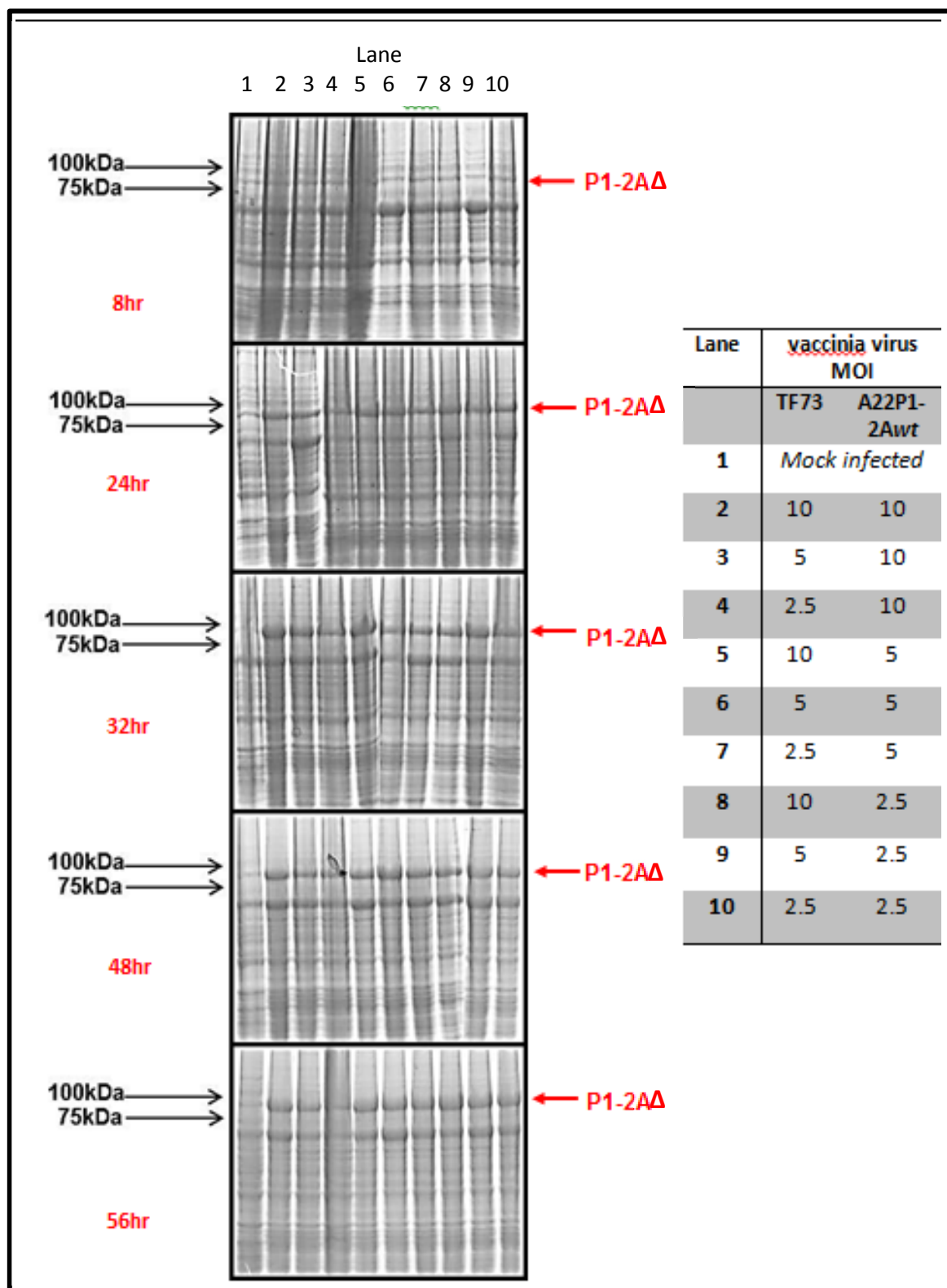


Figure 3.7 Optimization of the expression of recombinant P1-2AΔ from vaccinia virus dual infection. P1-2AΔ (88kDa) was expressed in RK13 cells for 8, 24, 32, 48 and 56 hours. After expression, cells were scraped into the medium, pelleted and boiled in SDS loading buffer. Lysates were applied to 10% SDS-PAGE gels which were subsequently stained with Coomassie R250 and then de-stained overnight (methods in sections 2.9 and 2.10).

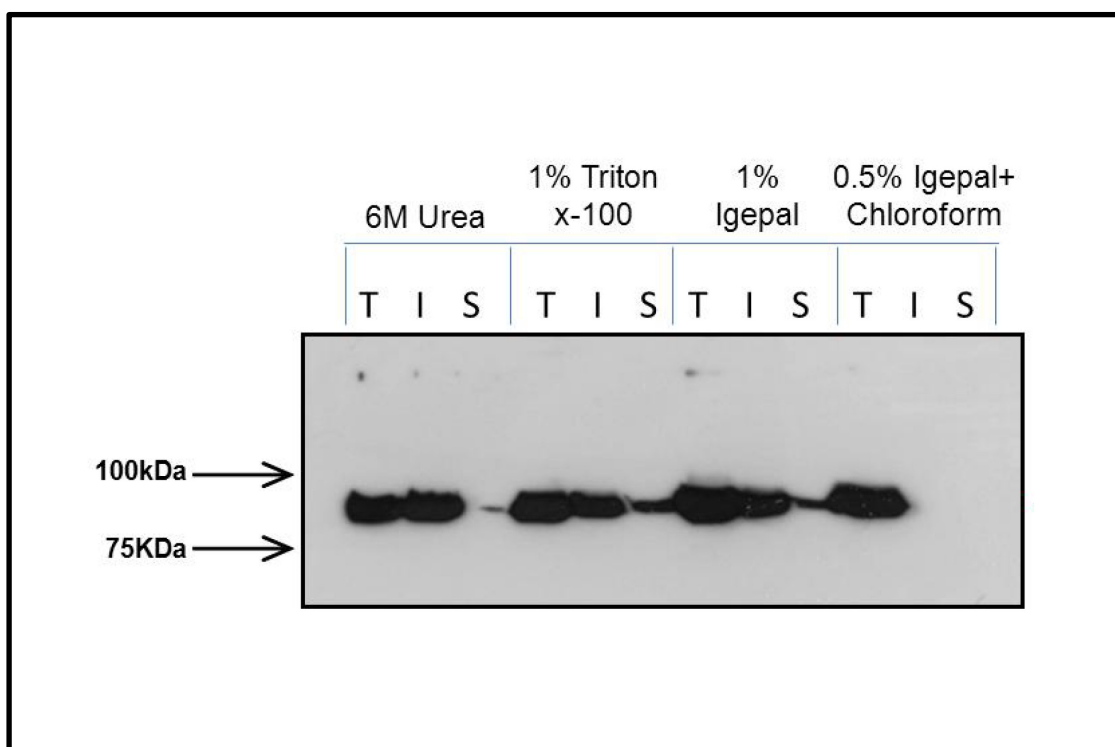


Figure 3.8: Effect of different extraction methods on the levels of soluble P1-2AΔ from vaccinia expression cultures. Vaccinia virus mammalian cell cultures expressing recombinant P1-2AΔ (88kDa) were aliquoted and centrifuged into cell pellets. The pellets were subjected to either 6M urea, 1% Triton X-100, 1% Igepal or 0.5% Igepal followed by chloroform extraction for 1 hour on ice before a total sample (T) was taken. The samples were then centrifuged to pellet the insoluble (I) lysate from the soluble (S) fractions before samples were run on 10% SDS-PAGE gels, transferred to nitrocellulose membrane, and probed with guinea-pig serum raised against A serotype virus. Detection was achieved by application of ECL reagent to the membrane and exposure to film (methods in sections 2.9 and 2.10).

experiments when the cells were treated with a mixture of 8M urea and detergent and left to lyse for longer, greater amounts of P1-2AΔ were solubilised. No P1-2AΔ was seen in the lanes corresponding to the insoluble or aqueous soluble fractions when the cells were treated with Igepal followed by chloroform extraction suggesting the protein remained in the organic phase. When the cell pellet was solubilised with 1% Triton X-100 or Igepal, the majority of P1-2AΔ remained in the insoluble pellet but a significant amount more was recovered in the soluble fraction. In these and future experiments, there appeared to be more insoluble P1-2AΔ than in the total sample, however this is believed to be an artefact of problems encountered when trying to take a representative sample for gel analysis from the highly insoluble pellets. Although only a proportion of P1-2AΔ was soluble using the conditions tested, it was thought likely that using larger expression cultures could circumvent this problem by increasing the overall yield and thus the overall amount of soluble P1-2AΔ if the solubility remained consistent in the scale up.

3.2.3.3 Purification of recombinant P1-2AΔ

Purification of P1-2AΔ from the soluble fraction of bacterial lysates was carried out by nickel ion affinity chromatography via the his⁶ tag at the C-terminus of P1-2AΔ. Samples were pumped through columns charged with Ni²⁺ ions, the bound proteins were washed before being eluted using a discontinuous gradient of increasing concentrations of imidazole. Samples were collected at every stage to analyse the success of the purification by separating the proteins by SDS-PAGE and detecting by WB. The majority of P1-2AΔ (88kDa) appeared in the flow-through, suggesting that it was not binding to the column (figure 3.9A). Although the column has the capacity to adsorb 40mg of his⁶ tagged protein, a reason for the lack of P1-2AΔ binding to the column may have been that the lysate was too concentrated, so, a twenty fold dilution was made of the soluble starting material but again P1-2AΔ was seen to run straight through the column without binding (figure 3.9B). A further suggestion was that the his⁶ tag may be buried inside the hydrophobic portions of P1-2AΔ and be

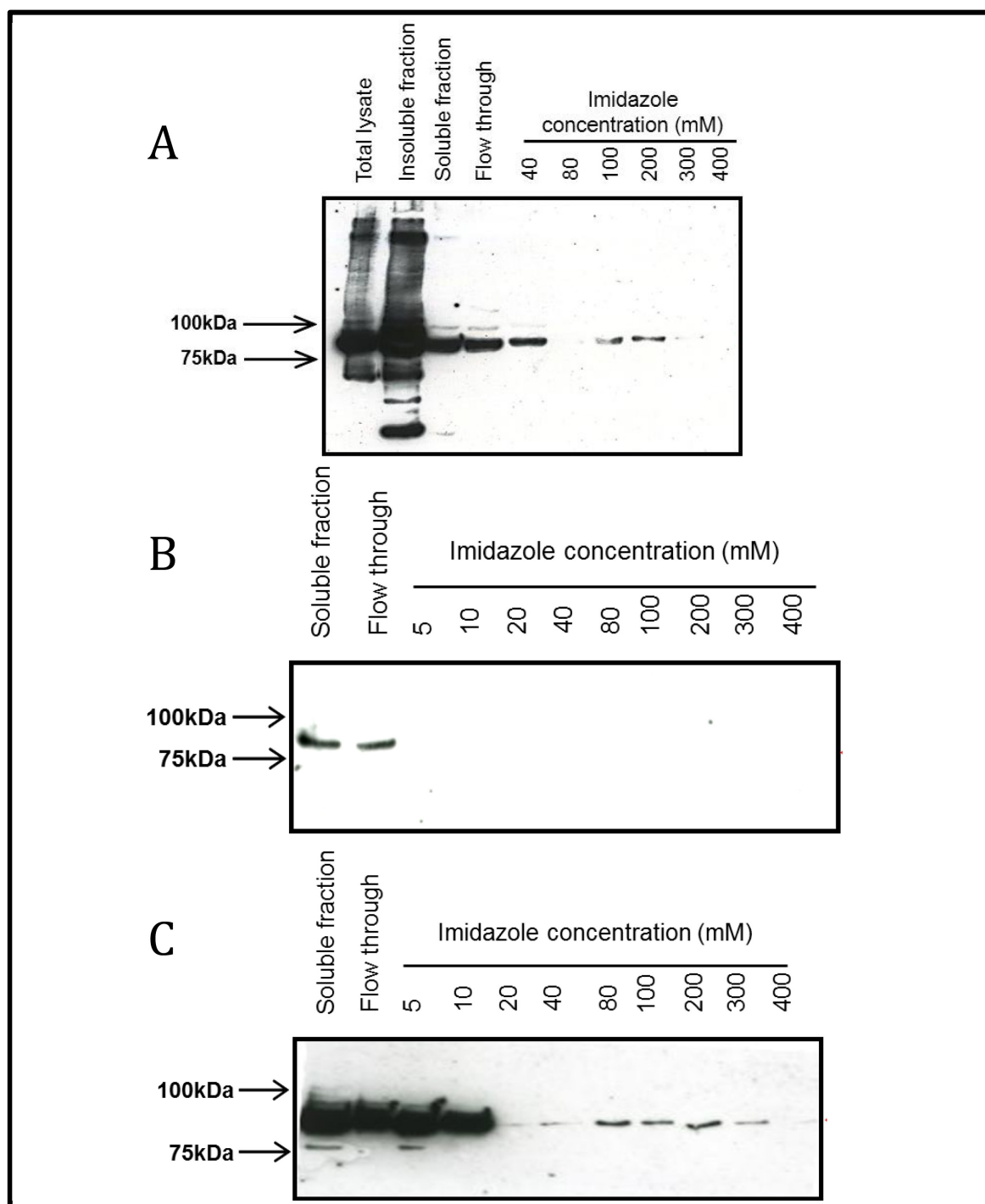


Figure 3.9: SDS-PAGE separation and WB analysis of the purification of P1-2A Δ from mammalian cells lysates on his-trap columns. **A.** Recombinant P1-2A Δ (88kDa) obtained from the soluble cellular fraction following lysis was run through a nickel column, washed and eluted in increasing concentrations of imidazole with fractions collected at each stage. **B.** To reduce the concentration of material loaded onto the column, the soluble fraction was diluted 20-fold and applied to the column. **C.** To determine whether the his⁶ tag was accessible to the column matrix, 8M urea was added to the lysis buffer before the sample was added to the column (methods in sections 2.6, 2.9 and 2.10).

inaccessible to the nickel ions bonded to the column. To test this theory, 8M guanidine hydrochloride was included in the lysis buffer and wash buffers on the column to denature the structure of P1-2AΔ and expose the tag if it was buried. Using 8M urea in the lysis buffer improved the solubilisation of P1-2AΔ so that about half of the material became soluble; however the majority of soluble P1-2AΔ again flowed straight through the column (figure 3.9C). Purification was also attempted on columns coated with cobalt²⁺ ions but no more P1-2AΔ was bound than in figure 3.9A (data not shown). Fractions were normalised to account for differences in volume before being analysed by WB as above.

A further explanation for the majority of P1-2AΔ not binding to the column was that the his⁶ tag had been cleaved off of the protein by a cellular protease. To test this, a further column elution was performed with the eluted fractions assayed by WB using guinea pig sera raised against A-serotype virus and also for the presence of the his⁶ tag on P1-2AΔ in parallel (figure 3.10A; upper and lower blots respectively). The eluted P1-2AΔ in this set of purifications was seen in the 400mM imidazole elution. The two sets of WBs performed using the α-his⁶ tag antibody or guinea-pig serum, gave fairly similar elution profiles. Both blots picked up P1-2AΔ in the pre-column soluble lysates as well as in the imidazole eluted fractions with the peak elution in 400mM imidazole with two fractions either side showing the shoulders of the elution. Interestingly, the P1-2AΔ band in the flow through was much fainter when using the α-his⁶ tag antibody for detection compared to the serum. With the other bands showing a relatively similar proportion in the two blots, it suggested that the flow through contained material that was not, or was incompletely tagged with six histidines coded for in the sequence. Lack of a his⁶ tag on P1-2AΔ could explain why a lot of the material was flowing straight through the column. The plasmid construct used to generate the vaccinia virus A22P12Awt had been sequenced to confirm the presence of the tag so the reasons why some of the material was lacking a his⁶ tag are unknown but suggestions are made in the discussion. Imidazole was present in the binding buffer to prevent non-specific interactions with the column to increase the

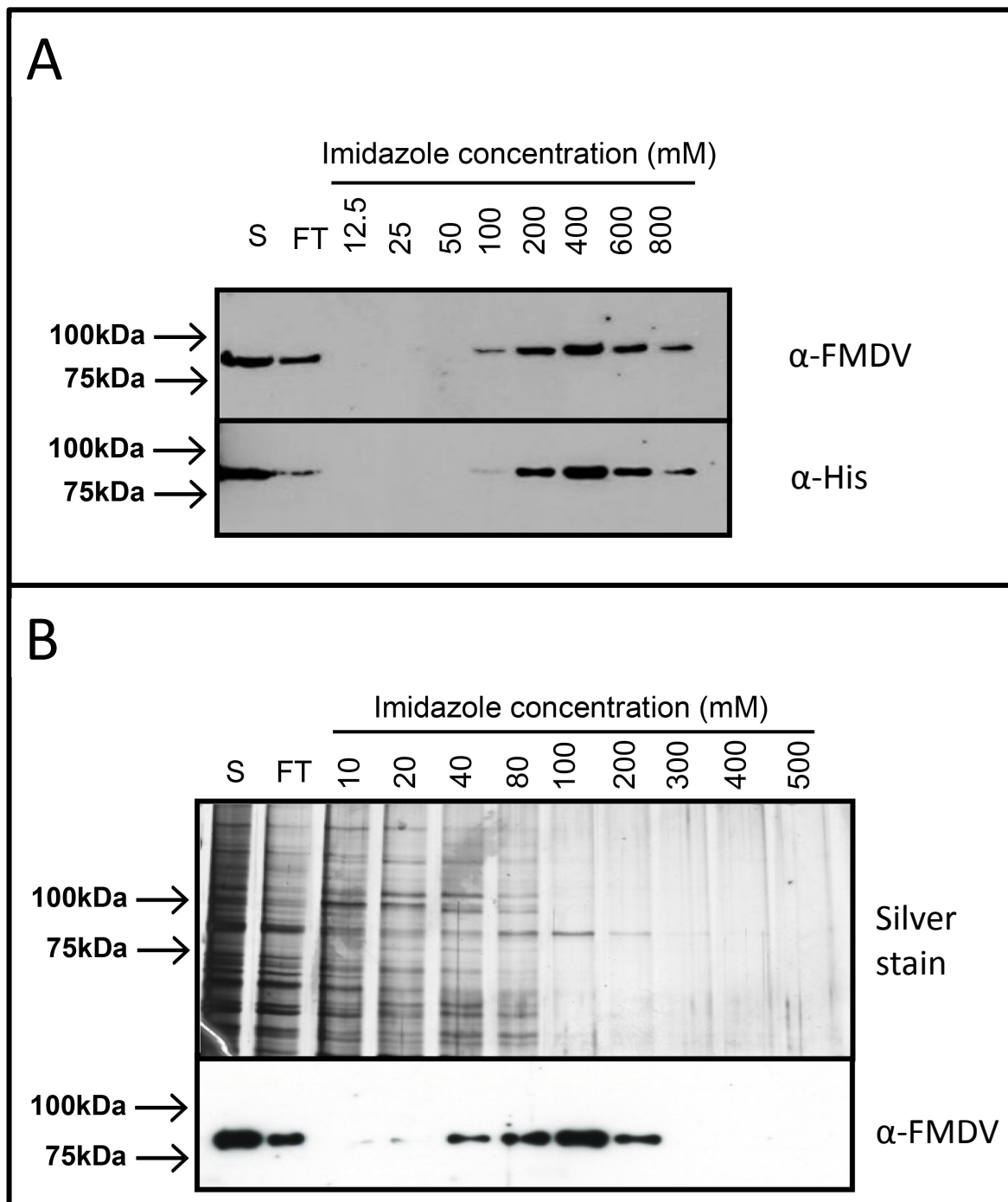


Figure 3.10: P1-2AΔ purification from mammalian cells lysates on nickel columns. To observe whether the his⁶-tag was present on P1-2AΔ, a further elution was performed with eluted fractions stained for the presence of the tag (Figure A, bottom box) and compared with the proportions of P1-2AΔ visualised using the hyperimmune sera (Figure A, top box). Soluble lysates from vaccinia virus expression cultures had 5mM imidazole added to them before they were applied to nickel columns for purification (B). To observe P1-2AΔ in the eluted fractions, five times more of the imidazole elutions had to be added to the gel than the input material. All samples were separated on 10% SDS-PAGE gels and either silver stain (B; top box), or probed in a WB for α-A serotype protein (A; top box and B bottom box) or α-his⁶ (A; bottom box) antibodies.

purity of the eluted protein. Purification experiments were carried out in which the concentration of imidazole in the binding buffer was reduced from the recommended concentration of 30mM to a nominal 5mM and the samples were analysed as before. While a significant proportion of P1-2AΔ was still flowing through the column without binding, a greater proportion had bound to the column (figure 3.10B Western blot) and the eluted material appeared pure when analysed by silver stain (figure 3.10B gel) with the peak of material contained in the 100mM imidazole elution. The difference in the concentration of imidazole required to elute P1-2AΔ was thought to be due to reducing the concentration of imidazole in the binding buffer, and that the material may be at a different concentration in the two lysates.

Despite several difficulties, the expression, solubilisation and purification of P1-2AΔ from mammalian cells infected with the recombinant vaccinia virus A22P12Awt was achieved. In future this process could be scaled up to express large amounts of material to study P1-2AΔ and its assembly into higher order structures such as pentamers using biophysical approaches that require larger amounts of recombinant material.

3.3 Discussion

This chapter has described the generation of a set of expression plasmids to produce *wt* and mutant forms of the P1 and P1-2AΔ protein of FMDV, and the expression and purification of recombinant FMDV 3C^{pro} from existing plasmid constructs. P1-2AΔ and 3C^{pro} were produced separately in order for the rate of P1-2AΔ processing and therefore assembly to be controlled. These reagents were used in the RRL expression and assembly assays described in chapters 4, 5 and 6 in order to study the early stages in the assembly process of FMDV in detail. In addition, recombinant vaccinia viruses have been generated from one of the *wt* expression constructs to develop a system for increased P1-2AΔ expression and purification that could be used to obtain structural information about P1-2AΔ.

3.3.1 3C^{pro} expression and purification

A wide range of systems are available for the overexpression of recombinant proteins. We chose to express FMDV 3C^{pro} in *E.coli* due to the method being well established in Professor Stephen Curry's lab, who had provided the expression constructs (Birtley and Curry, 2005, Sweeney et al., 2007, Zunszain et al., 2010). These plasmids encoded the 3C protease from FMDV A₁₀61 with the amino acid mutations C95K/C142A and C95K/C142T to enhance the protein's solubility while retaining its activity. 3C^{pro} was expressed and purified in an active form as evidenced by the fact that the band for 3C^{pro} was seen at 23kDa. In these constructs 3C^{pro} is expressed as a precursor polyprotein preceded by a truncated 3B1 and complete 3B2 and 3B3 proteins with a total molecular weight of 35kDa. Upon expression 3C^{pro} cleaves itself from the polyprotein resulting in the 23kDa band (Zunszain et al., 2010). While this initial activity in the bacteria is not a guarantee of 3C^{pro} activity after its purification, it was encouraging that the expected band pattern of an active 3C^{pro} was seen.

Our calculations for quantifying 3C^{pro} were obtained by Bradford assay followed by comparison against a standard curve. The CBB dye in the Bradford assay primarily binds to positively charged amino acids via electrostatic interactions but also to aromatic amino acids via hydrophobic interactions. Binding is strongest to arginine residues resulting in the biggest colour change, while binding to lysine and histidine is less strong, and the contribution to the colour change by tryptophan and phenylalanine is marginal. While Bradford assays are a standard and recognised method for quantifying protein, estimates of protein concentration can be skewed by differences in amino acid composition between the protein of interest (3C^{pro}) and the protein used to generate the standard curve (BSA). Sequences obtained from the NCBI protein database (3C^{pro}; PDB number 2J92_B, BSA; NCBI reference number NP_851335.1) were inserted into the ExPASy ProtParam tool (Gasteiger E. et al., 2005) to analyse their amino acid composition. Six histidine residues were added to the 3C^{pro} calculated sequence to account for the his⁶-tag. Table 3.2 shows the percentage of the two proteins that are composed of the important residues for the Bradford assay. Both the arginine

and lysine/histidine proportions are fairly similar in the 3C^{pro} and BSA being 4.7% to 4.3% and 12.6 to 12.7% respectively. The proportions are slightly less similar for the aromatic residues (3C^{pro}; 3.7%, BSA; 5.4%) but given that these only play a lesser role in the CBB colour change, it was thought that the two proteins were largely comparable and the extrapolated 3C^{pro} concentration was fairly accurate. We purified both 3C^{pro} proteins to a concentration of approximately 40μM which was a sufficient concentration (Sweeney et al., 2007) to optimise the cleavage assay conditions which are described in chapter 4.

Table 3.2. Accuracy of the Bradford assay for quantifying 3C^{pro} with BSA. The amino acid composition of different proteins can affect the accuracy of the Bradford assay. The relative proportions of the critical amino acids for the assay have been calculated from their sequences using the ExPASy ProtParam tool. Arginine has been shown to provide the biggest contribution, followed by the other positively charged amino acids lysine and histidine. Finally a minor contribution by the aromatic amino acids tryptophan and phenylalanine plays a role in the CBB colour change.

Amino Acid	Proportion of 3C ^{pro} (%)	Proportion of BSA (%)
Arginine	4.7	4.3
Lysine and Histidine	12.6	12.7
Tryptophan/Phenylalanine	3.7	5.4

3.3.2 Capsid precursor cloning and mutagenesis

Several considerations had to be taken into account when designing the strategy for expressing P1 and P1-2AΔ. Due to the omission of the L^{pro} sequence from our expression constructs, the start codon normally found at the start of this sequence had to be moved to precede the P1 sequence. This was done to avoid toxicity caused by L^{pro} to the translational machinery required to produce recombinant protein in mammalian cells and lysates. L^{pro} is not thought to have a role in assembly, it has been omitted successfully in other assembly studies, and its omission is required to generate recombinant vaccinia viruses (Belsham et al., 1990). In PV, 2A performs a similar function to FMDV L^{pro} and also has to be omitted in order to generate recombinant vaccinia viruses expressing PV P1

(Jewell et al., 1990, Turner et al., 1989). The authentic N-terminus of P1-2A is revealed after the removal of the N-terminal methionine by methionine amino-peptidase allowing myristoylation to occur. For this reason, it was decided to introduce an affinity tag at the C-terminus of P1 to aid in its purification and detection. A his⁶-tag was chosen as the affinity tag because at a size of only 6 amino acids, it is small enough to minimise any effect on the structure of the capsid precursor and can be purified using readily available nickel or cobalt columns and beads. Imidazole was used to competitively elute the his⁶-tagged protein of interest from the nickel column rather than eluting with a large pH change that may affect the protein of interest or by obtaining a competitively binding peptide when performing immuno-affinity purification. The small size of imidazole also allows its easy removal by dialysis. We decided against using a large affinity tag such as glutathione S-transferase (GST) or maltose binding protein (MBP) because P1-2A is already a large protein and the addition of a large modification might affect the yield of expression. Additionally, large modifications such as these could affect the correct folding of the capsid precursor (Zhao et al., 2013). Two sets of constructs were engineered to express at the C-terminus of P1; a 2-alanine residue spacer followed by the his⁶-tag and a stop codon, or the first four amino acids of 2A, a 2-alanine spacer, the his⁶-tag and then a stop codon. The first set of constructs allowed the his⁶-tag to be retained throughout the assembly process whereas the second set of constructs lost the tag upon 3C^{pro} cleavage. It was thought to be advantageous to have a set of constructs with an authentic P1 C-terminus by removing the his⁶-tag, although it has subsequently been shown that P1 with uncleaved 2A can assemble into empty capsids and can be tolerated by viruses (Gullberg et al., 2013b). However, the fact that there is a small effect on the levels of infectious virus does suggest that P1 without 2A is optimal for virus replication and this could be due to slight assembly differences.

FMDV viruses from serotypes A and O have known differences in their assembly. Differences have been seen in their ability to form empty capsids (Rweyemamu et al., 1979) and in the stability of those empty capsids (Doel and Baccarini, 1981). A-serotype empty capsids also have a large

proportion of VP4 and VP2 as opposed to intact VP0 (Curry et al., 1995), a feature that has also been reported in O serotype empty capsids (Gullberg et al. 2013a). To understand if any differences in these empty capsids were caused by differences in the ability of their capsid precursors to form pentamers, we generated P1 and P1-2AΔ sequences from A22 Iraq and O1 Manisa for comparison.

To perform mutagenesis on the P1 sequence, a vector was required that was smaller than the approximately 11kb of pBG200. pCR-Blunt II TOPO vectors were used due to their smaller size and for the ease of cloning PCR products into. We based our mutagenesis approaches on the knowledge that the cleavage sites in picornavirus species and genera are fairly conserved (Palmenberg, 1990, Blom et al., 1996, Castello et al., 2011), and the previous work of Ansardi and Morrow (1993), with input from Professor Stephen Curry.

The constructs were designed to allow their expression from a T7 promoter in RRLs but to also allow the generation of recombinant vaccinia viruses. The pBG200 transfer vector fits these criteria and as such was chosen as the destination vector for all of the constructs.

3.3.3 Purified recombinant P1-2AΔ from a vaccinia virus expression system

The multiple *wt* and mutant constructs described above were designed so that capsid precursor processing and assembly into pentamers could be modelled and analysed in a small scale RRL system. The pentamer to empty capsid step of assembly is fairly well characterised as requiring a sufficient concentration of pentamers, the correct temperature and ionic strength (Rombaut et al., 1991, Li et al., 2012). However the P1-2A, to cleaved P1, to pentamer assembly step appears to be more involved with the recent discovery of chaperone involvement (Geller et al., 2007, Mutsunguma et al., 2011, Tsou et al., 2013, Wang et al., 2013). Having biophysical data for the capsid precursor may allow the identification of how protein rearrangements facilitate pentamer

formation and to do this, a highly purified, highly concentrated source of the capsid precursor is required.

The recombinant vaccinia virus A22P12Awt was generated to evaluate this system as a means for producing P1-2AΔ. It was shown through a time-course study that the levels of P1-2AΔ expression were very high in the cells from this system with a strongly stained band present on Coomassie stained SDS-PAGE gels. Initial attempts to solubilise P1-2AΔ from these expression cultures only resulted in a fraction of the total P1-2AΔ becoming soluble even when using a range of detergents and chaotropic agents. Large insoluble clumps of protein were visible in P1-2AΔ expression lysates that weren't seen in parallel vaccinia virus infected cell cultures prepared in the same way. The most successful strategy adopted to obtain soluble P1-2AΔ was to use 8M Guanidine hydrochloride but still about half of the material remained insoluble. It appears that over-expression of P1-2AΔ resulted in large proportions being directed to, or, spontaneously forming highly insoluble aggregates inside the cells. This phenomenon has been noted before by other authors who have seen that picornavirus P1 is fairly insoluble when expressed to high levels without processing in *E.coli* and baculovirus infected insect cells (Lewis et al., 1991). P1-2AΔ insolubility could be explained by an overloading of the chaperone system described fully in the introduction and chapter 5. Molecular chaperones such as Hsp90 and Hsp70 protect hydrophobic regions of proteins from energetically favourable hydrophobic collapse and aggregation (reviewed in Hartl et al., 2011, Saibil, 2013). When cells are stressed i.e. in viral infection (as in the vaccinia virus infection here) or protein over-expression, the chaperone and protein turnover systems can be overloaded leading to the formation of amorphous aggregates (reviewed in Kopito, 2000). At low levels of expression and when chaperones are inhibited, unprocessed PV P1 is thought to be targeted for degradation (Macadam et al., 1991, Geller et al., 2007). Where large amounts of P1-2AΔ are being expressed in the background of a vaccinia virus infection, there is a distinct possibility that P1-2AΔ is overloading these systems. The P1-2AΔ protein does not multimerise with other P1-2AΔ molecules in the

assembly pathway until processed, but high levels of P1-2AΔ expression may force unordered P1-2AΔ multimerisation into the insoluble aggregates. Chapter 5 describes the requirement for the cellular chaperone Hsp90 in the FMDV lifecycle and the overexpression of Hsp90 in these expression cultures may be a strategy to avoid this problem.

A further possibility is that during virus infection, 3C^{pro} performs a function other than processing of P1-2A that aids solubility; and when P1-2A is expressed in *cis* these aggregates are absent. Although this effect could be just that of limiting over expression through its functions in disabling host-translation (Belsham et al., 2000, Li et al., 2001), novel functions of 3C^{pro} are being discovered frequently (Lei et al., 2011, Mukherjee et al., 2011, Qu et al., 2011, Lawrence et al., 2012, Lee et al., 2012, Lei et al., 2012, Wang et al., 2012b, Walker et al., 2013, Zhou et al., 2013, Du et al., 2014, Wang et al., 2014b), and the hypothesis that 3C^{pro} can directly or indirectly aid P1-2AΔ solubility is not implausible. The generation of a P1-2AΔ protein without any cleavage sites but with 3C^{pro} expressed in *cis* could be used to test this theory although sufficient controls would be necessary to account for any down regulation in expression caused by 3C^{pro}, such as using an attenuated form of 3C^{pro} or its expression down-regulated relative to P1-2A (Polacek et al., 2013, Porta et al., 2013b). It is also possible that the presence of the intact 2A protein or the 2A-2B junction as used by Lewis and colleagues (1991) is required by P1-2A to correctly fold, which may be one of the reasons that they were able to solubilise half of the P1-2A expressed from insect cells and we were not.

P1-2AΔ insolubility would not be a significant problem if enough of it could be solubilised from the cells and this material was in a native state to do the intended analyses, however the next challenge that was encountered was purifying a significant amount of the soluble P1-2AΔ. After extensive optimisation the amount of P1-2AΔ that could be purified by affinity chromatography using the C-terminal his⁶-tag was still limited, with a large proportion of the protein flowing straight through the column. The ability to purify some P1-2AΔ suggested that the his⁶ tag is present on at least a

proportion of the material. The presence of the tag in the plasmid constructs was confirmed by Sanger sequencing. There was the possibility that the tag was buried in the P1-2AΔ structure or obscured by a binding partner such as a molecular chaperone (Geller et al., 2007, Macejak and Sarnow, 1992) and therefore largely hidden from the affinity column, but a purification performed in the presence of 8M urea which would have exposed the tag, also failed to improve the purification. Another possibility is that the tag is no longer intact by the time of the purification and this is what is suggested by parallel WBs of the set of elution fractions (figure 3.10). In these blots, the proportions of all fractions looked the same in both blots apart from the material that had flowed through the column which was only faintly visible when using the anti-his mAb compared to the much stronger band seen when the blot is stained with the anti-FMDV sera. There was still a faint band present with the mAb which might indicate that only part of the his⁶ tag was missing which was not enough to be bound by the affinity column and was only partially picked up in the WB. This suggested that this material had lost some of the his⁶ tag between expression and purification. The ends of a protein are more susceptible to cellular protease digestion and whilst a complete protease inhibitor cocktail is added to the cells during lysis to prevent this happening, it appeared that enough of the tag was being lost to affect the purification of P1-2AΔ. The material in the flow through fraction was still migrating to the same position on the gel as the P1-2AΔ purified fractions that stained positive for the his⁶-tag suggesting very little of the protein had been degraded if this was the correct explanation for why not all P1-2AΔ was binding to the column.

Several complications were encountered when trying to produce a high yield of recombinant purified P1-2AΔ. To produce sufficient material for biophysical and structural analyses large expression cultures would have to be used to account for loss at the solubility and purification stages. Further optimisation was required and several of the strategies described above could be investigated for this purpose.

Chapter 4: Development of a cell free assay to analyse the requirements for the assembly of FMDV capsid pentamers

4.1 Introduction

While some parts of the FMDV capsid assembly process have been investigated extensively, we believe that there are areas of research within assembly that haven't been looked at in detail previously. The chapter describes the development of a cell-free assay to analyse FMDV pentamer assembly. The ability to separate the expression of FMDV 3C^{pro} and P1-2AΔ (as described in section 3.1.2) has allowed the development of an assay to accurately control and observe the processing of P1-2AΔ and its assembly into pentamers. The requirements for pentamer assembly in this assay are investigated, and then used to explore the requirements for myristoylation and 3C^{pro} processing of the FMDV P1-2AΔ protein in the pentamer assembly process.

4.1.1 Rationale for the systems that have been used to study assembly

In infected cells, P1 (enteroviruses) or P1-2A (aphthoviruses) is myristoylated, rapidly processed by 3C^{pro} and assembles into pentamers and capsids and is rarely seen. In attempts to analyse the transition from P1-2A into pentamers, recombinant systems have been used in several studies. The expression and purification of recombinant P1 and P1-2A in bacteria has been utilised by Goodwin et al. (2009) to study assembly but assembly in this system occurred relatively slowly (15 hours). Rabbit reticulocyte lysates (RRLs) have been shown to provide a convenient system for producing recombinant protein in an environment that facilitates the efficient replication, translation, and assembly of picornaviruses comparable to infected cells (Clarke and Sangar, 1988, Grubman, 1984, Palmenberg, 1982). RRLs have been used in this chapter to express P1 and P1-2AΔ precursors from which to analyse the requirements for pentamer assembly in a cell-free environment using exogenous 3C^{pro} (the generation of which is described in 3.2.1). The expression of proteins encoded by plasmids under the control of a T7 promoter are expressed authentically and rapidly in the RRLs. The constructs were generated in the experiments detailed in chapter 3 (described in section 3.2.2) and have allowed us to probe the intricacies of the assembly process.

4.2 Results

4.2.1 Assembly assay development

4.2.1.1 *Cell free P1-2AΔ is competent for processing by recombinant 3C^{pro}*

Chapter 3 describes the generation and quantification of recombinant, purified 3C^{pro} and the creation of plasmid constructs to express *wt* and mutant versions of P1 and P1-2AΔ. This section describes the use of these reagents in the development of a cell-free processing assay in RRLs. This assay was developed based upon the expectation that assembly requires processing at the VP0/3 and VP3/1 sites in P1. Therefore, concentrations of 3C^{pro} were required in this assay that would process in one hour all of the P1 or P1-2AΔ produced in a standard RRL transcription and translation (TnT). The TnT quick system from Promega (method in section 2.4.2.2) provides the necessary components for the transcription and translation of proteins from sequences of DNA under the control of a T7 promoter including a source of T7 RNA polymerase and ribonucleotide triphosphates (rNTPs) but without a source of methionine. The P1 and P1-2AΔ sequences for expression were cloned into pBG200 plasmids under the control of a T7 promoter for expression purposes (described in section 3.2.2). To detect expressed proteins ³⁵S-radiolabelled methionine was including into the reactions for incorporation into the amino acid sequences.

To test the ability of 3C^{pro} to process P1-2AΔ in this system, RRLs were programmed with P1-2AΔ expression constructs. A 2-fold serial dilution of 3C^{pro} was made in PBS and introduced in a minimal volume to the completed ³⁵S radiolabelled TnT RRL programmed with either of the two the wild-type constructs pA22P12Awt and pO1MP12Awt. Lysates were mock treated with PBS as a processing control. After 1 hour, the samples were separated on SDS-PAGE gels and radiolabelled proteins were visualised by fluorography (methods section 2.10; figure 4.1). A band of the expected size for FMDV P1-2AΔ (88kDa) was observed on the gel in the mock treated samples. In the samples incubated with 3C^{pro}, bands of the expected size for the cleavage products VP0, VP3 and VP1 were seen as well as bands consistent with the size of two intermediates, VP0-VP3 and VP3-VP1. In O-serotype viruses VP3 and VP1 are known to migrate separately and are visible as two bands. In contrast, in A-

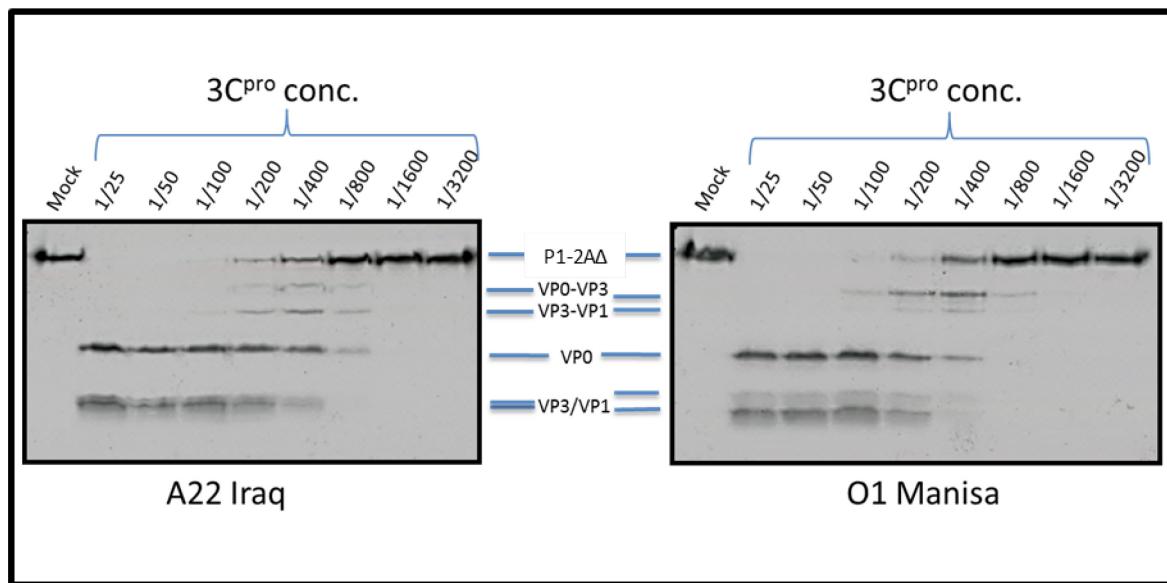


Figure 4.1 A one hour P1-2A Δ processing assay. A standard volume of completed RRL TnT assay, programmed with either pA22P12AwT (left-hand film) or pO1MP12AwT (right-hand film) was split into equal volumes and either mock treated or treated with a two-fold dilution series of 3C^{pro}. The samples were incubated at 37°C for 1 hour before they were prepared in Laemmli buffer and separated on 12% SDS-PAGE gels. The gels were soaked in fluorography reagent, dried onto Whatman paper and exposed to film at -80°C for a range of time-points. Where protein names are separated with a hyphen (-) they are uncleaved, separation with a forward stroke (/) indicates mature cleaved proteins.

serotype viruses, VP3 and VP1 co-migrate or migrate as a doublet depending upon the percentage of gel used. The RRL allows for ^{35}S -methionine incorporation to achieve labelling and therefore the intensity of the different protein bands is different depending upon how many methionine residues are encoded by the sequence. The band for VP0 is disproportionately intense compared with the bands for the other capsid proteins but this was expected to have labelled more strongly due to having more methionine residues to label than VP3 and VP1. In this one hour cleavage assay, the VP0-3 and VP3-1 intermediates were faintly visible at the 1/100 3C^{pro} dilution but the cleavage assays had gone to completion at the 1/50 dilution for the P1-2AΔ molecules from both A22 Iraq (figure 4.1 left-hand film) and O1 Manisa (figure 4.1 right-hand film). A dilution of 1/50 corresponds to a concentration of approximately $1\mu\text{M}$ and was used in all subsequent processing assays as the standard P1-2AΔ processing conditions in a RRL.

4.2.1.2 Sedimentation markers

Rate-Zonal sedimentation through a sucrose density gradients (SDGs) is an established method (Martin and Ames, 1961) for separating proteins based upon properties such as their mass, shape, density and the hydration state of the exposed amino acids (Marks, 2001). Ultracentrifugation through sucrose gradients was performed in 5mL tubes to allow for high speed centrifugation while retaining a tube path length long enough to be fractionated reproducibly. The sedimentation coefficient (S) is expressed in Svedberg units (S) with one Svedberg equal to 10^{-13} sec. It is standard practice to use proteins with known S to investigate the sedimentation of proteins with an unknown S . We have chosen to use the sedimentation marker proteins BSA and IgG which have known sedimentation coefficients of 4.6S and 7.1S as marker proteins to size unknown proteins in standard ultracentrifugation conditions (methods in section 2.8) In addition, a preparation of BEI inactivated FMDV A22 Iraq was dissociated into pentamers by treatment with an acidic buffer and run as a 12S marker protein control. Whether cleaved or uncleaved, P1-2AΔ (or P1) sediments at 5S and dissociated FMDV pentamers are known to sediment at 12S, therefore we chose to run the marker

proteins on 5-30% gradients which would give sufficient resolution to separate the 5S capsid precursor from the expected 12S pentamers (Goodwin et al., 2009).

After ultracentrifugation, the linear portion of the gradients were fractionated into 24 equal fractions and the fractions were assayed for the presence of each protein (by absorbance at 280nm for BSA and by ELISA for the presence of IgG or dissociated virus). For presentation purposes, the signal in each fraction was normalised by expressing it as a fraction of the total counts for that gradient (figure 4.2). The peak fraction for BSA with a known sedimentation of 4.6S was seen eight fractions into the gradient from the top (left to right in the figure). The peak fraction for IgG was 11 fractions into the gradient, and for pentamers, was 18 fractions which correspond to sedimentation coefficients of 7.1S and 12S respectively.

4.2.1.3 Linear regression of marker proteins

These data were used to plot a graph of expected sedimentation coefficients vs fraction number (figure 4.3). A line of linear regression was calculated from the three data points from which to extrapolate unknowns and forced through the coordinates $x=1, y=0$ to account for fractions with a sedimentation of 0 that were in the first fraction. From the equation of the linear regression line ($y = 0.7014x$), it was predicted that material sedimenting at 5S, such as unprocessed and processed P1, would peak in fraction 8. Due to findings that will be discussed later on in this chapter and in chapter 6, the position on the gradient for 14S poliovirus pentamers was also calculated to peak in fraction 20 or 21.

4.2.1.4 Unincorporated radiolabel removal

Proteins translated in the cell-free system were radiolabelled by incorporation of ^{35}S -methionine.

When reactions were analysed by SDS-PAGE the large amount of residual free-radiolabel in the reaction was effectively removed by allowing it to run off of the bottom of the gel. In contrast, initial

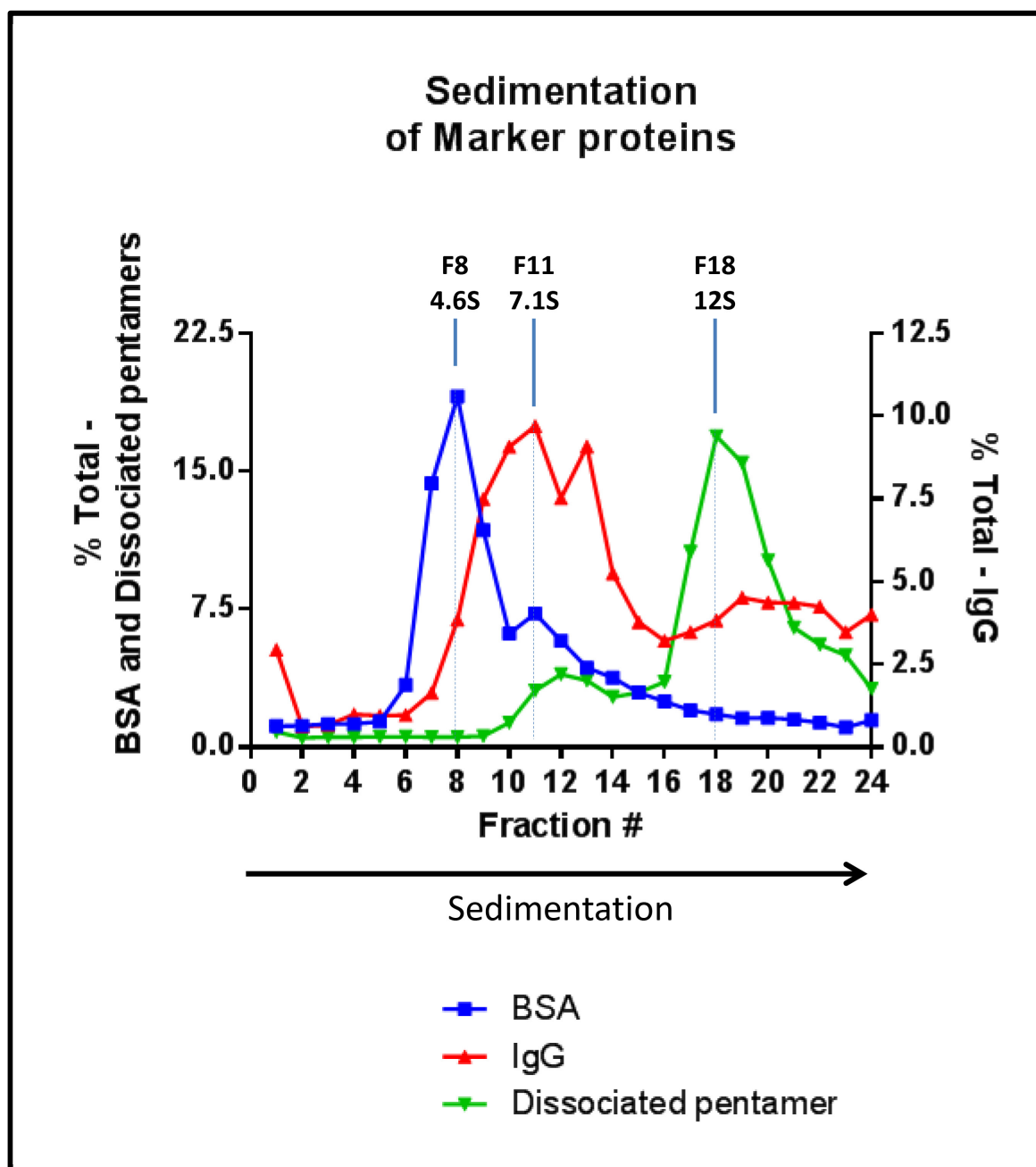


Figure 4.2. Sucrose density gradients of sedimentation markers. BSA (blue), IgG (red) and acid dissociated virus (green) were used as markers with expected sedimentation coefficients of 4.6S, 7.1S and 12S respectively. Samples were centrifuged through 5-30% sucrose gradients loaded onto an SW55Ti rotor. Gradients were fractionated using a Biocomp gradient fractionator into 24 equal fractions (as described in methods 2.8). The BSA gradient fractions were analysed spectrophotometrically at a wavelength of 280nm and the IgG and 12S pentamers fractions were analysed by ELISA and read at 492nm. Absorbance values for each fraction were normalised by expression as a percentage of the total signal for that gradient.

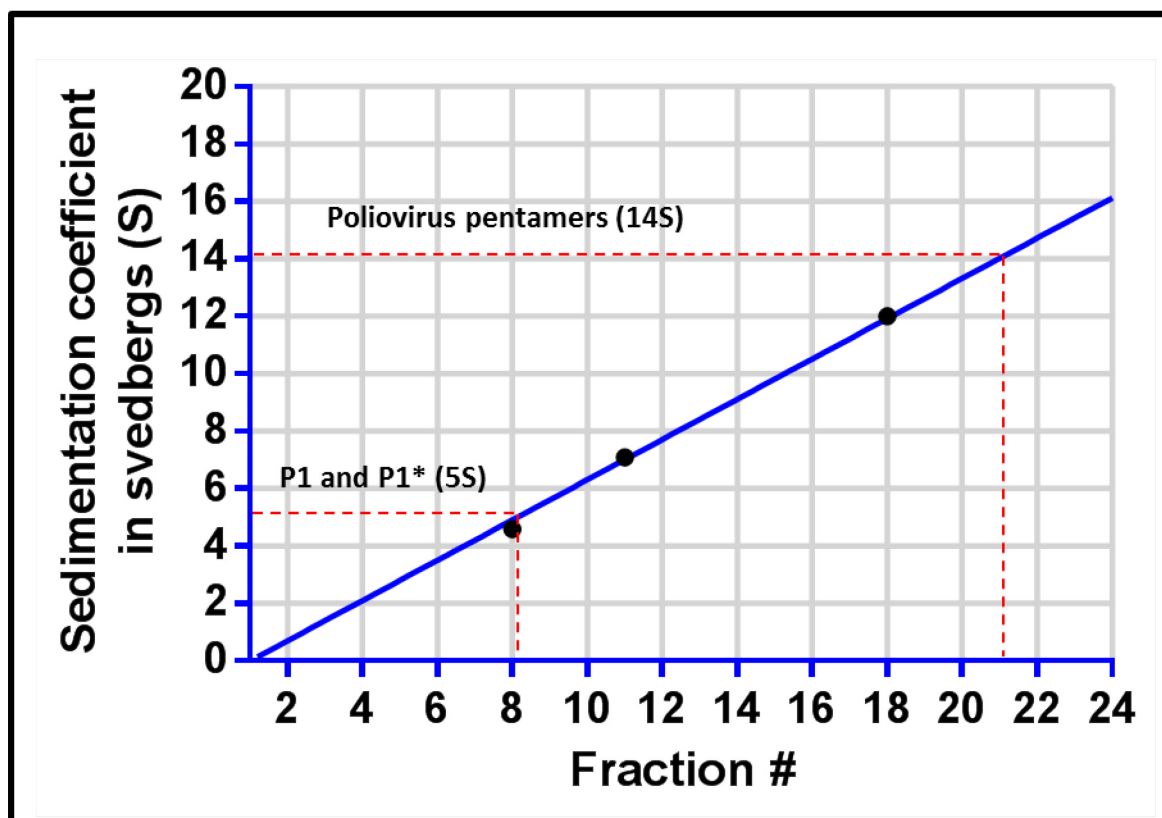


Figure 4.3. Linear regression of sucrose density gradient marker proteins: extrapolation of fractions for expected components. The fraction that each sedimentation marker had travelled to was plotted against its known sedimentation coefficient and linear regression analysis was performed on the data points with the line forced through coordinates $x=1, y=0$. From the equation of the regression line, the fractions where we expected to see unprocessed P1-2AΔ (P1) and processed P1 (P1*) or 14S poliovirus pentamers were calculated (shown using red hashed lines - - -).

attempts to analyse these samples by SDGs gave a very high signal at the top of the gradient which appeared to obscure some of the expected peaks on the gradient (data not shown). To overcome this issue, the ability of either mini dialysis devices or size exclusion spin columns to remove free ^{35}S -met was investigated (methods sections 2.6.3.2 and -3 respectively). The spin column has a 6kDa MW cut off and uses the principle of size exclusion during centrifugal elution of the large P1-2AΔ molecule while retaining small free radiolabelled amino acids. The dialysis device has a 10kDa MW cut off to allow the retention of large proteins such as P1-2AΔ during dialysis. To compare these two methods, P1-2AΔ was expressed using pA22P12Awt (table of constructs; table 3.1). The sample was divided into two, with one half mock treated and the other treated with 3C^{pro} as per the optimal processing conditions described in figure 4.1. After processing, both processed and unprocessed samples were divided in two again with one sample from each put through spin columns and the other set dialysed. Samples were separated by sedimentation through sucrose gradients and fractionated as before. The fractions were mixed in scintillation fluid, read on a scintillation counter and the counts were plotted without normalisation to give an indication of the yield from each system (figure 4.4). In the unprocessed samples (figure 4.4A) a peak was observed between fractions 7 and 8 that was in the expected position of P1-2AΔ. The peak in the spin column sample had about one third of the amount of counts as the sample that had been dialysed. In the spin column sample there was also a larger tail of background radiation at the top of the gradient. In addition to the P1-2AΔ peak in the samples processed with 3C^{pro} samples (figure 4.4B), a second peak was observed in fractions 19 and 20 of both the dialysed and spin column treated samples assumed to be pentamers. Again the P1-2AΔ peak was smaller and the level of background higher in the spin column treated samples compared with the dialysed samples and given these results, it was decided that dialysis would be used to remove free-radiolabel in future experiments.

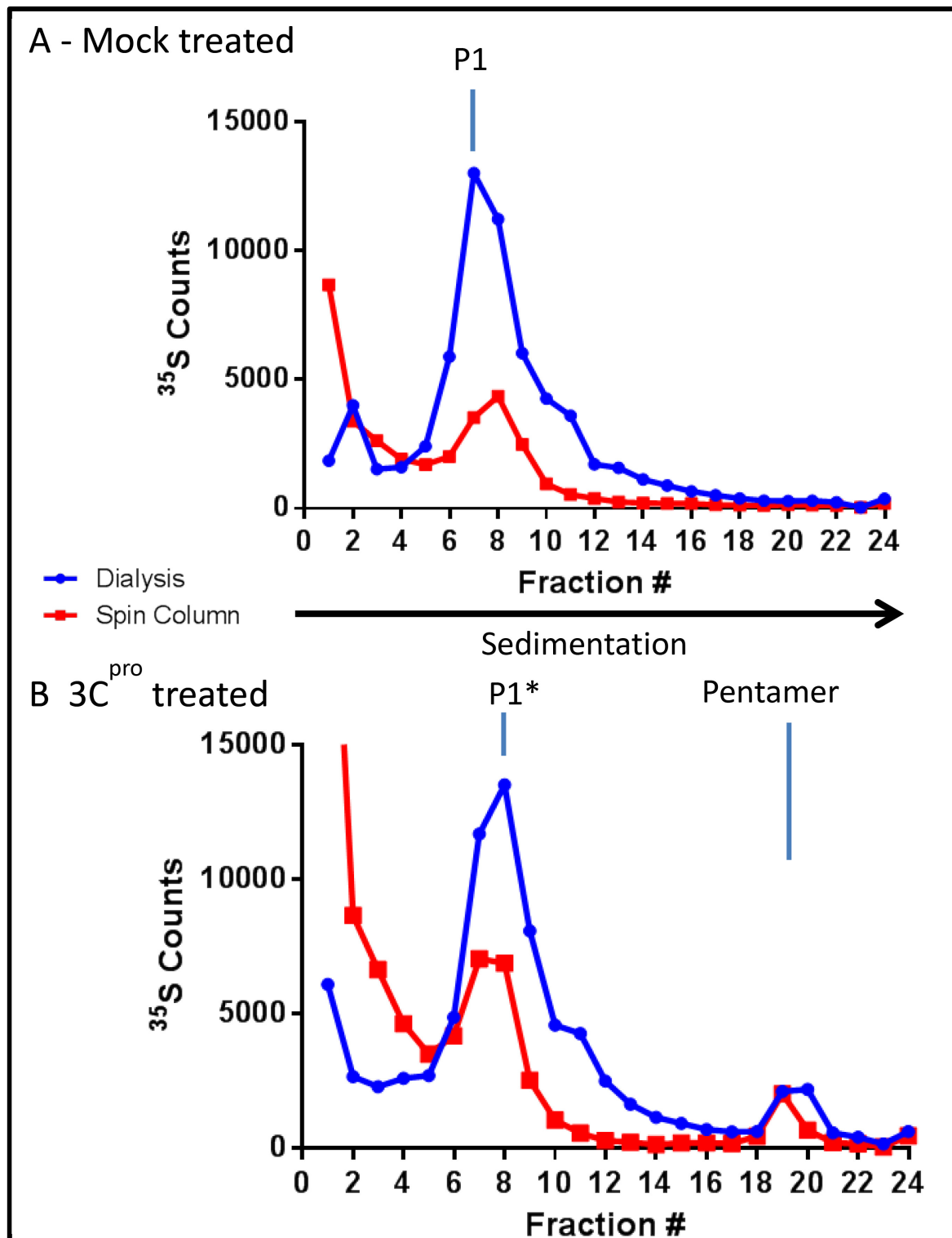


Figure 4.4. Determination of best method to remove free radiolabel from TnT; spin column or dialysis cassette. It was necessary to remove unincorporated ^{35}S from the TnT reactions to before the improve the resolution of the gradients to analyse assembly of P1. The two methods trialled were Pierce polyacrylamide packed spin columns (red lines) or mini dialysis cassettes (blue lines). Unprocessed (top graph) and 3C^{pro} processed P1 samples from a TnT reaction programmed with pA22P12Awt were subject to either of these methods before ultracentrifuged on 5-30% sucrose density gradients, fractionated and analysed (described in the methods for radiolabelled samples). Peaks have been labelled as the expected product for that position based upon marker protein data.

4.2.2 Cell-free assay requirements for pentamer assembly

4.2.2.1 *Pentamer assembly requires sufficient P1-2AΔ concentration and a component of the RRL*

During the initial development of the pentamer assembly it was noticed that there were sometimes differences in the number of counts for the P1-2AΔ and P1 peaks obtained before and after processing. One suggestion for this was that processing the cleavage sites in P1-2AΔ may increase the lability of the individual capsid proteins allowing their termini to fold into the conformation required for assembly. In this rearrangement, changes in the structure of P1-2AΔ, such as an increased burial of hydrophobic residues, could decrease the adsorption of the protein to plastic tubes and the dialysis membrane, therefore accounting for the loss of processed material. Because of this, the effect of performing dialysis before processing to obtain a uniformly concentrated P1-2AΔ reagent which could then be split into separate conditions post-dialysis was assessed. To do this, a TnT reaction was programmed with either pA22P12Awt or pO1MP12Awt before the samples were split in half with one half processed with 3C^{pro} before dialysis as previously performed and one half dialysed before processing. Subsequently the samples were ultracentrifuged, fractionated and analysed as for previous TnT experiments. Pentamers were present on the gradient (fraction 20) for both the A22 (figure 4.5 upper graph) and O1M (figure 4.5 lower graph) samples that had been processed before dialysis, whereas no pentamers were seen in the samples that had been processed after dialysis. The levels of P1-2AΔ in the samples that were processed after dialysis were also about a third less than in the samples that had been processed before dialysis.

The lack of pentamer assembly in samples of P1-2AΔ that had been dialysed before processing was seen in multiple experiments. This was thought to be a result of either there being too low a concentration of P1-2AΔ to facilitate assembly due to loss during dialysis, or as a result of the loss of a critical component for assembly during dialysis, or a combination of both. To test the first of these theories, TnT reactions were programmed with either pA22P12Awt or pO1MP12Awt before a dilution series of these samples was made in un-programmed RRL. The samples were then dialysed, ultracentrifuged, fractionated and analysed as for previous TnT experiments (figure 4.6). In both

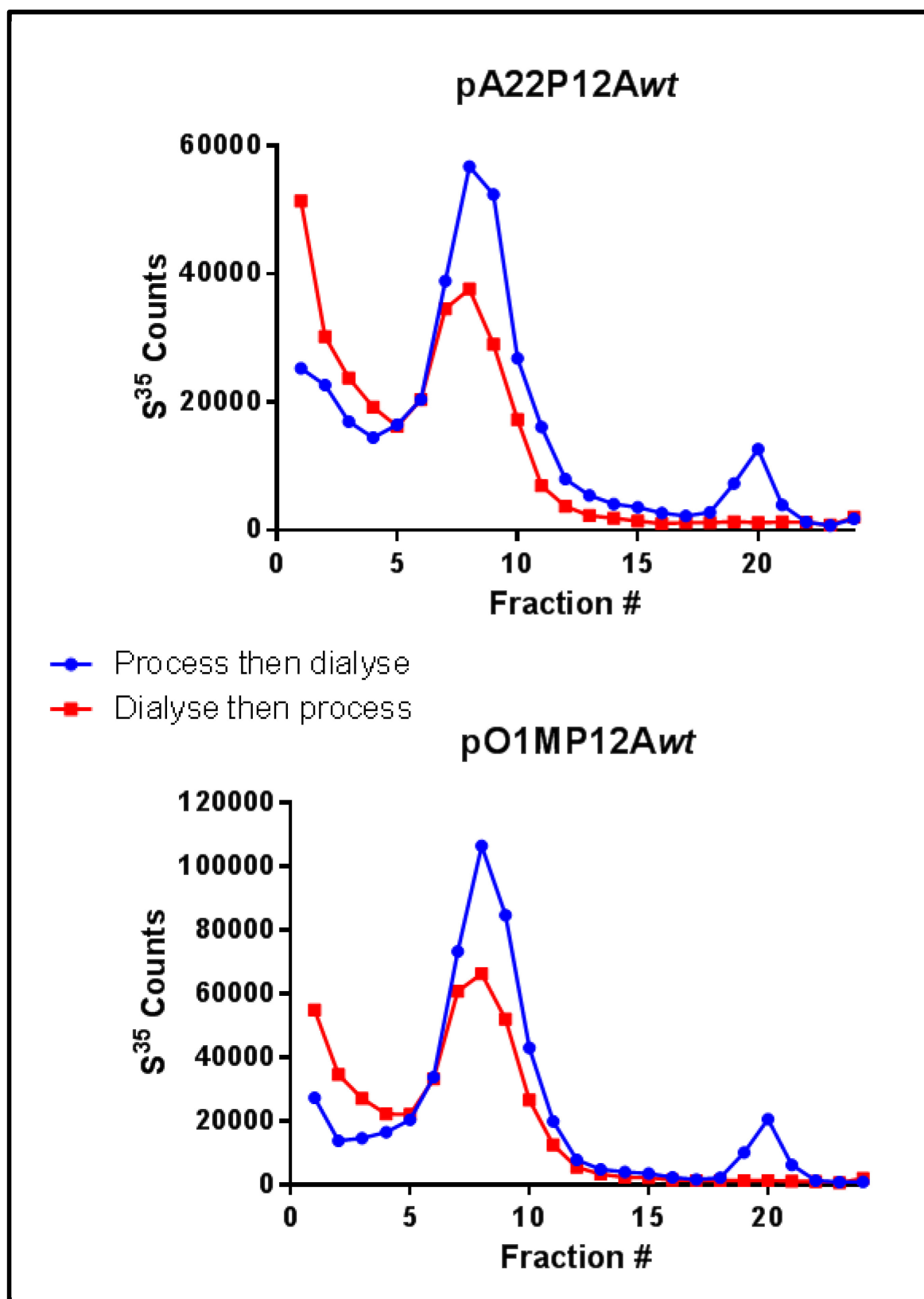


Figure 4.5. Determining the optimal order for the processing and dialysing of assembly assays. The effect on pentamer assembly of processing P1-2AΔ before (blue lines) or after (red lines) dialysis was compared. TnT lysates were programmed with either pA22P12Awt or pO1MP12Awt, before processing and dialysis were carried out in the order specified. These reactions were ultracentrifuged through 5-30% sucrose density gradients, fractionated and analysed as described in methods section 2.8.

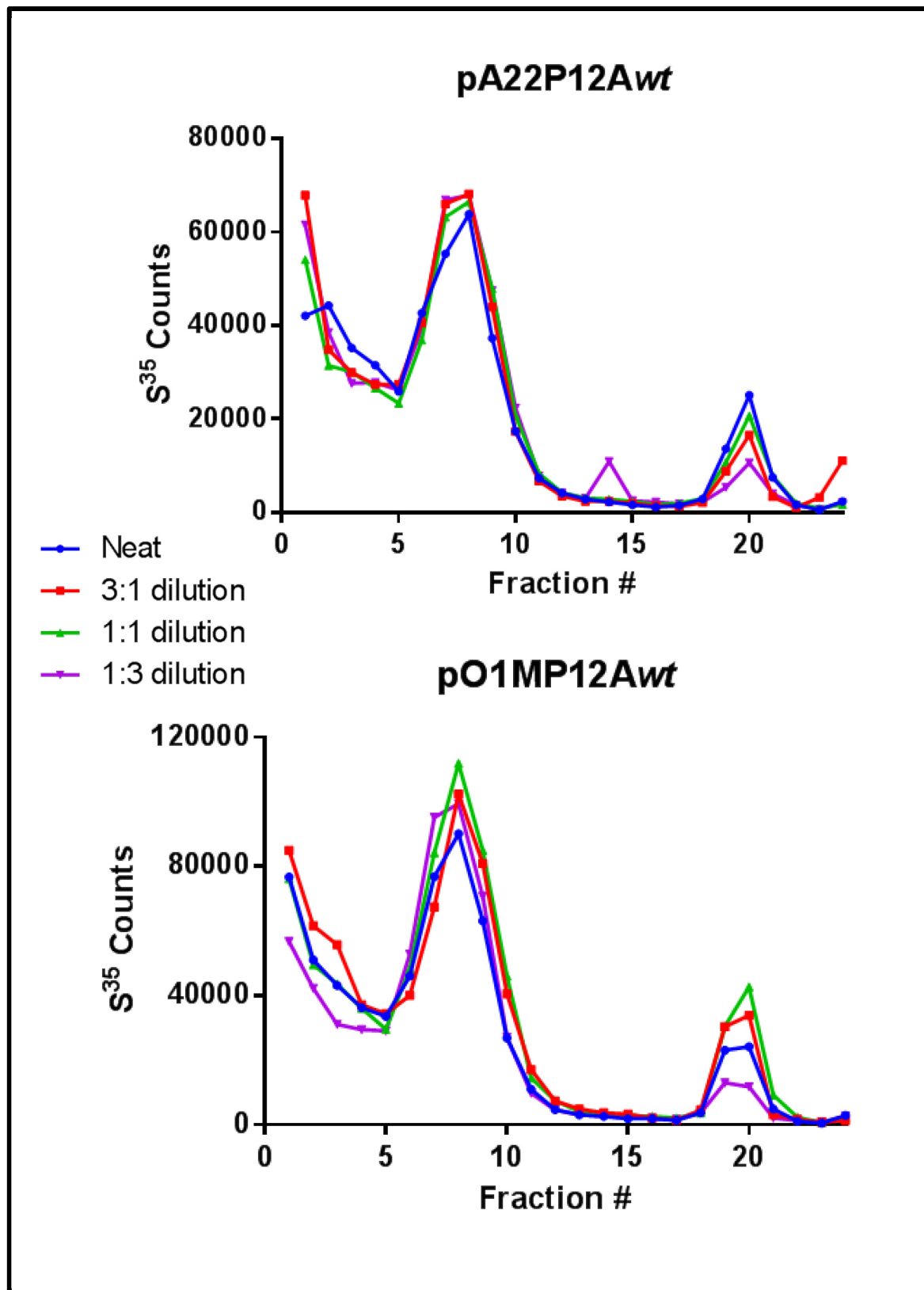


Figure 4.6. Effect of dilution on P1 before processing. TnT lysates programmed with either pA22P12Awt (top graph) or pO1MP12Awt (bottom graph) were diluted to a range of concentrations in unprogrammed RRL prior to processing with 3C^{pro}. Subsequently, these reactions were dialysed, ultracentrifuged on 5-30% sucrose density gradients, fractionated and analysed as described previously (*) indicates an error in the handling of this gradient.

instances, dilution of the TnT by a ratio of 1:3 in unprogrammed lysate was insufficient to completely abrogate pentamer assembly (and at the 25 and 50% dilution in O1M pentamer assembly was slightly enhanced). In figure 4.5, the P1-2AΔ counts (fractions 6-11) were approximately 30.9% for A22 and 30.3% for O1M less for the samples that had been dialysed before processing. Given that the volume of the reaction in the dialysis cassettes was not seen to change greatly during dialysis, this suggested the lack of pentamer formation upon processing of dialysed P1-2AΔ was not caused solely by the reduction in P1-2AΔ concentration. This implied that at least one component of the lysate, which is lost during dialysis against PBS through a 10kDA MW membrane, is also required during P1-2AΔ processing for the assembly of pentamers. However, a 1:3 dilution of both A22 (figure 4.6 upper graph) and O1MP12A (figure 4.6 lower graph) programmed lysates with unprogrammed lysate did have the effect of reducing pentamer assembly suggesting that pentamer formation is more efficient at a higher concentration of P1-2AΔ.

Having shown that dialysis of P1-2AΔ before processing prevented its assembly into pentamers, attempts to obtain a uniform P1-2AΔ reagent were abandoned and it was decided to proceed with dialysis after processing.

4.2.2.2 P1-2AΔ does not have to be cleaved co-translationally to assemble into pentamers

The optimisation so far had revealed that in the RRL assembly assay P1-2AΔ could be processed, assemble into pentamers and subsequently dialysed and sedimented through SDGs (for analysis). In the SDG analysis, P1-2AΔ always remained the largest peak on the gradient. It did not appear that the assembly of pentamers reduced the amount of P1-2AΔ. This was difficult to explain as pentamers were expected to be formed from P1-2AΔ. This led to a hypothesis that the only molecules of P1-2AΔ that were able to assemble were those co-translationally processed by 3C^{pro} i.e. only P1-2AΔ that had been translated after the addition of 3C^{pro} and not P1-2AΔ accumulated during the initial 1.5hr TnT reaction. To test this, a TnT reaction was programmed with pO1MP12Awt with

$3C^{pro}$ added from the start of the reaction, compared with a standard reaction ($3C^{pro}$ added after the completion of the 1.5 hour reaction) and an uncleaved control. Half of the reaction with $3C^{pro}$ added from the start was put on ice after the TnT to stop the reaction and the other half was placed at 37°C for one hour as per the standard assembly reaction conditions. The reactions were separated by SDS-PAGE or SDGs and analysed (as described in methods sections 2.8 and 2.9) (figure 4.7). In both of the $3C^{pro}$ treated samples P1-2AΔ was processed normally and formed pentamers (figure 4.7A). The sample that had been processed during the TnT reaction had a large P1-2AΔ peak and a small pentamer peak (figure 4.7A green line). During the extra hour of incubation (figure 4.7A purple line) approximately one third of the radioactive signal had shifted into the pentamer peak. This suggested that P1-2AΔ made during the TnT did not have to be co-translationally cleaved to be competent for assembly into pentamers.

This was confirmed with a further set of experiments in which samples were treated with RNase A or cyclohexamide (CHX) after the TnT had run to completion but before processing. RNase A and CHX degrade RNA transcripts and inhibit translation respectively so were used to stop the TnT reaction producing any more protein during the one hour assembly incubation. RNase and CHX were included from the start of the reaction as negative controls and a standard assembly reaction was included as a positive control for pentamer formation. The samples were analysed by SDS-PAGE and SDGs (as described in methods sections 2.8 and 2.9). When completed TnT reactions were treated with either RNase A or CHX before processing, pentamer formation still occurred and was largely comparable to the control condition (figure 4.7B) confirming that P1-2AΔ does not have to be co-translationally processed to assemble into pentamers.

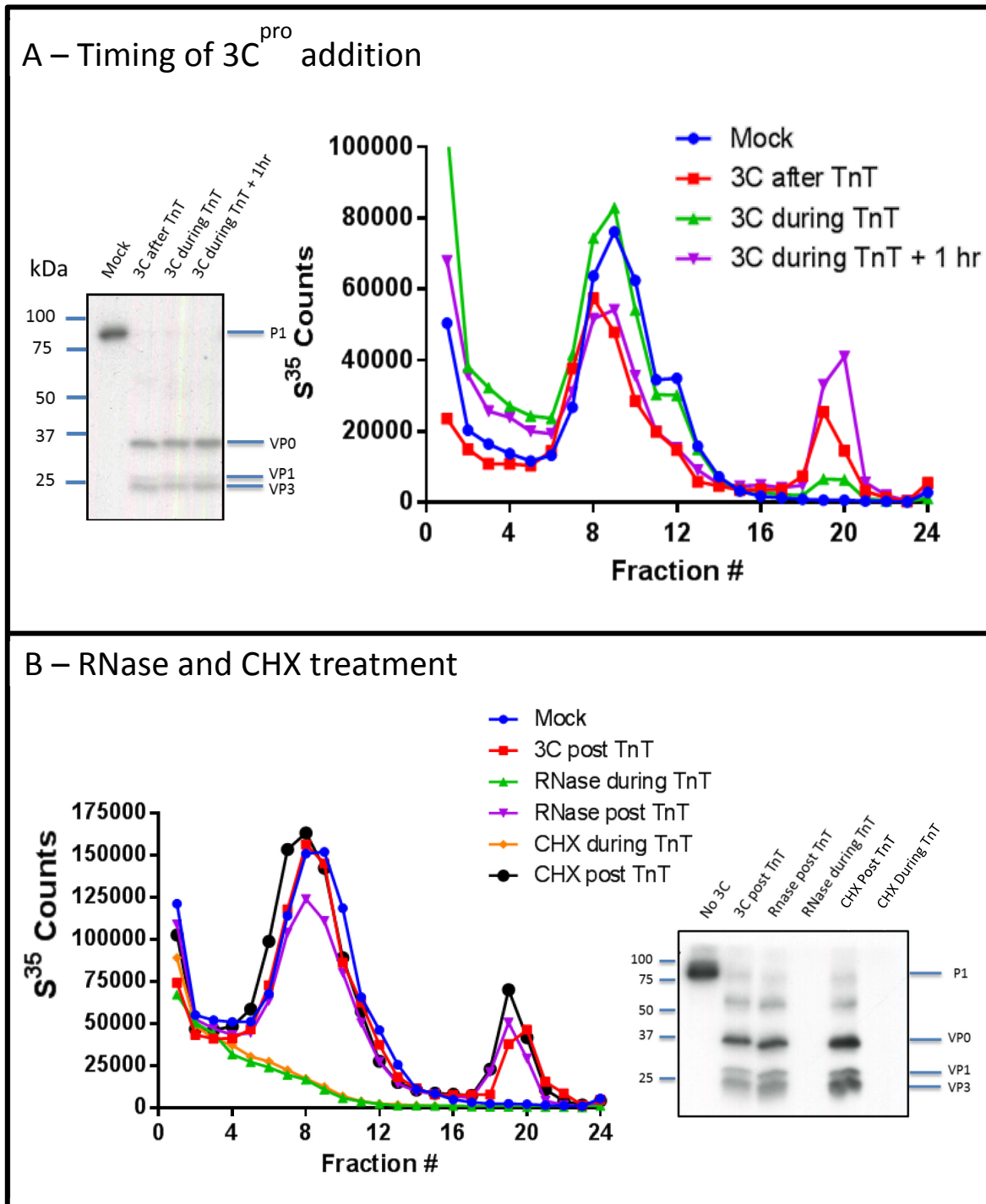


Figure 4.7. P1-2AΔ does not have to be processed co-translationally to assemble into pentamers. *In the top panel;* 3C^{pro} was added to a pO1MP12Awt programmed lysate either during the TnT reaction (green) or after the reaction had finished (red). In addition, the “during” sample was left for a further hour (purple). A mock treated negative control reaction was also performed (blue). *In the bottom panel;* the lysate was either mock treated (blue) or processed with 3C^{pro} following the TnT reaction. The processed reactions either had no further treatment (red), or were treated with 10U/mL RNase A during (green) or after the TnT before processing (purple), or 0.1% CHX during (orange) or after the TnT reaction before processing (black). After treatment, these reactions were analysed by both 12% SDS-PAGE, dialysed, ultracentrifuged on 5-30% sucrose density gradients, fractionated and analysed as described in methods sections 2.8, 2.9 and 2.10.

4.2.3 Myristoylation signal mutants at the N-terminus of FMDV VP4 lack myristoylation, do not affect 3C^{pro} processing of P12AΔ 3C^{pro} but abrogate assembly into pentamers

The N-terminus of P1-2A is co-translationally modified by the addition of the fatty acid myristic acid in a process known as myristoylation (section 1.5.6). In PV, myristoylated pentamers are preferentially selected for inclusion into more stable higher order structures (Moscufo et al., 1991) and myristoylation is required in order to form infectious virus particles (Marc et al., 1991, Marc et al., 1990), due to a lack of pentamer assembly (Ansardi et al., 1992). However, Ansardi et al. (1992) also showed that when recombinant unmyristoylated PV P1 moieties are complemented with a wt poliovirus infection, higher order complexes are formed that travel through a sucrose cushion. Recombinant VP2 is also present in these samples suggesting that the unmyristoylated precursors can undergo the maturation cleavage normally seen in viruses. This suggests that when myristoylated P1 precursors are provided in excess, unmyristoylated material can enter the assembly pathway but evidence of this material in viruses from sucrose gradients is lacking.

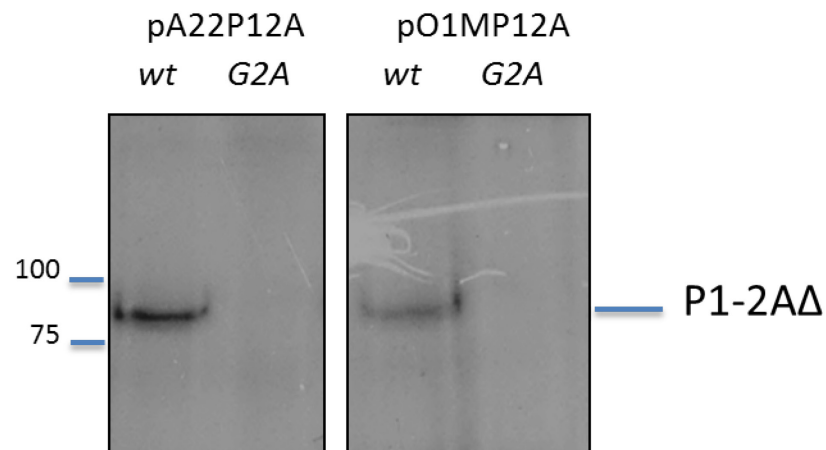
The removal of the myristoylation signal from FMDV P1-2A has been shown to prevent the assembly of recombinant empty capsids (Abrams et al., 1995). Despite the structural differences observed that affect the interactions of myristyl-VP4 in pentamers of enteroviruses and FMDV, Goodwin et al (2009), found that cleaved, unmyristoylated, recombinant FMDV P1-2A could assemble into higher order structures, but that these sedimented at faster rates than expected. An aberrant and unstable 55S assembly product has also been observed in PV mutants which lacked key myristate interacting residues in the N-terminus of VP3 (Moscufo and Chow, 1992). The reasons for these unusual assembly products in these examples are unknown.

To investigate whether myristoylation is required for the process of pentamer assembly in FMDV, P1-2AΔ myristoylation signal mutants were generated (as described in chapter 3). Initially we wanted to confirm that the G2A substitution that we had made would prevent the myristoylation of P1-2AΔ. To do this, a TnT assay was programmed with either pA22P12Awt, pA22P12AG2A,

pO1MP12Awt or pO1MP12AG2A. Instead of radiolabelling with ^{35}S -methionine, we added unlabelled methionine to the reaction and instead labelled with ^3H -myristic acid. The samples were analysed by SDS-PAGE and fluorography (Figure 4.8A). A band of the expected size for P1-2AΔ (approx. 88kDa) was observed in the *wt* samples but was not seen in the myristoylation signal mutants indicating that this substitution is effective at preventing myristoylation.

To further characterise these P1-2AΔ mutants, we assessed their ability to be processed by 3C^{pro}. There is conflicting evidence from the literature that suggests that a lack of myristoylation affects processing, with some authors suggesting that a lack of myristoylation causes less efficient processing (Abrams et al., 1995, Krausslich et al., 1990, Marc et al., 1989), contrasting with others who have found that processing occurs as efficiently (Ansardi et al., 1992, Goodwin et al., 2007). We programmed TnT lysates with the same plasmids as in figure 4.8A, but the reactions were labelled with ^{35}S -methionine. Upon completion of the TnT reaction, the lysates were split into two with half processed by 3C^{pro}, and the other half mock treated for one hour, before the samples were analysed by SDS-PAGE and fluorography (figure 4.8B). In all of the untreated samples, a band of the expected size for P1-2AΔ was observed which demonstrated that although P1-2AΔ with the myristoylation signal mutant was not able to be labelled with myristic acid, it was still expressed as expected. Upon processing with 3C^{pro} the myristoylation signal mutants were cleaved to the mature capsid proteins VP0, VP3 and VP1 that migrated to the same position on the gel as the *wt* samples. A difference that was noted between the samples was that the proteins in wild-type samples gave a slightly weaker signal on the gel than the myristoylation mutants.

A – G2A mutation mutant myristoylation phenotype



B – TnT reaction performed in the presence of ^{35}S -methionine

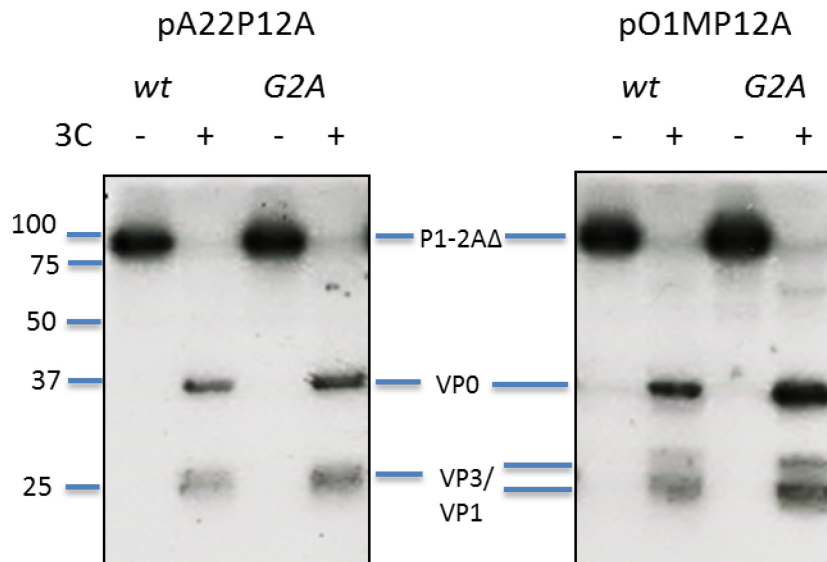


Figure 4.8. Myristoylation of P1-2AΔ can be prevented but does affect P1-2AΔ processing. *In panel A:* ^3H -myristic acid was added to a TnT reaction programmed with either pA22P12Awt, pA22P12Amyr, pO1MP12Awt or pO1MP12Amyr. *In panel B:* These plasmids were also programmed into conventional ^{35}S -methionine labelled reactions, subsequently split in two with one half processed with 3C^{pro} and the other half mock treated. The reactions were analysed by 12% SDS-PAGE and fluorography as described in methods section 4.9 and 4.10.

The sedimentation of these samples were also analysed by SDG. The gradient profile analysis of these samples is initially presented to express each gradient fraction as a percentage of the total counts on the gradient. This is to allow the comparison between the gradient profiles of the *wt* samples and the myristoylation signal mutants. The raw counts will be presented and described afterwards due to interesting observations when analysing by this method. In both the *wt* A22 (figure 4.9 top graph) and O1M (figure 4.9 bottom graph) samples, a peak representing P1-2AΔ was present in all samples, and pentamers were also present in the samples processed by 3C^{pro}. In contrast, no pentamers were present in the myristoylation mutant samples when processed, suggesting that myristoylation was required for pentamer formation. When the raw counts of these gradient fractions were plotted on graphs, it was evident that in both the A22 (figure 4.10 top graph) and O1M (figure 4.10 bottom graph) samples, the *wt* samples had far less ³⁵S counts than the myristoylation signal mutant samples which was a result of a P1-2AΔ peak that was approximately four-times smaller. This trend was evident in both sets of samples and was also present in additional experiments with these samples (data not shown). Whether this is as a result of higher levels of transcription and translation or an effect of the experimental process is unknown but the reasons for, and implications of this will be speculated upon in the discussion to this chapter.

4.2.4 The FMDV P1-2AΔ 3C^{pro} cleavage sites VP0/3 and VP3/1 can be cleaved independently but mutants lacking either cleavage junction cannot form pentamers

It is generally accepted, that processing of P1 by 3C^{pro} is required for EC and virion morphogenesis to proceed (Arnold et al. (1987), and reviewed in Ansardi (1996) and Putnak and Phillips (1981b)). This is supported by the crystal structures of a number of picornavirus virions (Hogle et al., 1985, Rossmann et al., 1985, Luo et al., 1987, Acharya et al., 1989, Fry and Stuart, 2010) and ECs (Basavappa et al., 1994, Curry et al., 1997) in which the termini of the capsid proteins are separated in their structure. This cleavage is thought to be required in order for slight structural changes to occur that allow inter-protomer interactions in the stabilisation of pentamers (Ansardi et al., 1996).

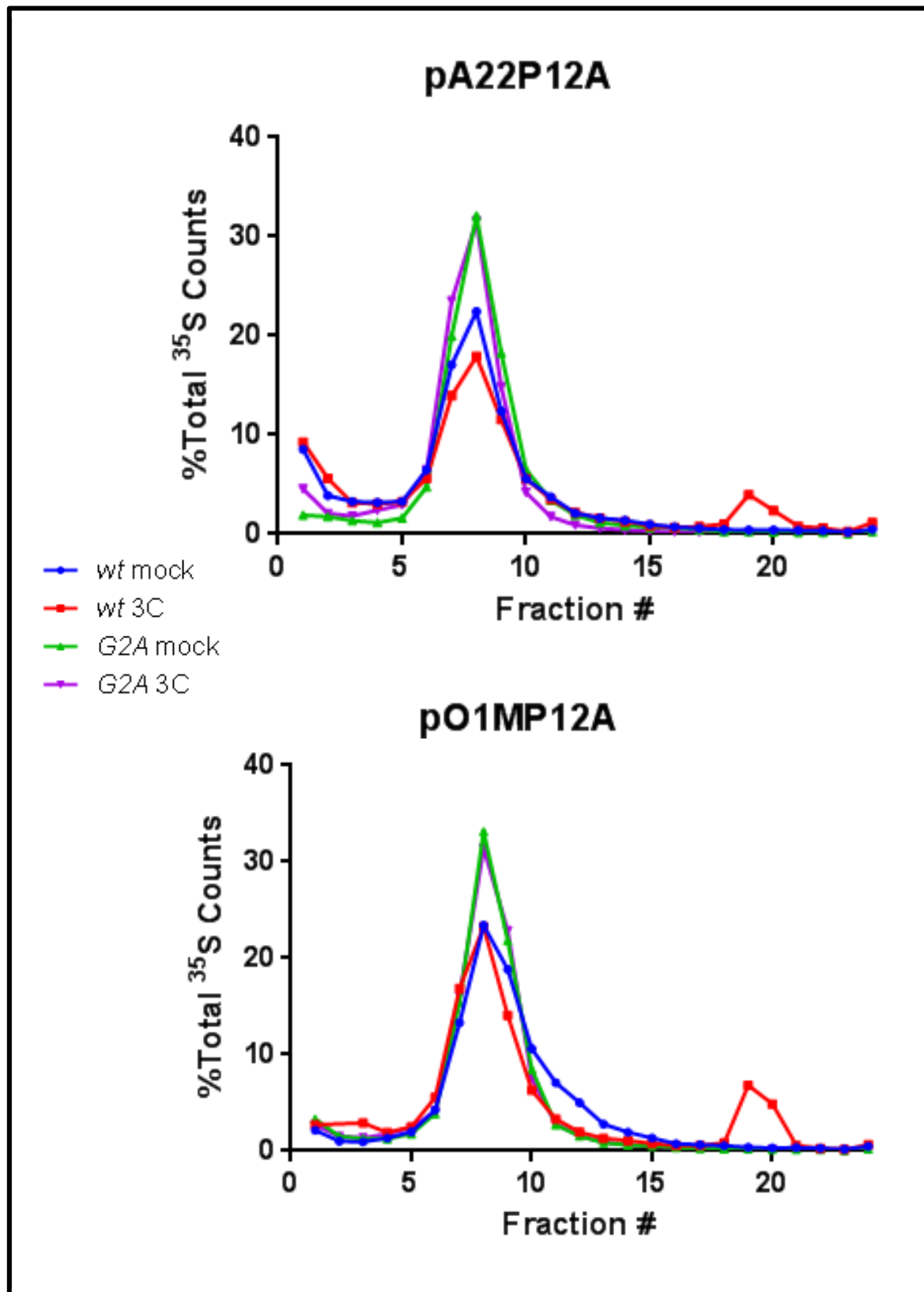


Figure 4.9. Myristoylation of P1-2AΔ is required for pentamer formation. Samples from figure 4.8B containing processed or unprocessed lysates programmed with pA22P12Awt, pA22P12AG2A (top graph), pO1MP12Awt or pO1MP12AG2A (bottom graph) were dialysed and centrifuged through 5-30% SDGs, fractionated and analysed (as described in methods section 2.8). Scintillation counts for each fraction were normalised by expressing them as a percentage of the total signal for that gradient.

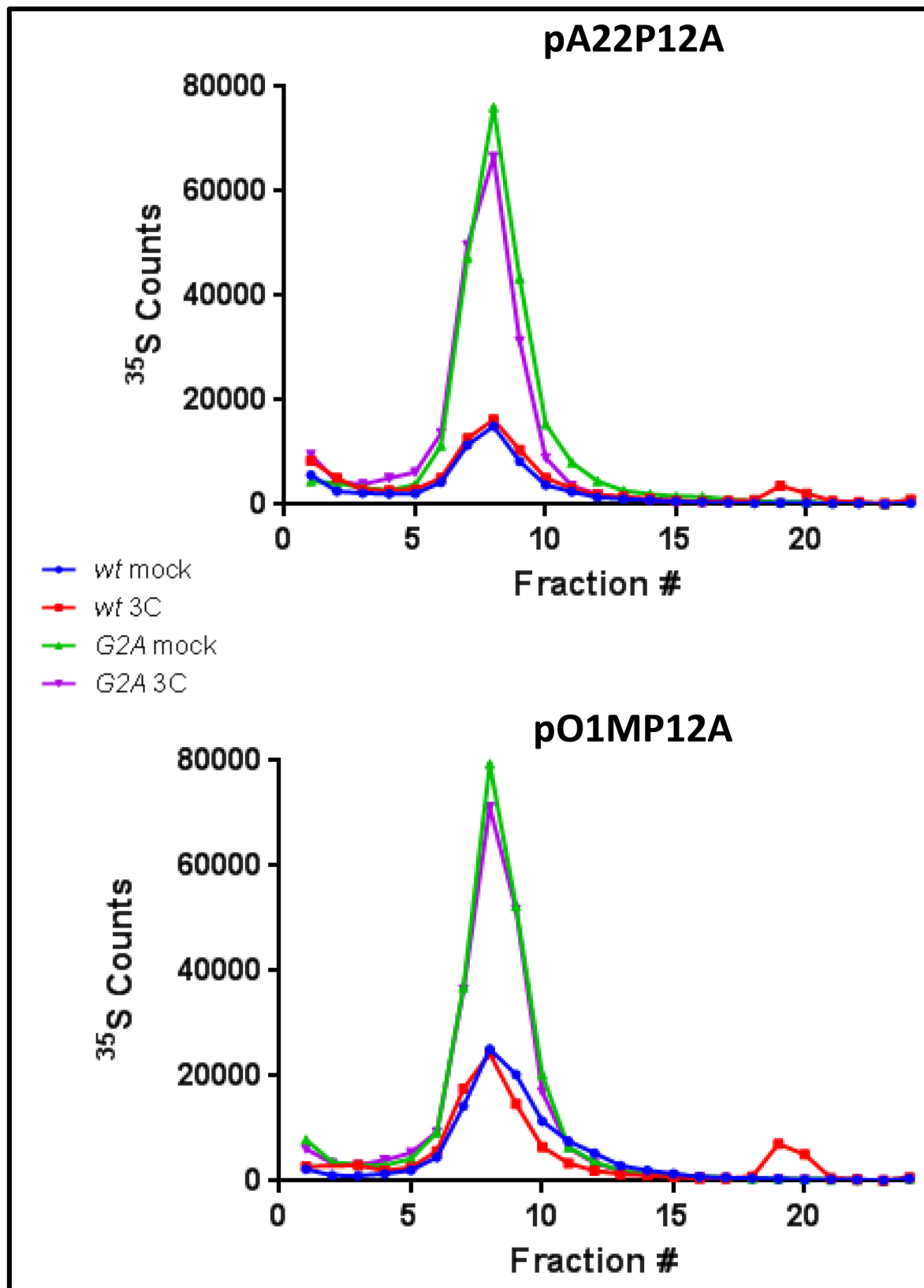


Figure 4.10. Un-myristoylated P1-2AΔ is expressed or retained to a much higher level during expression and assembly assays. The raw scintillation counts of the SDG samples from figure 4.9 are plotted here with the samples from the pA22P12A programmed lysate shown in the top graph and the pO1MP12A shown in the bottom graph.

A vast pool of evidence suggests that assembly of pentamers requires processed P1, however, there have been several reports suggesting that HAV and EMCV can assemble 13.4S and 14S pentamers without 3C^{pro} processing (Borovec and Anderson, 1993, McGregor and Rueckert, 1977). Cleavage at the VP3/1 junction has been shown to be required for the assembly of EMCV pentamers (Parks and Palmenberg, 1987).

In PV, the precursor protein 3CD is more efficient at cleaving P1 than 3C alone (Ypma-Wong et al., 1988, Jore et al., 1988). Indeed, PV 3C^{pro} has been shown to only cleave the VP3/1 junction in the presence of 3D or when at non-physiologically high concentrations (Nicklin et al., 1988, Ypma-Wong et al., 1988). 3D may also enhance FMDV 3C^{pro} processing at the VP3/VP1 and VP1/2A junctions in FMDV (Ryan et al., 1989) but processing can occur efficiently in systems lacking 3D^{pol} (e.g. Abrams et al., 1995). It also appears that the P1-2A structure is important for its processing. C-terminal truncations of FMDV VP1, that still produce 163 of the wt 211 aas cannot be processed at the VP3/VP1 junction and the VP2/VP3 junction is processed less efficiently (Ryan et al., 1989). The analogous truncation in PV P1 renders both of these sites un-cleavable (Ypma-Wong and Semler, 1987). This suggests that part of the structure of the approximately 50 aa C-terminus of VP1 is required for correct processing of the upstream protease sites. Processing of the VP1/2A junction in FMDV serotype O viruses can be influenced by mutation to a VP1 residue (E83K) both distant in amino acid sequence and distant in 3D structure to the cleavage junction in the capsid (Gullberg et al., 2014). This is also true of the mutation VP1 M54I which inhibits VP3/1 cleavage but is distant from it in the capsid structure (Escarmis et al., 2009). These examples suggest that the structure of the unprocessed P1-2A precursor undergoes significant rearrangement in order to assemble into pentamers which further reinforces the idea that obtaining its structure may provide interesting insights into the assembly process (chapter 3) and for the possibility of chaperone involvement in its re-folding (chapter 5).

To understand the contribution of the individual P1-2A processing sites in the assembly of pentamers, plasmids were designed to individually knock-out the VP3/1 and VP0/3 cleavage sites in the P1 sequences of A22 Iraq and O1 Manisa (chapter 3). The TnT lysates were programmed with these plasmids before they were split into two aliquots with one processed by 3C^{pro} and the other mock treated. Samples of these reactions were separated by SDS-PAGE and visualised by fluorography. The mock treated *wt* and cleavage site *ko* samples gave bands of the correct size for P1-2AΔ (~88kDa) (figure 4.11A). Upon processing with 3C^{pro} the *wt* samples had three bands (VP0, VP1 and VP3; largest to smallest). When the VP0/VP3 *ko* mutant was processed, a predominant band was seen with apparent molecular weight of 60kDa and a second band was seen for VP1. This mutant was expected not to cleave at the VP0/VP3 junction and as such, the 60kDa this band was in the expected position for uncleaved VP0-VP3. A very faint band in the expected position for VP0 was also present in this sample suggesting a small amount of residual cleavage at this substituted recognition site had occurred. Given that VP0 gives the strongest signal in these experiments due to its higher methionine content (relative to the other mature capsid proteins), it was thought that this very low level of cleavage at the VP0/3 junction containing the substitution would not be sufficient to alter the conclusions of these experiments. Upon cleavage of the pA22P12A_3/1 and pO1MP12A_3/1 mutants, P1-2AΔ was shown to be processed into two proteins, one of which was in the correct expected for VP0 and the other with apparent molecular weight of approximately 55kDa. This second protein was in the expected position for uncleaved VP3/1.

These samples were subsequently separated on SDGs to analyse the sedimentation of the proteins in the sample. The unprocessed P1-2AΔ samples were seen to sediment to the position expected of 5S on the gradient (figure 4.12 blue lines, green and orange lines). Upon processing a peak for pentamers was also seen in the gradient of the *wt* sample (figure 4.12 red lines). Upon processing, neither the A22 Iraq nor O1 Manisa P1-2AΔ sequences containing the VP3/1 substitution were able to assemble into pentamers (figure 4.12 purple lines). The O1 Manisa VP0/3 *ko* mutant also had no

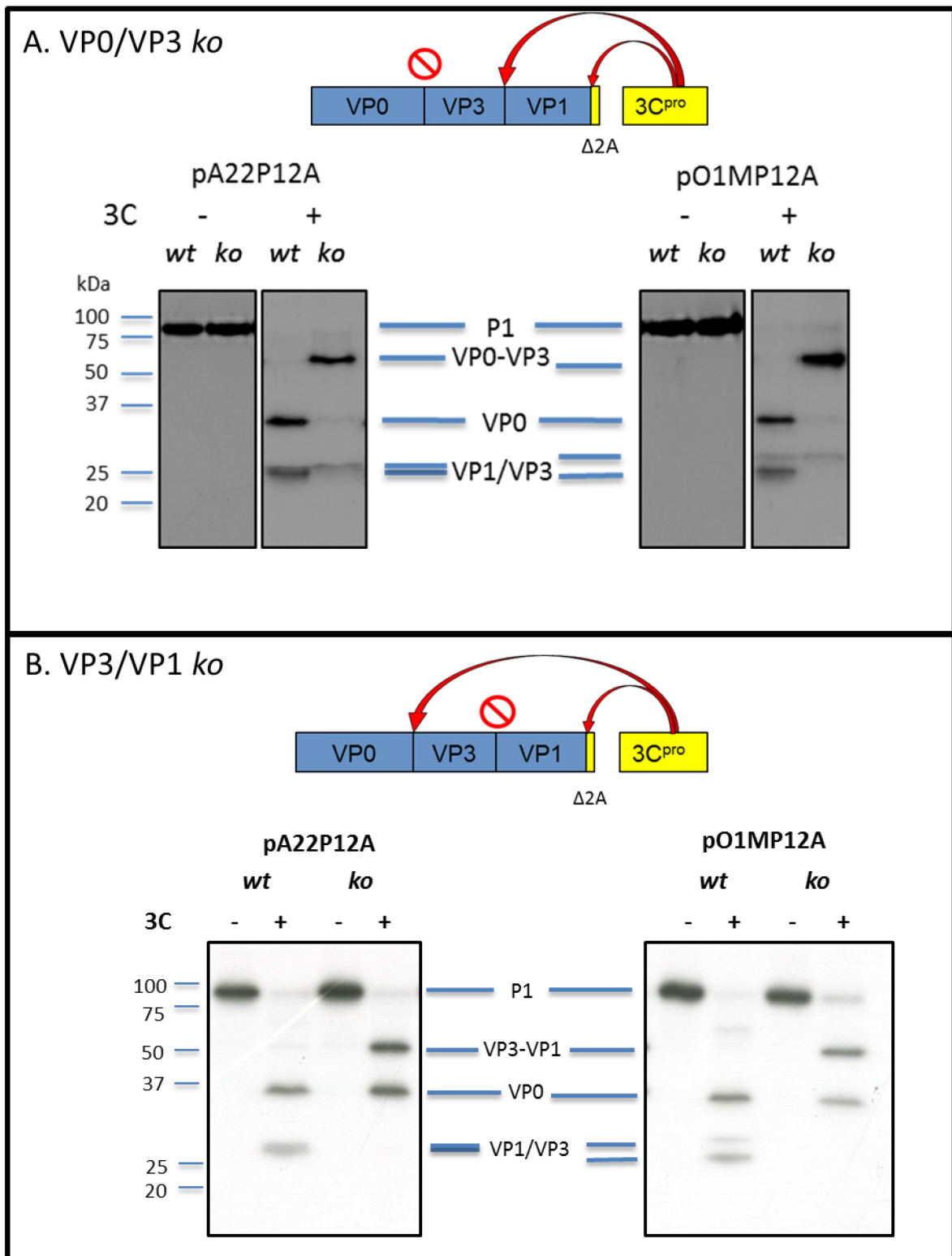


Figure 4.11. Mutations at the VP2/VP3 and VP0/VP1 3C^{pro} cleavage sites prevent their cleavage. TnT lysates were programmed with either pA22P12Awt, pA22P12A_0/3, pO1MP12Awt, or pO1MP12A_0/3 (panel A) or pA22P12Awt, pA22P12A_3/1, pO1MP12Awt or pO1MP12A_3/1 (panel B). In each case, the reactions were divided into two halves with one processed with 3C^{pro} and the other mock treated. The reactions were analysed by 12% SDS-PAGE and fluorography (described in methods sections 2.9 and 2.10). Where protein names are separated with a hyphen (-), they are uncleaved, separation with a forward stroke (/) indicates mature cleaved proteins.

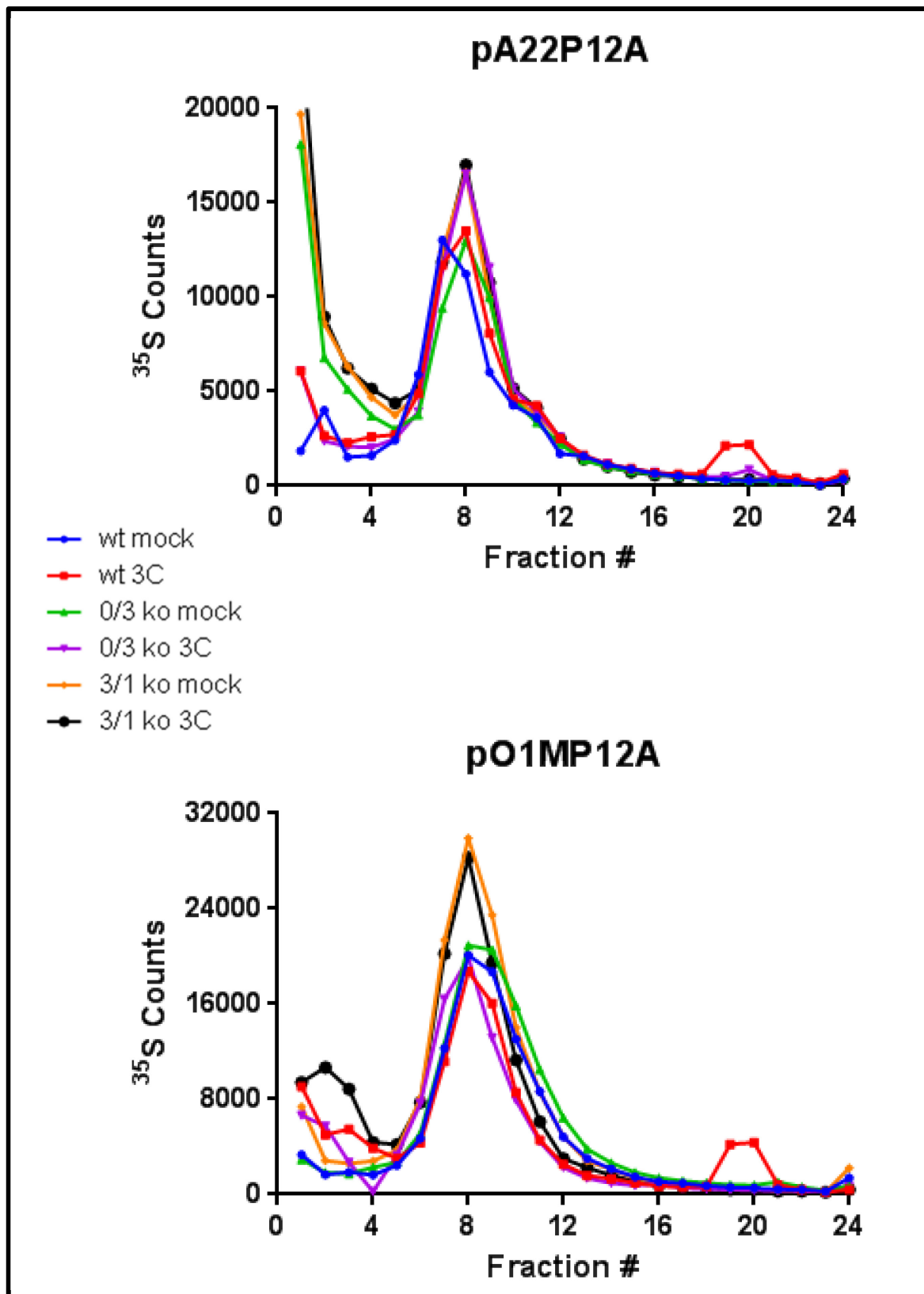


Figure 4.12. Preventing 3C^{pro} processing of either the VP0/3 and VP3/1 cleavage sites in P1-2AΔ prevents the assembly of pentamers. Samples from figure 4.11 containing processed or unprocessed lysates programmed with pA22P12Awt, pA22P12A_0/3 or pA22P12A_3/1 (top graph) or pO1MP12Awt, pO1MP12A_0/3, pO1MP12A_3/1 (bottom graph) were dialysed and centrifuged through 5-30% SDGs, fractionated and analysed as described in methods section 4.8. The raw scintillation counts are plotted.

visible pentamer peak (figure 4.12, black lines lower graph) but the corresponding A22 Iraq version of this mutant gave a very low signal in the pentamer position (figure 4.12, black lines upper graph). For the fractions in the pA22P12A gradient that represented the position for pentamers (fractions 18-21), the area under the curve has been calculated (table 4.1) and shows that the pentamer peak in the VP0/3 *ko* sample is over five times smaller than the pentamer peak in the *wt* sample.

From these experiments it was seen that substitutions could be made at both the VP0/3 and VP3/1 cleavage junctions in P1-2AΔ to prevent cleavage by 3C^{pro} or make the cleavage far less efficient. The processing profiles also revealed that the junctions could be processed independently of each-other indicating, at least in these cell-free processing assays that both junctions were available to the active site of 3C^{pro} in the uncleaved structure. Substitutions to prevent processing also largely prevented pentamer assembly. The exception to this was in the case of the A22 VP0/3 cleavage in that a small amount of cleavage had taken place at the site containing the substitution and a small pentamer peak was visible on the gradient for this sample. The reasons for, and implications of this are debated in the discussion for this chapter.

4.3 Discussion

This chapter has described the development and application of an assay to analyse the early stages of the FMDV assembly process. We have used the multimerisation of the FMDV structural subunit into pentameric capsid precursors as a measure of successful assembly and investigated the underlying control points required for it. The results presented here show that P1-2AΔ can be expressed in RRLs, the protein has the correct sedimentation of 5S and, upon processing by 3C^{pro}, can assemble into structures with the same protein composition and sedimentation as virus-derived pentamers. The expression, processing and assembly assays were performed in RRLs and the outputs were analysed using SDS-PAGE and SDGs. RRLs are known to provide an authentic environment for the processing and assembly of picornavirus proteins (Boege et al., 1986, Grubman

Table 4.1. Comparison of pentamer peaks from the pA22P12A samples figure 4.12. The scintillation counts from the gradients in figure 4.12 were analysed in order to compare the sizes of pentamer peaks in the processed *wt* and *VP0/3 ko* samples. The positions deemed to contain the peak and shoulder of the pentamer were fractions 18-21. The mean was calculated for the background in these positions (i.e. the samples where there had been no pentamer formation: *wt* mock, *VP0/3 ko* mock and *VP3/1 ko* both mock and $3C^{pro}$ treated), removed from the average counts in the pentamer containing samples before the ratio of the two sets of counts were compared.

	<i>Wt</i>		<i>VP0/3 ko</i>		<i>VP3/1 ko</i>	
Fraction number	Mock	3C	Mock	3C	Mock	3C
18	396	626	387	452	416	431
19	311	2122	315	507	349	379
20	291	2188	272	855	307	343
21	310	582	262	303	288	312
Average counts	327	1379.5	309	529.25	340	366.25
Average counts for samples without pentamers	$(327 + 309 + 340 + 366.25) / 4 = 335.563$					
Difference in size of pentamer <i>wt</i> : <i>VP0/3 ko</i> (Background adjusted)	$(1379.5 - 335.563) / (529.25 - 335.563) = 5.3898$					

and Baxt, 1982, Grubman et al., 1985, Shih et al., 1979, Palmenberg, 1982). RRLs have proven a useful tool for the analysis of FMDV assembly, validated by their ability to accurately replicate the assembly pathway seen in viral infection. For example, in infection, the L^{pro} is autocatalytically removed from the polyprotein (Strebel and Beck, 1986) to reveal the N-terminal glycine of P1 which is myristoylated by host N-myristoyl transferase. The L^{pro} sequence was omitted from our expression constructs because it has been shown not to be required for assembly and can be detrimental to levels of recombinant protein expression. The start codon for translation initiation of the polyprotein is contained within the L^{pro} sequence and so a start codon was inserted into the DNA sequence at the 5' end of the P1 sequence which resembles the arrangement in poliovirus. In mammals, the enzymes N-aminopeptidase and N-myristoyl transferase are responsible for the removal of this initiating methionine and addition of myristic acid to the N-terminal glycine residue of P1 respectively (Towler et al., 1987, Wilcox et al., 1987, Dorner et al., 1982). It was shown that the P1-2AΔ that was

expressed in the RRL is myristoylated (figure 4.8A), demonstrating that both of these enzymes are present and active on the correct substrate.

4.3.1 Development of the processing and assembly assay

The first stage in the development of the assembly assay was to identify conditions in which 3C^{pro} could process all of the P1 in the TnT assay in one hour. Uncleaved P1 in EMCV has been established to have a half-life of 6.7 minutes before being processed into mature proteins (Butterworth and Rueckert, 1972). We assumed that FMDV would have roughly similar processing kinetics to EMCV and therefore chose the timing of the assay to reflect this. According to these figures (which are a rough estimate due to the inherent differences between a virus infection and the *in vitro* system), 99% of P1 in a natural infection would be processed after 47 minutes. Conditions were chosen for the assay that resembled these figures and so it was decided to use an amount of 3C^{pro} that could process both internal cleavage sites in P1-2AΔ in a one hour assay at 37°C. In PV, pentamer assembly has also been identified to occur rapidly (within 7-10 minutes of the addition of radiolabel to PV infected cells (Putnak and Phillips, 1981a)), and so it was expected that the one hour assay could achieve both the processing and assembly of P1-2AΔ. These processing conditions were used in all of the processing and assembly assays and proved to be conditions at which pentamer formation could always be detected. The levels of pentamer formation did differ slightly between assays but were fairly consistent between samples run at the same time and the trends that were seen were consistent for repeats of the same experiment. Pentamers have been reported to readily adhere to surfaces (Putnak and Phillips, 1981a) and perhaps this was the reason for the slight variation observed. All attempts were made to standardise the experimental process but these slight differences could be due to small changes in the experimental procedure such as changes in the volume of lysate between samples, differences between experiments in the efficiency of the blocking agent and the temperatures used to prevent proteins adsorbing to plastic surfaces and/or slight variations in the output of the TnT reaction.

Increasing the levels of assembly were also investigated in time-course experiments by increasing the duration of the assembly reaction to determine if this would produce increased levels of pentamer assembly. By performing parallel gradients suitable for detecting either pentamers or capsids, attempts were made to detect empty capsid formation. We observed pentamer formation was optimal after one hour of assembly reaction but that by 4 hours the pentamer peak had disappeared and a small peak was detectable in approximately the expected position for ECs on the gradient. However the four hour sample also had a larger 5S peak suggesting that the loss of pentamers may have been their disassembly. These findings were not reproducible and so were omitted from the data in this chapter, but it is possible that capsids are being formed in the assay but are on the limit of detection and require a higher concentration of pentameric subunits in order to assemble (Li et al., 2012, Rombaut et al., 1991, Putnak and Phillips, 1981a). This contrasts to findings by Palmenberg and colleagues (1982) who found that EMCV pentamer assembly increased by increasing the incubation periods up to 15 hours. The reasons why only a small proportion of cleaved P1 was competent for assembly into pentamers are not known but could also be due to the requirement for a higher concentration of subunits, or a limitation of the cell-free assay.

SDGs were used to separate assembly components, according to their sedimentation. The sedimentation coefficients of our unknown proteins were extrapolated using molecular weight markers with known sedimentation coefficients from literature (reviewed in Marks, 2001). This facilitated the identification of P1-2AΔ, cleaved P1 and pentamers on our assembly gradients. The pentamers that were assembled during our experiments migrated a couple of fractions further into the gradient than the 12S sedimentation marker (dissociated pentamers obtained from acid treated inactivated FMDV comprised of VP2, VP3 and VP1) and were in a position more similar to 14S poliovirus pentamers. It was thought that perhaps FMDV assembly and disassembly pentamers could have a different sedimentation properties and this theory was developed further in chapter 6.

To remove background counts from the assembly reactions, it was necessary to perform a clean-up step prior to SDG analysis. Pierce polyacrylamide spin columns and mini dialysis cassettes were evaluated for their ability to remove background counts from the sample and to retain the proteins of interest. It was found that the dialysis cassettes gave a lower background signal at the top of the gradient possibly due to the more stringent conditions for depleting the lysates of unincorporated radiolabel that are afforded during the dialysis compared to the spin columns. The dialysis cassettes also retained more of each of the proteins of interest than the spin columns showing larger peaks for both cleaved and uncleaved P1-2AΔ and pentamers. The spin columns possibly suffer in this regard due to their larger surface area for proteins to adsorb to, in comparison to the dialysis cassettes. Interestingly, when analysing the P1-2AΔ myristoylation mutants, far more unmyristoylated P1-2AΔ was retained during dialysis than the corresponding myristoylated sample suggesting that the myristate moiety also increases the adsorption of P1-2AΔ to the plastic dialysis cassette. Despite being a much more time-consuming technique, dialysis was chosen as the method by which we would remove unincorporated ³⁵S-methionine from our samples prior to SDG analysis.

4.3.2 Requirement for a sufficient P1-2AΔ concentration and a lysate component for pentamer assembly

Due to slight differences in the quantities of protein between samples after dialysis, attempts were made to standardise the P1-2AΔ reagent by performing dialysis before processing. It was reasoned that the difference in P1-2AΔ retention might be due to different adsorption characteristics in cleaved and uncleaved P1-2AΔ. This may be due to the freeing of the protein termini in the cleaved form allowing a greater degree of protein rearrangement and amino acids with less adsorptive characteristics that could interact with the plastic surfaces. Dialysis of P1-2AΔ prior to processing experiments resulted in less P1-2AΔ being retained after dialysis, but, more importantly also completely prevented the assembly of pentamers. The reasons why pentamer assembly is prevented if P1-2AΔ is dialysed before processing could be explained by the requirement for a certain concentration of P1-2AΔ (demonstrated in subsequent experiments), that goes below a threshold

value due to P1-2AΔ loss during dialysis. A second hypothesis is that a molecule required for assembly is lost during dialysis through the 10kDA MWCO membrane. If FMDV assembly requires a chaperone (Geller et al., 2007, Tsou et al., 2013, Wang et al., 2013) or reduced glutathione (Ma et al., 2014a, Thibaut et al., 2014) similar to that which has been described for the assembly of other picornaviruses, then the loss of ATP during dialysis may have been the component responsible for preventing pentamer assembly and this is explored further in chapter 5. Additionally Li and colleagues, (2012), demonstrated that ionic conditions in the assembly buffer are also important to facilitate assembly, although this is thought to be controlled for by providing physiological levels of sodium, potassium and divalent cations in the dialysis exchange buffers. Evidence to refute the loss of a small molecule component during dialysis is provided by the work of Goodwin et., al. (2009) and Guo et., al. (2013) who showed that FMDV P1 and P1-2A are still able to assemble when processed after purification into a standard buffer.

It was noticed when performing the SDG analysis on processed samples, that the largest peak in all of the samples was that of P1-2AΔ or cleaved P1 as opposed to pentamers. It is unclear whether during virus infection, a surplus of unassembled P1 remains in the cells as most studies do not look for this specifically, although P1 can be still be observed in infected cells after the assembly of some virions (Putnak and Phillips, 1981a). A hypothesis suggesting that P1-2AΔ produced during the TnT reaction was not competent for assembly if not processed by 3C^{pro} immediately was put forward. To test this, 3C^{pro} was added either from the start of the TnT or after completion, and the TnT assay was also stopped with either RNase or CHX prior to processing. These data suggested that P1-2AΔ did not have to be cleaved co-translationally to assemble into pentamers as pentamer formation was equivalent in all conditions. Given the requirement for a threshold concentration of P1-2AΔ to facilitate assembly it was reasoned that P1-2AΔ at sufficient concentrations could assemble into pentamers but when the pool of remaining P1-2AΔ had been depleted sufficiently, the remainder was at too low a concentration to assemble. An alternative hypothesis suggested that the pentamer

peak was not representative of the total amount of pentamers in the sample as they too have a tendency to adsorb to surfaces (Putnak and Phillips, 1981a). To confirm these suggestions a future experiment might look to concentrate and quantify P1-2AΔ material prior to processing to observe if the efficiency of assembly is improved and to determine what the concentration threshold required for assembly is, although, perhaps given that only small amounts of protein are produced in the RRL system, this type of experiment may be better performed with P1-2AΔ produced in the vaccinia virus expression system. It was also notable that including 3C^{pro} during the assembly reaction did not seem to have a detrimental effect on the levels of expression, as has been demonstrated in other systems, due to the ability of 3C^{pro} to disable host translational machinery (Polacek et al., 2013, Porta et al., 2013b). This has been shown to be because the rabbit eIF4G protein is not cleaved by 3C^{pro} (Strong and Belsham, 2004) (section 1.5.4), possibly due to reduced specificity for the cleavage junction e.g. the reduced specificity of PV 3CD for the VP2/3 site due to an unfavourable proline in the P-4 position relative to the scissile bond (section 1.5.5) (Krausslich et al., 1990, Nicklin et al., 1988).

4.3.3 P1-2AΔ myristoylation is required for FMDV pentamer assembly

Myristoylation of the N-terminus of P1 occurs co-translationally and is required for the assembly of FMDV capsids (Abrams et al., 1995) and PV pentamers (Ansardi et al., 1992). The precise role of the myristate moiety in assembly is better understood in PV because the crystal structure reveals hydrogen bonding between myristic acid and capsid proteins at the 5-fold axis of symmetry suggesting that it provides structural support to the virus (Chow et al., 1987, Hogle et al., 1985). The myristate groups are in the same location but are disordered in the FMDV structure suggesting that they could be playing a slightly different role (Acharya et al., 1989). Where recombinant systems have been used to analyse the assembly of processed P1 that is unmyristoylated, aberrant assembly products have also been observed at 17S in FMDV (Goodwin et al., 2009) and 55S in PV (Moscufo and Chow, 1992). There have also been conflicting reports of the role for the myristic acid group in

proteolytic processing of P1 or P1-2A (Krausslich et al., 1990, Marc et al., 1989, Goodwin, 2007, Abrams et al., 1995, Ansardi et al., 1992). The effect of preventing myristoylation on the processing of P1-2AΔ by 3C^{pro} and on the assembly of pentamers in FMDV was investigated. Recombinant unmyristoylated P1-2AΔ was successfully expressed in RRLs by the mutation of the sequence encoding glycine to alanine (G2A) at the P1 N-terminus (Krausslich et al., 1990, Marc et al., 1989, Goodwin et al., 2009). The processing of unmyristoylated P1-2AΔ was investigated and it was found that P1-2AΔ was processed with the same efficiency as the *wt* myristoylated P1-2AΔ proteins. As discussed above, this finding splits the opinion of previous studies but in our RRL assay, using an amount of 3C^{pro} that is at a low enough concentration not to be detrimental to expression in the TnT, it was clear that processing was not affected. The theory given for processing being less efficient in unmyristoylated poliovirus P1 is of a required interaction between the N-terminus of VP0 and 3CD, but, whether this is true of FMDV is not known. We next analysed these samples on SDGs and showed that unmyristoylated P1-2AΔ did not assemble into pentamers, or assembly products with an altered sedimentation coefficient. This suggests that while structurally there are differences in the positioning and interactions of the myristate moiety in PV and FMDV, it is probably performing a similar function in stabilising protomer-protomer interactions in the assembly of pentamers. The reasons for the aberrant assembly products seen previously by other authors is not known, but it is suggested by Goodwin et al., (2009), that an unmyristoylated pentameric structure forms a closed “umbrella” shape that increases its sedimentation through sucrose. An interesting finding from this experiment was that the unmyristoylated P1-2AΔ was either expressed or was retained to a much higher level than myristoylated P1-2AΔ. Myristoylation has been shown by others to reduce the yield of recombinant VP0 (Brauntingam et al., 1993) but whether this effect is a result of an increased capacity of the ribosome to translate protein in the absence of co-translational myristoylation, or an effect of increased adsorption to plastic surfaces caused by the binding of myristate is not known.

4.3.4 Processing of P1-2AΔ by 3C^{pro} is required for FMDV pentamer assembly

Evidence from the crystal structures of different picornaviruses demonstrates that both the VP0/3 and VP3/1 cleavage sites in P1 have to be processed in order for assembly to occur (Acharya et al., 1989, Hogle et al., 1985, Luo et al., 1987, Rossmann et al., 1985). This is because the N-terminus of VP3 is required in the formation of the β -annulus at the five-fold axis of symmetry in the virus structure that is some distance from the C-terminus of VP0 (VP2). While it is assumed that cleavage of P1 is required to initiate the assembly cascade only a few studies have examined these events in detail. In EMCV, lack of VP3/1 processing prevented pentamer assembly (Parks and Palmenberg, 1987) and 14S pentamers only contain fully processed P1 (Palmenberg, 1982). PV may require a sequential cleavage of the two sites to achieve assembly, cleaving firstly at the efficiently cleaved VP3/1 site and secondly at the less efficiently cleaved VP0/3 site (Reynolds et al., 1992). A mutation at the VP0/3 site to increase its efficiency is lethal to the virus (Blair et al., 1993) suggesting that the staggered cleavage of these sites is a mechanism used by the virus in order to achieve structural rearrangements required for assembly. FMDV also performs processing in a staggered manner; at VP3/1, then VP0/3, then VP1/2A (Grubman et al., 1995) and it is clear that the structure of P1-2A is important for this staggering as mutations at sites distant in sequence to the cleavage site can affect P1-2A processing (Gullberg et al., 2014, Ryan et al., 1989). The relationship between the staggered cleavage and a requirement for a molecular chaperone (chapter 5) is hypothesised in the final discussion (chapter 7).

To investigate the effect that the individual cleavage sites have on the ability of FMDV to assemble into pentamers, mutants were made to prevent cleavage at either of these junctions. The substitutions made at the VP3/1 junctions were successful in preventing cleavage at this site, whereas there appeared to be a faint band in the processing assay of the VP0/3 mutants suggesting that although very inefficient, the substitutions made at this site to prevent cleavage, had instead just made it less efficient. The 3C^{pro} of aphthoviruses are able to cleave a wider range of sequences

than the 3C^{pro} of members of the enteroviruses and cardioviruses (Palmenberg, 1990, Birtley et al., 2005, Blom et al., 1996, Castello et al., 2011), and so perhaps the design of our substitutions at this site were too conservative. Nevertheless, the substitution at this site almost completely prevented the formation of pentamers. Interestingly though, a very small pentamer peak was observed in the A-serotype mutant but not the O. This was the only evidence for a difference in the pentamer formation seen between the representative strain from the A and O viruses and could suggest that A22 Iraq can form pentamers more efficiently than O1 Manisa but this will require further work. The small pentamer peak observed in the VP0/3 knockout suggests that a small amount of assembly had occurred. Given the small amount of cleavage compared to the wildtype, this maybe suggests that the concentration dependence for pentamer assembly is not reliant upon cleaved P1-2AΔ but on the total P1-2AΔ concentration. This could also indicate that the VP3/1 junction is the important cleavage to get P1-2AΔ into a state whereby, upon cleavage of the VP0/3 junction, assembly is rapid. This could be investigated with mutants that slow cleavage but don't stop it or by low 3C levels over a longer time period. None of the mutants containing a substitution at the VP3/1 junction were able to form pentamers. These experiments demonstrate that both of the cleavage sites in P1-2AΔ need to be processed in order to assemble pentamers. It also shows that both sites can be independently processed by 3C^{pro} showing that structural rearrangements of P1-2AΔ are not necessary after the "first" cleavage to allow the enzyme to access the second site. FMDV 3C^{pro} does not require 3D^{pol} in order to efficiently process P1-2A, unlike in poliovirus, where 3D^{pol} is thought to provide a substrate recognition function (Blair 1991). This restriction can be overcome, however when using high concentration of 3C^{pro} (Nicklin et al., 1988). Previous discussions in this chapter validate the concentrations of 3C^{pro} that we are using and therefore our findings here.

Chapter 5: Processed capsid precursor requires the chaperone Hsp90 to assemble into pentamers

5.1 Introduction

This chapter describes the investigation of the requirement for Hsp90 in the lifecycle of FMDV and the evidence to demonstrate that the processed capsid precursor is a client protein of Hsp90, and that this interaction is required for the assembly of pentamers.

5.1.1 Picornavirus morphogenesis is more complex than the term “self-assembly” implies

The multimerisation of the picornavirus capsid is often described by the term “self-assembly”, meaning that the viral structural proteins are able to assemble without external factors. This has been demonstrated to be true in the case of empty capsid assembly from pentamers, which have been shown to require nothing more than a sufficient pentamer concentration, the correct ionic strength and physiological temperature of the solution, and time to assemble (Li et al., 2012, Rombaut et al., 1991, Putnak and Phillips, 1981a, Palmenberg, 1982). The biological role of empty capsids is debated, but it is fairly well accepted that pentamers are a biologically relevant intermediate, by interacting with, and encapsidating RNA in the assembly of virions (Verlinden et al., 2000). The process of RNA encapsidation has been demonstrated to require interactions between the viral structural proteins and the viral non-structural protein 2C (Liu et al., 2010), and in some cases the abundant cellular tri-peptide glutathione (Ma et al., 2014a, Thibaut et al., 2014). The ability of the structural sub-unit (P1) to form higher order structures such as pentamers is also no longer seen as a self-assembly event in enteroviruses, with the recent discovery of the requirement for a cellular chaperone for P1 processing (Geller et al., 2007) and capsid assembly (Tsou et al., 2013, Wang et al., 2013). The lifecycle of FMDV has many characteristics in common with the lifecycle of enteroviruses, but a role for a chaperone has not yet been identified and will be investigated in this chapter.

5.1.2 Protein folding and chaperones

As described in chapter 4, pentamer assembly requires the VP0/3 and VP3/1 junctions in P1 to be processed by 3C^{pro} resulting in the separation of the termini between these bonds in its crystal

structure (Acharya et al., 1989). The capsid precursor protein must overcome a significant folding barrier, in its rearrangement after processing, in order to form a structure that is present in the virion. Protein folding is often explained in terms of an energy folding landscape, whereby unfolded proteins “explore” a range of conformations and molecular interactions in the formation of intermediates on the pathway to correct folding. During this “exploration”, interactions between amino acid side chains found in the correctly folded protein are assumed to infer increased stability compared with non-native contacts and allow transition to the next state by reducing the landscape of conformations that the protein can then adopt. This continues until the correct structure is obtained (reviewed in Jahn and Radford, 2005). The folding of small (<100 amino acids), single domain proteins is rapid and the number of misfolded intermediates is few. However, larger proteins such as the capsid precursor can take much longer (Herbst et al., 1997) and have a much larger set of intermediates that can be adopted. Long amino acid chains have an increased tendency to undergo hydrophobic collapse and form compact conformations in which there is a greater free-energy barrier required in order for them to “explore” different conformations (Vendruscolo et al., 2003) which may aid in the formation of the correct secondary and tertiary structures (reviewed in Jahn and Radford, 2005).

Cells have protein quality control mechanisms enabling them to fold, unfold and degrade proteins. One such example of a cellular quality control mechanism is the heat shock proteins (Hsps) which are some of the most abundant molecular chaperones in a cell and are up-regulated in conditions of stress such as heat shock or starvation (Pelham, 1986, Ellis, 1987). Two of the most important Hsp families are Hsp70 and Hsp90 with the number representing the approximate molecular weight of the family member. Members of the Hsp70 and 90 families can fold proteins into their correct conformation, assist in their maturation and trafficking, and help regulate cellular pathways. However, unlike many proteins, molecular chaperones do not show strong specificity for their client proteins and are instead required in the specific folding of a wide range of proteins which includes

exogenous proteins such as recombinant proteins and viral proteins (reviewed in Ellgaard and Helenius, 2003). They tend to bind transiently to hydrophobic amino acid side chains preventing the mis-folding and aggregation of the protein while it finds the correct folding interactions that would have been energetically unfavourable without chaperone binding (reviewed in Hartl et al., 2011).

Hsp70 and 90 have ATPase activity (Lewis and Pelham, (1985), and Obermann et al., (1998) respectively) and go through cycles of conformational rearrangements upon the binding and hydrolysis of ATP at an ATP binding site on the chaperone. Client proteins bound to the chaperone are released upon ATP hydrolysis, after which, in its ADP bound state, the chaperone has a high affinity for substrate binding. ADP is replaced by ATP and the cycle can repeat itself (reviewed in Mayer, 2010). Members of the Hsp70 and 90 families have a number of domains which are required for their function. Hsp70 has two domains, one of which infers its ATPase activity, and the other which binds to the substrate required for folding. The substrate binding domain consists of a channel into which the substrate sits, and an α -helical lid structure that closes over the substrate (Zhu et al., 1996). Hsp90 molecules in the cytosol form dimers which are connected at their C-terminal domains (Minami et al., 1991, Minami et al., 1994) but only homo-dimers are functional (Wayne and Bolon, 2007). Hsp90 dimers also have a middle domain and an N-terminal domain, the latter of which gives it its ATPase activity and transiently dimerizes when bound to ATP (Ali et al., 2006). All domains contain sites for the binding of co-chaperones. A diverse range of co-chaperones are required for Hsp70 and 90 to perform their function. Both require nucleotide exchange factors (NEFs) to release ADP and recruit ATP, and to stimulate ATP hydrolysis e.g. the activator of Hsp90 ATPase (Aha1) and p23 co-chaperones of Hsp90. Co-chaperones can also control chaperone localisation and recruit substrates to the chaperone binding site. For example, the Hsp90 kinase recruiter known as Cdc (cell division cycle homolog) 37 or the Hsp70 co-chaperone family Hsp40 (reviewed in Röhl et al., 2013, Tzankov et al., 2008).

Valbulas and colleagues (2010) define molecular chaperones as “any protein that interacts, stabilizes, or helps a non-native protein to acquire its native conformation, but is not present in the final functional structure”. The transition of the capsid precursor into this assembly competent state would therefore seem to be an appropriate target for chaperoning.

5.1.3 Rationale for studying the role of Hsp90 in the FMDV life-cycle

As explained in sections 1.5.3 and 1.5.4, viruses have the ability to hijack a range of cellular components including the host’s translational machinery and membranes. There is a growing pool of evidence that many DNA, positive and negative sense RNA and double stranded RNA viruses also use the host chaperone machinery (reviewed in Mayer, 2005, Knox et al., 2011, Geller et al., 2012), and in particular Hsp90 for parts of their lifecycle. Many viruses are highly sensitive to Hsp90 inhibitors at non-cytotoxic concentrations (e.g. Geller et al., 2007, Nakagawa et al., 2007, Liu et al., 2009) which could be due to a number of factors, which Geller et., al. (2012) speculate upon:

1. The limited viral genome size requires proteins to perform multiple tasks which can make them large, with multiple domains which are likely to require chaperones to be folded correctly.
2. Viruses need to produce a large amount of protein in a short time period which may saturate the constitutive folding machinery and require different types and increased amounts of chaperone assistance.
3. Capsid proteins are susceptible to aggregation and mis-folding, so chaperones may hold precursors in a soluble ready-state for capsid assembly.
4. Chaperones can also buffer the effect of deleterious mutants produced in the viral swarm allowing viruses to further “explore” the sequence space.

There is increasing evidence that the picornavirus life cycle requires cellular chaperones. Hsp90 inhibitors reduce the propagation of PV, HRV, CVB3, EV71 and TMEV in cell culture and of PV and EV71 in small animal models (Geller et al., 2007, Mutsunguma et al., 2011, Tsou et al., 2013, Wang

et al., 2013). siRNA knockdown of Hsp90 also inhibits EV71 propagation in cell culture (Tsou et al., 2013, Wang et al., 2013) and this inhibition may be due to a requirement for Hsp90 β rather than α . While, picornavirus NSPs appear to be resistant to Hsp90 inhibitors meaning there is no effect on polyprotein translation and genome replication (Geller et al., 2007, Tsou et al., 2013) there is evidence that Hsp70 and 90 are required to chaperone the structural proteins. The first indication of this was in the research of Macejak and Sarnow (1992), who demonstrated that an interaction between Hsp70 and the P1 of PV and CVB1 extended the half-life of P1 but that the interaction was lost upon incubation with ATP. Geller et al. (2007) subsequently showed that PV P1 was also a client of Hsp90 by its co-immunoprecipitation with Hsp90 and the Hsp90 nucleotide exchange factor p23. Inhibition of the Hsp90 ATPase function prevented co-IP with p23 but not Hsp90 which along with the other data suggests that P1 folding follows a canonical Hsp90 folding pathway; from translation at the ribosome to folding by Hsp70 and finally to folding by Hsp90 in a p23 and ATP dependent manner. In addition, Hsp90 interaction was required for processing of PV and HRV P1 by 3C^{pro} (Geller et al., 2007) which, as described in chapter 4, is a critical step in the ability of P1 to multimerise and form pentamers. The interaction between Hsp90, and the P1 of PV and EV-71, was also required to protect it from proteasomal degradation (Geller et al., 2007, Tsou et al., 2013).

HAV is the only picornavirus, thus far, to exhibit different characteristics. HAV is relatively insensitive to Hsp90 inhibitors when compared with poliovirus (with an IC₅₀ to Hsp90 inhibitors almost 20 times greater) and it has been suggested that HAV P1 may use an alternative processing and assembly pathway that has been adapted intentionally for slow growth kinetics (Aragonès et al., 2010). FMDV more closely resembles members of the enterovirus than HAV in many aspects of its lifecycle and so the possibility that FMDV proteins are clients of Hsp90 was investigated.

5.1.4 Probing Hsp90-FMDV interactions using pharmacological agents

Interactions between FMDV and Hsp90 can be investigated using pharmacological inhibitors of Hsp90. Hsp90 is upregulated in many cancers to buffer the deleterious effects of reduced nutrients, proteotoxic stress, hypoxia and genetic instability and as such, a range of compounds have been developed as anti-cancer agents targeting Hsp90 (Neckers and Workman, 2012). One such compound, is the benzoquinone ansamycin, geldanamycin (GA) which is highly similar in structure to the twisted structure that ATP adopts in the ATP binding site of Hsp90 (Roe et al., 1999) making it a highly specific competitive inhibitor (reviewed in Jhaveri et al., 2012). A water soluble and less toxic derivative of GA known as 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (Hollingshead et al., 2005, Li et al., 2010), has been used in many of the experimental studies presented here to investigate whether there is a requirement for Hsp90 in the FMDV replication cycle.

5.2 Results

5.2.1 The Hsp90 inhibitor 17DMAG causes a reduction in the growth of FMDV in BHK21 cells

5.2.1.1 17DMAG prevents virus induced cellular toxicity and is non-toxic to cells at these concentrations

To determine whether there was a requirement for Hsp90 in the growth of FMDV in a cell-culture model, cell-lines of baby hamster kidney (BHK21) and pig kidney (IB-RS-2) were used. These cell-lines are known to be permissive to FMDV, and provide an environment where high titres of FMDV can be produced (Mowat and Chapman, 1962, De Castro, 1964, Ferris et al., 2002). Initially the toxicity of 17DMAG in these cell lines was tested to rule out any confounding effect that the drug was having on the cells independently of an effect on the virus. To do this, cell viability assays were performed (Viral ToxGlo™; methods 2.12) which quantify reductions in metabolic activity in cells caused by viral infection or toxic drugs, by measuring ATP levels in cell lysates.

Firstly BHK21 or IB-RS-2 cells were treated with a quadruplicate dilution series of 17-DMAG in culture media covering a range of concentrations from 100pmol to 640µM. From the previously

published data investigating the effect of GA on picornaviruses, 17DMAG was expected to be effective and non-toxic at concentrations of around 0.5-2 μ M (Geller et al., 2007, Tsou et al., 2013). The cells were incubated with the drug for 9 hours to represent the upper limit of time that would be required for a single cycle viral infection. At 9 hours, the cells were lysed with the ATP detection reagent in the Viral ToxGlo™ kit before the luminescence counts of the samples were read using a luminometer (figure 5.1A). Mock treated cells were used as a control and all luminescence readings were expressed as a percentage of the control. At a concentration of 40 μ M, 17DMAG treatment reduced cell viability by about 50% in the IB-RS-2 (red line) and 40% in the BHK21 (blue line) cells. Higher concentrations of drug were used on the BHK21 cells and concentrations of 80 μ M and above were shown to cause up to a 99.8% reduction in the cell viability at 640 μ M. The drug did not appear to have any negative effect on cell viability in either cell line when used below 20 μ M. Small amounts of variation were seen in the BHK21 cells treated with concentrations below 20 μ M suggesting that 17DMAG could be having a positive effect on the cell viability. While there is a possibility that pharmacological inhibition of Hsp90 at certain concentrations could have positive effect on cell viability, there was no clear trend to support a hypothesis and this result was thought to have been caused by slight inconsistencies in the assay.

To accommodate any combined effects on cellular toxicity of using FMDV with 17DMAG in the following experiments, it was thought important to analyse the effect on cellular viability, when using a combination of the two. The toxicity limit in this assay was calculated to be 40 μ M and so concentrations of drug were used that were below this limit in inhibition assays. Six wells of BHK21 cells were either mock treated with culture media or treated with either 1 μ M or 10 μ M 17DMAG diluted in culture media and incubated for 30 minutes at 37°C followed by 30 minutes at 4°C. At one hour post-treatment, half of the cells in each condition were mock treated (replenished with cold media and 17DMAG) and the other half of the wells were infected with chilled FMDV virus at MOI1

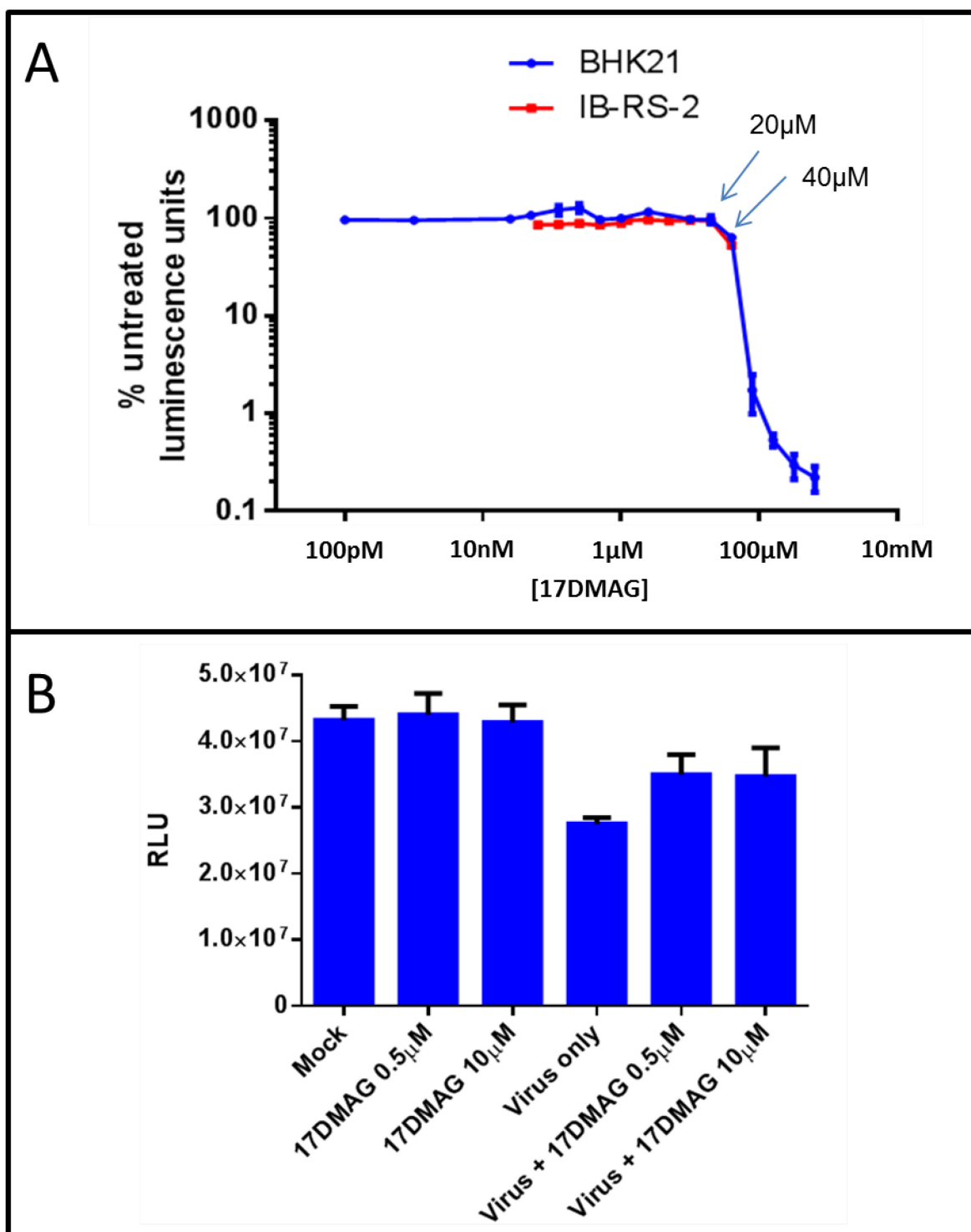


Figure 5.1. 17DMAG cellular toxicity assay. A dilution series of 17DMAG was prepared in media without phenol red indicator and added in quadruplicate to cultures of BHK21 and IB-RS-2 cells grown in media without phenol red. Cells were incubated for 8 hours at 37°C after which time, the cells were lysed with viral ToxGlo ATP detection reagent before the emitted photons were read using a luminometer (method 2.12). Readings for the 17DMAG treated samples were expressed as a percentage of readings from untreated cells (A). Additionally BHK21 were mock treated or pre-treated with 17-DMAG for one hour before being mock infected or infected with FMDV A22 Iraq at an MOI of 10 prepared in media containing the same concentration of drug. Plates were incubated at 37°C for 8 hours before the samples were prepared as above and the luminescence readings plotted (B).

in the presence of 17DMAG and incubated at 4°C for 30 minutes to allow the virus to adsorb to the cells. The cells were then placed at 37°C to standardise the point of virus entry and incubated for a further 7.5 hours at 37°C before being lysed with the Viral ToxGlo™ ATP detection reagent. The luminescence counts of the samples were then read using a luminometer (figure 5.1B), and one-way ANOVA statistics using a Tukey multiple comparisons test were applied to the data using Graphpad Prism software. No significant differences were observed between the uninfected mock and either of the 17DMAG treated samples confirming the results from figure 4.1A that these concentrations are not toxic to the cells. When mock treated cells were infected with FMDV, there was a highly significant ($p \leq 0.001$) 38% reduction in the luminescence signal for cellular viability. While there was a slight but significant ($p \leq 0.05$) drop in the 17DMAG treated and infected conditions when compared to the treated uninfected samples, treatment with either 1µM or 10µM 17DMAG resulted in observable but not significantly ($p \leq 0.1$) reduced toxicity. Pre-treatment of the cells with 17DMAG appeared to protect the cells from this virus induced toxicity, reducing the toxic effect of the virus from 38% to approximately 21%.

These data demonstrated that treating BHK21 and IB-RS-2 cells with concentrations of 17DMAG 20µM and lower were non-toxic to the cells and that 17DMAG treatment BHK21 cells at these concentrations partially protected the cells from FMDV induced toxicity caused by cytopathic effect (CPE), suggesting that Hsp90 was required by FMDV in a part of its lifecycle.

5.2.1.2 Pharmacological inhibition of Hsp90 reduces FMDV titre in a cell culture model

An experiment was designed in parallel with the above viability experiment to analyse the effect of the Hsp90 inhibitor 17DMAG on the titre of FMDV in cell culture. BHK21 cells were mock treated or pre-treated with 0.5µM or 10µM 17DMAG in triplicate before FMDV A22 Iraq at MOI 1 was adsorbed to the cells in the presence of 17DMAG as above. After 8 hours incubation the samples were lysed by freeze thawing. Virus titration was performed by Dr. Amin Asfor using tissue culture infectious dose (TCID) 50. The virus infected cells that were treated with 17DMAG showed over a log

(1.2 log₁₀ for 0.5µM and 1.1 log₁₀ for 10µM) reduction in virus titre (figure 5.2A) which were both significantly different ($p \leq 0.01$) to the mock treated infected control cells infected with virus when the data were analysed by one-way ANOVA in Graphpad software. There were no significant differences observed between the different concentrations of 17DMAG treatment. This demonstrated that inhibition of Hsp90 by 17DMAG had a direct effect in reducing FMDV growth in cell culture.

To further investigate this, an experiment was devised which was performed by Dr. Amin Asfor. This measured the reduction in FMDV titre caused by 17DMAG over the course of a growth curve. The experiment above was repeated but multiple timepoints for each sample were analysed. At 0, 2, 4, 5, 6 and 7 hours post infection (hpi), the cells were frozen and lysed for titration. Titres at each time point on the growth curve were analysed by TCID₅₀ (figure 5.2B) and analysed by two-way ANOVA using Graphpad Prism software. From 4 to 7hpi, the FMDV titres obtained from 17DMAG treated cells were significantly different ($p \leq 0.01$) to the mock treated and infected cells. At 7 hours post infection FMDV from 17DMAG treated cells had a titre approximately 0.55 log₁₀ lower than the control cells. This demonstrated that inhibition of Hsp90 by 17DMAG reduced the replication of FMDV.

An additional experiment was performed to ascertain whether the requirement for Hsp90 was serotype specific. FMDV A22 Iraq and O1 Manisa virus stocks used for this experiment were obtained from Dr. Sarah Gold at Pirbright. BHK21 cells were either mock treated or pre-treated with 0.5µM 17DMAG before adsorption with either virus. Cells were infected with either a low MOI (0.1) or high MOI (10) to analyse if there was a difference between the requirements for Hsp90 in a single cycle (8 hour) high MOI infection or a multiple cycle (24 hour) low MOI infection. At these time

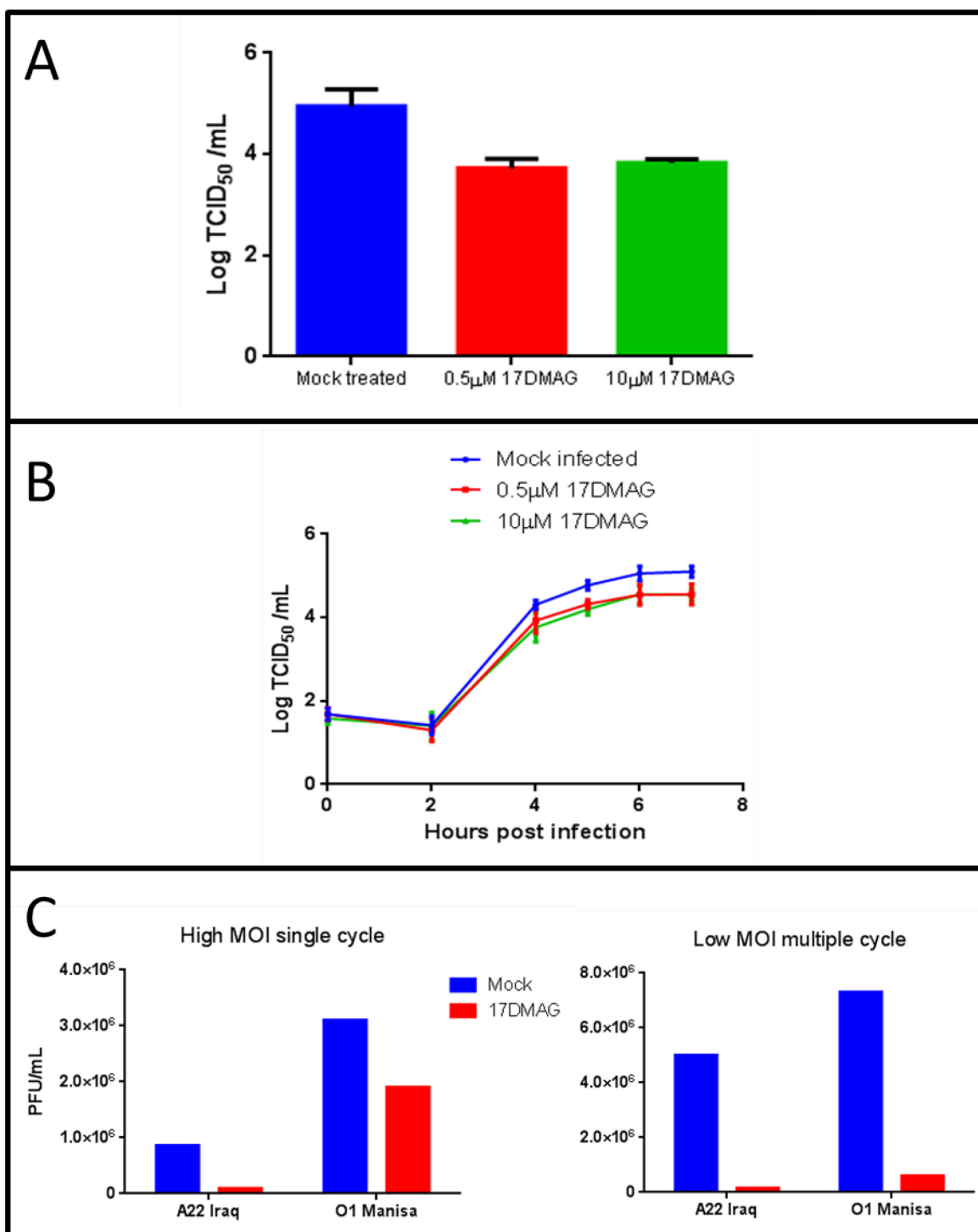


Figure 5.2 FMDV titration in the presence of 17DMAG. Three experiments were performed to assess the effect of inhibition of Hsp90 with 17DMAG. In all cases, BHK21 cells were either mock treated or pre-treated with 17-DMAG before adsorption with virus at 4°C. Cells were infected with A22 Iraq at MOI 1 and incubated for eight hours before they were lysed and the viruses titrated by TCID₅₀ (A). Cells were infected with A22 Iraq at MOI 1 and incubated for 0, 2, 4, 5, 6 or 7 hours before they were lysed and the virus titrated by TCID₅₀ (B). Cells were infected with A22 Iraq or O1 Manisa at an MOI of 10 (left-hand graph) and lysed after 8 hours or an MOI of 0.1 (right-hand graph) and lysed after 24 hours at which point titrations were performed by plaque assay (C). Joseph Newman devised all experiments and performed experiments A and C. Amin Asfor helped devise experiments A and B, performed experiment B and the titrations in experiment A (methods section 2.11).

points, the cells were frozen and lysed before the FMDV titres were analysed by plaque assay (figure 5.2C). In the single cycle, high MOI infection, the titre of A22 Iraq was reduced by one $\log_{10}$ compared to a reduction of $0.12\log_{10}$ in the O1 Manisa titre. The drop in titre from the low MOI infection was more pronounced in both viruses with the A22 Iraq titre $1.35\log_{10}$ lower and the O1 Manisa titre $1.13\log_{10}$ lower. This demonstrated that strains from two different serotypes in FMDV were affected by inhibiting Hsp90 with 17DMAG but that A22 Iraq virus appeared more sensitive to this inhibition than O1 Manisa. The effect of the reduction in titre was more pronounced in the multiple replication cycle infection suggesting that the virus had not been able to compensate for Hsp90 inhibition through mutation in this time period.

5.2.2 The Hsp90 inhibitor 17DMAG has no effect on FMDV RNA replication or translation
The experiments presented above demonstrated that inhibition of Hsp90 with 17DMAG decreased the levels of infectious virus that could be produced from cell culture. It has been shown that Hsp90 inhibition of EV71 does not cause a reduction in the levels of viral RNA in the cell (Tsou et al., 2013) and does not affect the activity of a poliovirus luciferase replicon (Geller et al., 2007). Both of these data suggest that proteins required for RNA replication do not have a requirement for Hsp90.

To investigate whether FMDV RNA replication or translation were affected by inhibition of Hsp90, a FMDV luciferase replicon was employed. The replicon encodes the sequence for Renilla-luciferin 2-monooxygenase inserted into the sequence of FMDV strain O1 Kaufbeuren under the control of a T7 promoter. The luciferase gene replaces a large section of the O1K coding region for the structural proteins so that the sequences for VP2 and VP3 are completely absent, and only approximately 30 amino acids of the N- and C-terminal amino acids of VP4 and VP1 respectively are encoded. Upon transfection of RNA transcribed from the replicon into permissive cells, a luciferase signal can be detected which corresponds with the replication of the genome.

Frozen RNA transcribed from this replicon plasmid was obtained from Emma Howes at Pirbright. Replicon RNA was transfected into IB-RS-2 cells that had been mock treated or pre-treated with 3mM guanidine hydrochloride (GuHCl) or 0.5 μ M 17DMAG in triplicate. GuHCl, has been shown to inhibit the replication of several viruses at concentrations which are non-toxic to cells (Rightsel et al., 1961, Brown et al., 1966). It was used as a negative control for replication in this experiment. Transfected cultures were incubated for 5 hours with luminescence readings taken every half an hour from one hour post-transfection and plotted using Graphpad Prism software (figure 5.3). A two-way ANOVA was also performed on the data in the software. In the untreated and 17DMAG treated samples, none of the data points showed any significant differences as the luciferase signal increased throughout the assay. From 3.5 hours post-transfection and onwards the luciferase signal from the GuHCl treated condition levelled off and then decreased demonstrating the fate of the luciferase signal in the absence of RNA replication. From 3.5 hours onwards the luciferase readings from the GuHCl negative control differed significantly ($p \leq 0.001$) from the other two samples. This data set has shown that 17DMAG has no effect on the expression of luciferase from an FMDV replicon demonstrating that the drug was not interfering with FMDV replication or translation in its ability to reduce the titre of virus in culture. This implicated virus entry or assembly as the point at which the drug was acting, the latter of which was analysed in the next set of experiments.

5.2.3 Inhibition of Hsp90 with 17DMAG prevents the assembly of FMDV pentamers independently of proteolytic processing in a cell free system

This chapter has so far presented findings that demonstrate that 17DMAG protected cells from toxicity caused by FMDV, which may in part have been due to restricting the growth of virus in culture but was not caused by inhibiting the ability of an FMDV replicon to translate from the FMDV IRES or replicate the genome. Geller et., al. (2007) have shown that inhibition of Hsp90 with GA appeared to inhibit PV P1 processing by 3C^{pro} in a cell-free model and targeted P1 for degradation by the proteasome in a cell-culture model. In contrast to this, Wang and colleagues (2013), showed that

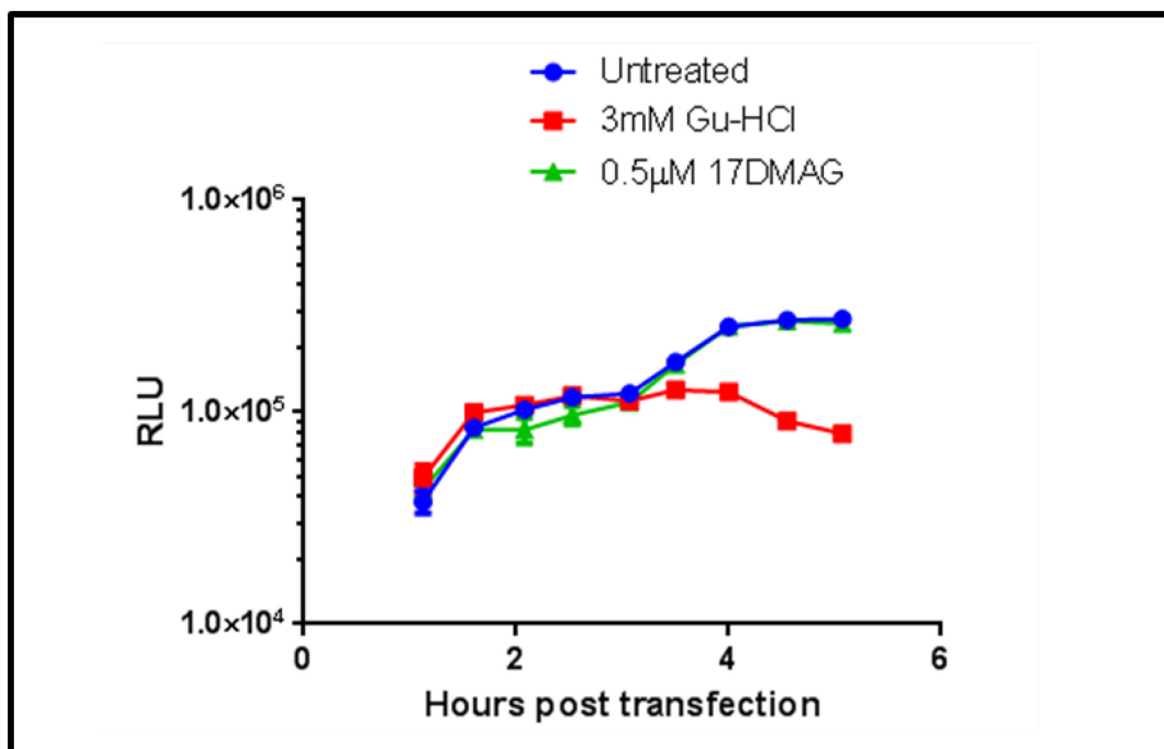


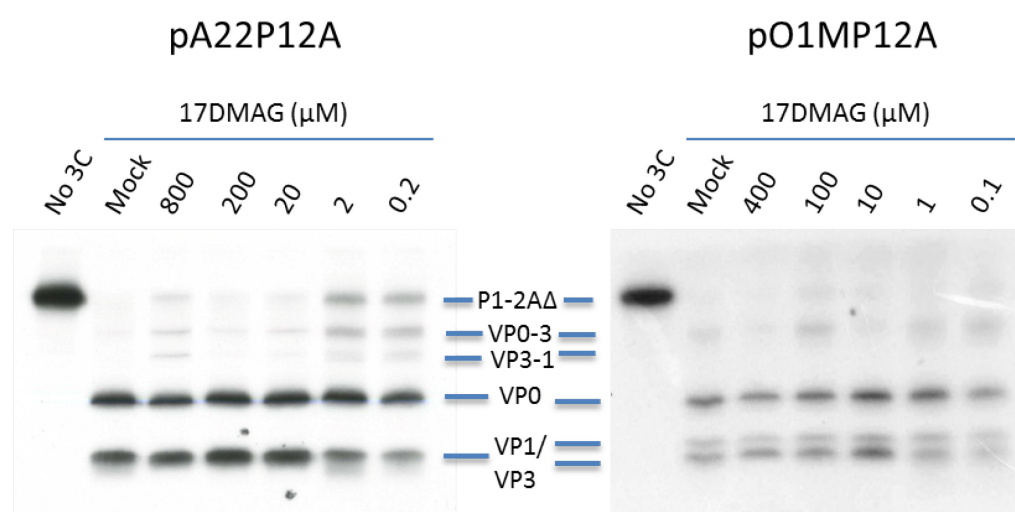
Figure 5.3 The effect of 17DMAG on an FMDV replicon. IB-RS-2 cells were mock treated or treated with 3mM GuHCl or 0.5μM 17DMAG in triplicate for 30 mins at 37°C before FMDV replicon RNA was transfected into the cells using Lipofectamine 2000 transfection reagent. Luciferase readings were taken on a luminometer every half an hour from 1 hour to 5 hours post transfection (methods section 2.13). The mean luminescence counts (RLU) for the triplicate samples were plotted in Graphpad Prism software.

while a lower level of mature capsid proteins were visible following treatment with GA, inhibition of the proteasome resulted in the protection of VP3 and VP1 from degradation in an EV71 cell culture model. This suggested that Hsp90 was required by the mature capsid proteins, after processing, to avoid proteasomal degradation of mis-folded proteins.

The cell-free RRL system described in chapter 4 was used to determine the effect of Hsp90 inhibition on proteolytic processing and pentamer assembly in FMDV. Initially the effect of 17DMAG on P1-2AΔ processing was investigated. RRLs were programmed with pA22P12Awt or pO1MP12Awt followed by treatment with a range of concentrations of 17DMAG, processed with 3C^{pro} and then analysed by SDS-PAGE (figure 5.4A). A non-processed, mock drug treated control was included to give the position of P1-2AΔ and a processed, mock drug treated control was included to give the positions of mature capsid proteins. The concentrations of 17DMAG used in these experiments were far higher than could be used in the previous cell-culture model due to the problems of toxicity in culture being avoided in the RRL. The range of 17DMAG concentrations tested covered the range that was used in the previous cell-culture studies but very high concentrations of 800μM for A22 and 400μM for O1M were used to exaggerate any effect that 17DMAG was having. Interestingly, very little difference was seen between the cleavage profiles treated with 17DMAG and the untreated, processed samples with most samples showing complete cleavage in both the A22 and O1M samples. Faint traces of intermediate cleavage products were seen in the 2μM and 0.2μM conditions in A22 but this did not follow a trend in any of the other samples and so there did not seem to be any effect of 17DMAG on P1-2AΔ processing.

It was thought that maybe the 3C^{pro} concentrations that were being used were too high and thus were masking an effect of the drug that was evident in cell culture but not in the recombinant system. To investigate this, the processing assay was repeated but using half the concentration of 3C^{pro} (approx. 0.5μM) for processing because it was reasoned that comparing the levels of

A – standard 3C^{pro}



B – low 3C^{pro}

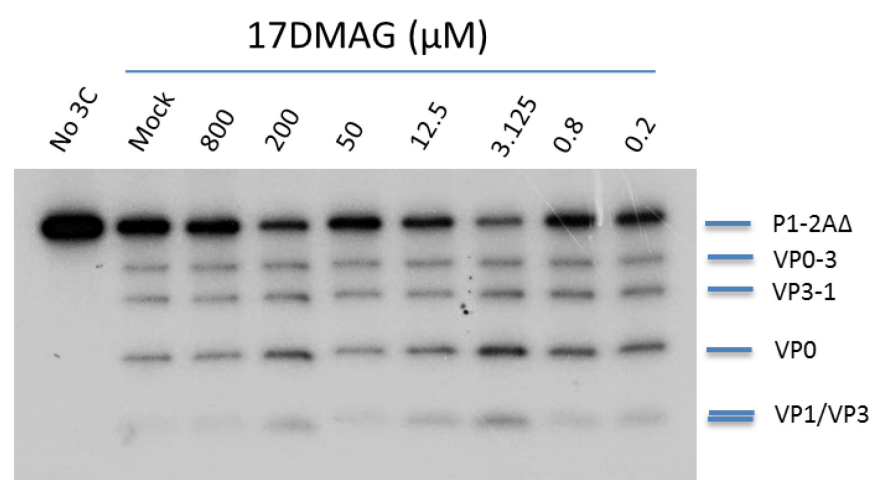


Figure 5.4 17DMAG does not affect the proteolytic processing of P1-2AΔ by 3C^{pro} in a cell-free assay. TnT lysates were programmed with pA22P12Awt or pO1MP12Awt and incubated for 1.5 hours at 30°C. After completion of the TnT, the lysate was divided into incubated with a dilution series of 17DMAG in PBS, or PBS alone (mock and no 3C). 3C^{pro} was added to all reactions at a final concentration of 1μM apart from the no 3C control and the reactions were incubated for a further hour at 37°C (A). This process was repeated for the pA22P12Awt sample but using half the concentration of 3C^{pro} (B). All reactions were then separated on 12% SDS-PAGE gels, soaked in 1M sodium salicylate and exposed to X-ray film at -80°C have been separated to isolate specific sample sets from other experiments on the same films. Where protein names are separated with a hyphen (-), they are uncleaved, separation with a forward stroke (/) indicates mature cleaved proteins.

intermediate processing products would give a better indication about whether 17DMAG was affecting the processing of P1-2AΔ (figure 5.4B). However, again while there were very slight differences in the strength of the signal for the intermediate processing products, they were observed to be largely similar with no consistent trend that 17DMAG was affecting the ability of 3C^{pro} to process P1-2AΔ even at the very highest concentrations of 200μM and 800μM.

If Hsp90 was indeed required in the assembly pathway, it appeared that a stage after P1-2AΔ processing or during synthesis must be affected. To test this hypothesis, processed A22 samples from the experiment in figure 4.4A were dialysed and then ultracentrifuged through SDGs. The fractionated gradient was analysed by scintillation counting and graph plots were obtained by using Graphpad Prism software (figure 5.5). At a 17DMAG concentration of 800μM the ability of P1-2AΔ to assemble into pentamers was completely prevented. This value was used as a baseline that was taken away from the total signal for the pentamer peaks (fractions 19-21) in the other samples and the % reduction in pentamers compared to the mock treated sample was calculated (table 5.1). In the concentration range that was effective and non-toxic in cell culture (0.2- 20μM), there was a 26-34% reduction in the size of the pentamer peaks compared to the control. At a 17DMAG concentration of 200μM, pentamer formation was reduced by 45% and at 800μM there were no pentamers. These data are representative of several experiments which showed a reduction in pentamer formation in the presence of 17DMAG suggesting that Hsp90 is required by P1-2AΔ at a stage after processing in order to assemble into pentamers.

5.2.4 Replacement of reduced glutathione and ATP is insufficient to restore factors lost in dialysis required for pentamer assembly

Having demonstrated a requirement for Hsp90 in the assembly of FMDV pentamers, a question from chapter 4 was readdressed. In chapter 4 it was shown that if the assembly assay was dialysed before P1-2AΔ was processed with 3C^{pro} the ability to assemble pentamers was removed. Two hypotheses were put forward to explain this finding. The first of which was that the concentration of P1-2AΔ was

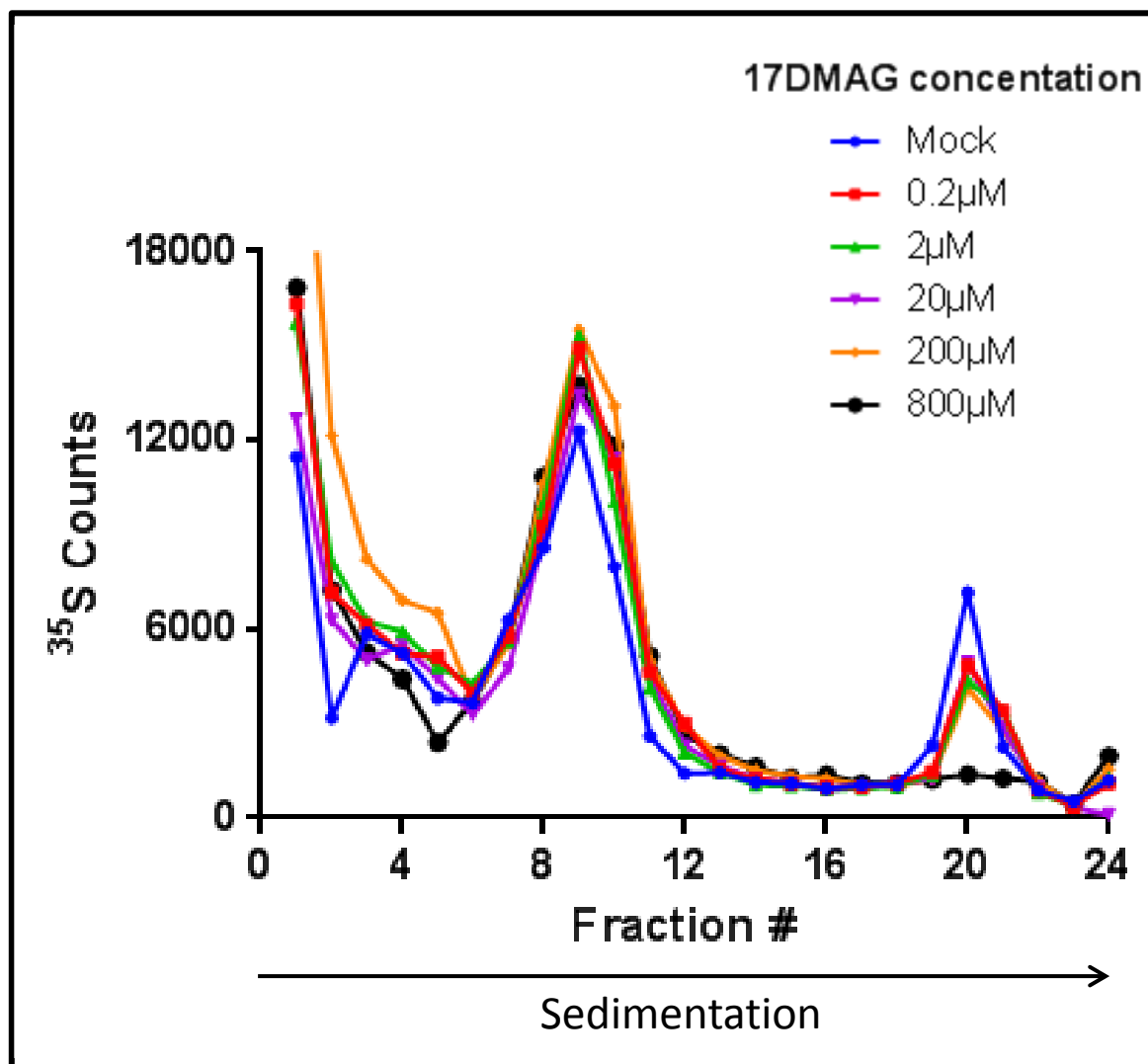


Figure 5.5 The effect of 17DMAG on the assembly of pentamers. Samples were taken from the A22 TnT reactions from figure 4.4A and dialysed three times against 500mL PBS. They were then diluted and ultracentrifuged through a 5-30% SDG for 6 hours at 55000 rpm in an SW55Ti rotor at 10°C. The gradient was fractionated into 24 approximately 200µl fractions using a Biocomp fractionator into 1mL scintillation fluid in scintillation vials. These were then counted on a scintillation counter and the counts plotted as a percentage of the total counts for that gradient using Graphpad Prism software.

Table 5.1. Comparison of pentamer peaks from the gradient fractions in figure 5.5. The scintillation counts from the gradients in figure 5.5 were analysed in order to compare the sizes of pentamer peaks in the dilution series of 17DMAG treated samples. The positions deemed to contain the peak and shoulder of the pentamer were fractions 19-21. The counts were calculated for the background in these positions (i.e. the samples where there had been no pentamer formation; the 800 μ M condition) and removed from the counts in the pentamer containing samples before the pentamer counts in each sample were expressed as a percentage of the counts in the mock treated sample.

	17DMAG (μM)					
Fraction number	Mock	0.2	2	20	200	800
19	2303	1476	1393	1246	1263	1232
20	7161	4835	4370	4960	4116	1390
21	2259	3404	3411	2886	2782	1269
Total counts	11723	9715	9174	9092	8161	3891
Total minus background	7832	5824	5283	5201	4270	0
% mock treated pentamer formation	100	74	67	66	55	0

reduced if dialysed first and was therefore at a sub-optimal concentration for pentamer assembly after dialysis. The second theory was that a component was being lost during dialysis that was required for pentamer assembly. Given the requirement for Hsp90 in pentamer assembly that has been demonstrated in this chapter, it was hypothesised that ATP which is fundamentally required by Hsp90 to fold substrates, could be the component that was lost. Additionally, several groups have reported that reduced glutathione is required by several picornaviruses for assembly (Ma et al., 2014a, Thibaut et al., 2014) and that this interaction may stabilise protomer-protomer interfaces in pentamer assembly.

To test these hypotheses, the dialysis experiment from chapter 4 (figure 4.5) was repeated and an additional condition was included whereby ATP and reduced glutathione were returned to the dialysed lysate before processing to investigate their ability to restore pentamer assembly. Plasmids pA22P12Awt and pO1MP12Awt were programmed into TnT lysates before being split into the three conditions. The control conditions were, the standard pentamer assembly conditions in which P1-

2AΔ is processed before dialysis, and the negative control in which P1-2AΔ is dialysed before processing. Ten micro molar reduced glutathione (GSH) and 10mM ATP were replaced in the additional dialysed sample to represent physiological levels of these compounds. The samples were then ultracentrifuged through 5-30% SDGs and the fractions analysed by scintillation counting and plotted using Graphpad Prism software (figure 5.6). The P1-2AΔ sample that had been processed before dialysis assembled into pentamers, but addition of ATP and GSH failed to permit pentamer assembly and the gradient profile of these samples resembled the negative control sample. Interestingly, while the amount of P1-2AΔ in the samples that failed to assemble was reduced compared to the pentamer assembling control, in the A22 negative control the levels of P1-2AΔ were not very much lower. This may suggest that the reduced concentration of P1-2AΔ is not the reason, or at least isn't the only reason, why these samples cannot assemble into pentamers implying that a further factor is required for assembly that was not accounted for in these experiments. This will be discussed further below.

5.3 Discussion

5.3.1 Summary

Why should viruses have evolved to require the use of cellular chaperones for their assembly?

Picornavirus structural proteins have the same requirements for their capsids as the structural proteins of viruses in many families (Geller et al., 2012). Picornavirus capsids are composed of 60 copies of a hetero-tetrameric capsid protomer that is required to encapsidate and protect the genome from the intra- and extra-cellular environments but be labile enough to undergo structural alterations upon re-entry into new cells (Hogle, 2002). The complexity of generating such structures and the limited size of viral genomes has required viruses to evolve interactions with host-cell components in order to achieve these goals. In the absence of such control mechanisms, incorporation of misassembled capsid components can infer a dominant negative effect on the virus

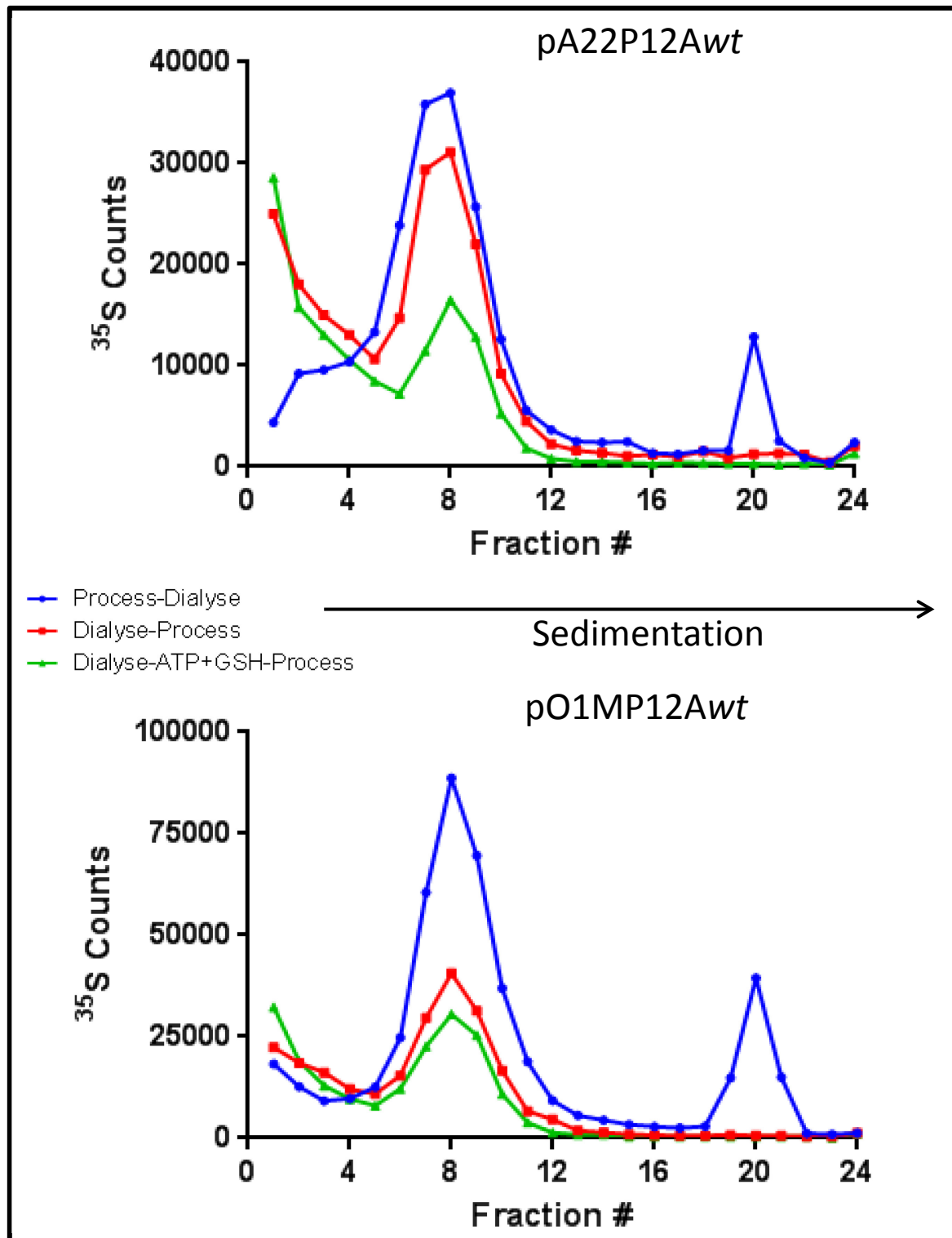


Figure 5.6 ATP and GSH are not able to restore the ability of dialysed P1-2AΔ to assemble into pentamers. TnT lysates were programmed with pA22P12Awt or pO1MP12Awt and incubated for 1.5 hours at 30°C. After completion of the TnT, the lysate was divided three equal parts with one part processed by 1μM 3C^{pro} before dialysis (blue), one processed after dialysis (red) and 10μM reduced glutathione and 10mM ATP added to the third part before processing (green). The samples were then sedimented through SDGs before they were fractionated and counted as in figure 5.6 and the ³⁵S counts were plotted using Graphpad Prism software.

(Crowder and Kirkegaard, 2005). The Hsps are the cell's protein folding machinery and it appears that many picornaviruses are able to hijack Hsp90 for their own benefit.

This chapter presented data to investigate the interaction between the molecular chaperone Hsp90 and FMDV. The experiments demonstrated that the Hsp90 inhibitor 17DMAG is able to protect cells from FMDV induced toxicity, which may in part, be due to its ability to reduce the productivity of FMDV infection, and reduce virus titres. The reduction in virus titre was not due to an observable effect on the ability of an FMDV replicon system to replicate itself which also implied that translation was not the target of the inhibition. The process that was sensitive to Hsp90 inhibition was that of assembly, where it was shown that 17DMAG treatment of a cell-free assembly system reduced the ability of P1-2AΔ to assemble into pentamers. This sensitivity was not due to a reduced ability of P1-2AΔ to be processed by 3C^{pro} but was due to a process which affected the multimerisation of processed P1-2AΔ into pentamers. The replacement of the mandatory Hsp90 co-factor ATP or a small molecule co-factor required for some enteroviruses to assemble (GSH) (Ma et al., 2014a, Thibaut et al., 2014) in a dialysed sample of P1-2AΔ was insufficient to restore the ability of P1-2AΔ to assemble after processing.

5.3.2 The picornavirus requirement for cellular chaperones

The toxicity assay data showed that 17DMAG could be used at concentrations below 40μM for at least up to 9 hours without causing toxicity to either the BHK21 or IB-RS-2 cell lines used for the experiments. At concentrations of 0.5μM and 10μM, 17DMAG was able to reduce the toxicity caused by FMDV infection of the cells. While this result was not quite statistically significant, the effect was clear from the data and with a larger sample size it was felt that this could be demonstrated. The toxicity assay measures the levels of ATP in the cells to give an indication of their metabolic function and therefore their health. Infection with picornaviruses uses up cellular resources and damages the cells through events such as inhibition of host-protein synthesis

(Belsham, 2000, Li, 2001), the use and rearrangement of cellular membranes for viral replication purposes (Limpens et al., 2011, Midgley et al., 2013b) and the lysis of cells upon virus release (reviewed in Richards and Jackson, 2013). These types of mechanisms used by the virus account for the reduced ATP signal from virus infected cells. It was shown when using 17DMAG that virus titres were reduced which is likely to be the reason for the reduced cellular toxicity observed.

17DMAG was seen to cause up to a $\log_{10}$ reduction in virus titre when cell cultures were treated prior to infection with FMDV. This suggested that the reasons for reduced toxicity when using 17DMAG were that viral processes in the cells were being inhibited. The reduction in titre was seen from 4 hours post infection in an FMDV growth curve, was shown at two drug concentrations (0.5 μ M and 10 μ M), in two strains of FMDV representing serotypes A and O and in a high MOI (10) and low MOI (0.1) single or multiple cycle infections respectively. A single replication cycle of FMDV is known to be between 4-7 hours and it was expected that if the Hsp90 was required by the virus then the effect would be evident in this time period. The effect of inhibition was evident from 4 hours onwards but the inhibitory effect of the drug in the growth curve appeared less than in the single time-point and multiple timepoint experiments. Hsp90 is a highly abundant protein in mammalian cells, so two concentrations of drug were used to investigate if a higher concentration of drug would produce a greater reduction in viral titre. This did not appear to be the case, however, with very similar levels of inhibition achieved with the different drug concentrations. Hsp90 has six functional Hsp90 genes; *HSP90AA1* (Hsp90- α_1), *HSP90AA2* (Hsp90- α_2), *HSP90N* and *HSP90AB1* (Hsp90- β) which are found in the cytosol with *HSP90AB1* mainly constitutively expressed and the others, which are highly inducible. *TRAP1* is the gene that encodes an Hsp90 paralogue found in the mitochondrion and similarly *HSP90B1* expresses the form of Hsp90 known as GRP-94 which is found in the endoplasmic reticulum. Each gene has also been found to have several splice variants and polymorphic forms (Chen et al., 2005, and reviewed in Sreedhar et al., 2004). Interestingly, EV71 has been shown to only require Hsp90- β but not α forms of the chaperone (Tsou et al., 2013, Wang et

al., 2013). It is possible that 17DMAG targets, or can only access a subset of Hsp90 at these concentrations and so increasing the concentration has no enhanced effect against FMDV which could use a range of subsets of Hsp90 or different chaperones. This may also explain why in the experiments described here and in the published literature the inhibition of Hsp90 by GA or GA derivatives appears to result in a variable degree of inhibition in viral infection and cell-culture models (Geller et al., 2012, Tsou et al., 2013, Wang et al., 2013). In future experiments, determining the relative contributions of the various Hsp90 subsets using siRNA knockdown could elucidate the precise mechanism. Over-expressing specific Hsp90 isoforms in cells could also be used to investigate the viral mechanisms better.

Interestingly, in primary cell lines or animal models experimentally infected with PV (Geller et al., 2007) the reduction in virus titre caused by inhibition of Hsp90 is far greater. Transformed cells are far more susceptible to Hsp90 inhibitors than primary cells due to the presence of highly active, multichaperone complexes that have a high affinity for ansamycin drugs (Kamal et al., 2003). This appears to be paradoxical to the findings for PV. Perhaps, in transformed cells there are antagonistic systems that require Hsp90 that can both inhibit and facilitate virus infection. Calf thyroid primary cells (BTY) are highly sensitive to FMDV (Snowdon, 1966, Ferris et al., 2006) and it would be interesting to compare the interaction of the virus with Hsp90 in these cells.

The MOI of infection may also play a role in the requirement for chaperones. Differences in the MOI of infection can result in differences to the outcome of infection. Schulte and Andino (2014) showed that in individual cells, changes in MOI did not affect the number of PFUs obtained from that cell but did affect the number of viral genome copies. The author suggests that this might be due to the fact that in high MOI infection there may be multiple competing foci of infection using up resources such as ribosomes, energy, nucleotides, membranes, chaperones. The different levels of drug inhibition that were observed in the infections at different MOIs could be explained by differences in the

availability of chaperones to virus singly infecting or multiply infecting cells and this could be investigated further. The multiple cycle low MOI infection showed that the FMDV titre could not recover to untreated levels in the presence of the Hsp90 inhibitor. This was in agreement with Geller et al. (2007) who demonstrated that after multiple passages in the presence of GA in cell culture or in an animal model, PV was still as sensitive to the drug as the untreated controls. It was inferred from this that PV has a critical requirement for Hsp90 that cannot be overcome easily by mutation. Similar experiments with FMDV would need to be performed to confirm this finding but the low MOI data presents preliminary data for this. While the toxicity of 17DMAG had not been tested over a 24 hour period, it was thought that because the drug was being used 80 times more dilute than the toxicity limit at 9 hours, that toxicity would not be a problem. However, this would need to be confirmed experimentally.

5.3.3 Characterising the requirement of Hsp90 for FMDV P1-2AΔ

An FMDV replicon was used to further define at which point in the FMDV lifecycle Hsp90 was required. The replicon contained all the viral proteins apart from the majority of the structural proteins which were replaced by a reporter luciferase gene. This replicon RNA is translated and replicates itself like wild-type virus and therefore allows the quantification of these processes in a cell-culture system. FMDV translation is initiated from a type II IRES which is different to the type I IRES of PV which is insensitive to 17DMAG (Geller et al., 2007). It was shown that luciferase production was unaffected by 17DMAG treatment demonstrating that Hsp90 is not required for either of these processes to occur.

Contrasting reports exist as to the requirement for Hsp90 in P1-2A processing. Geller et al. (2007) demonstrated a requirement for Hsp90 in the processing of PV P1 but Wang et al. (2013) suggest that for EV71, Hsp90 is required in order to chaperone processed P1. It was surprising that there would be a different mechanism in the picornavirus family for Hsp90 usage due to the conservation

of viral capsid structure and maturation (with the exception of HAV (Aragonès et al., 2010)). The cell-free system was utilised to investigate a requirement for Hsp90 in FMDV P1-2AΔ processing. No inhibition of processing was detected in this system in P1-2AΔ derived from an A22 or O1M sequence indicating that FMDV did not require Hsp90 for processing. The effect of 17DMAG on processing in cell-culture was not investigated but would be an interesting experiment to perform. Gullberg et al. (2014) have identified mutants in the individual processing sites in P1-2A and it would provide an interesting topic of study in future to see how these individual sites were affected by chaperone inhibition. The P1/2A junction in particular has not been investigated by us and while it appears that P1 is still able to assemble into pentamers *in vitro* without any 2A sequence (Goodwin et al., 2009); Hsp90 might influence the efficiency of the cleavage at this site.

This chapter demonstrated that FMDV pentamer assembly was affected by Hsp 90 inhibition. Concentrations of 17DMAG that reduced the titre of virus by one log₁₀ in cell culture were only effective at reducing pentamer assembly in the cell-free system by between 26-34%. Perhaps this level of pentamer inhibition is sufficient to cause a disproportionate effect on the virus because it lowers the pool of pentamers below a threshold concentration required for virus assembly (Li et al., 2012, Rombaut et al., 1991). An alternative explanation is that the cell culture experiments were pre-treated with 17DMAG whereas the cell-free reactions were treated after the translation of P1-2AΔ. The canonical model for Hsp function in the cytosol is that nascently translated polypeptides are protected from misfolding by Hsp70 chaperone complexes before being cycled into an Hsp90 folding pathway (Otto et al., 2005, Jaiswal et al., 2011, and reviewed in Hartl and Hayer-Hartl, 2002). Perhaps pretreatment of the lysate with 17DMAG would disrupt P1-2AΔ interactions with Hsp90 at an earlier stage and cause a greater inhibition.

Experiments in chapter 4 demonstrated that processing P1-2AΔ after dialysis prevented its assembly into pentamers. Two hypotheses for this were that either due to sample loss in the dialysis cassettes,

a P1-2AΔ concentration required for efficient assembly was lost or that a cellular co-factor required for assembly was dialysed out of the sample. ATP is required as a co-factor for Hsp90 function and reduced glutathione has been shown to facilitate pentamer assembly in some enteroviruses (Ma et al., 2014a, Thibaut et al., 2014). However, returning ATP and GSH to dialysed lysates did not recover the ability of P1-2AΔ to assemble although subsequent information has suggested that the concentration of GSH used in these experiments was three orders of magnitude too low (Piątek et al. 2009). Additionally, the PBS solution used to dialyse the samples did not contain Mg^{2+} which is required for ATP hydrolysis in chaperone function (Lissin et al., 1990) and this experiment would need to be repeated using these conditions to confirm if there was an effect. Interestingly though in this experiment, the loss of dialysed P1-2AΔ was not as great as in previous experiments, suggesting that an unidentified co-factor may still be required. Experiments should be performed to correct for GSH and Mg^{2+} concentrations to determine whether further co-factors are required for P1-2AΔ assembly.

This chapter successfully determined that Hsp90 is required for the formation of FMDV pentamers. The requirement for Hsp90 in combination with the proteolytic processing requirements identified in chapter 4 is discussed in the final discussion (chapter 7).

Chapter 6: An engineered 'maturation cleavage' generates recombinant pentamers with different properties

6.1 Introduction

Recombinant pentamers can be engineered to contain a novel cleavage site in VP0. Processing of this site mimics the maturation cleavage event producing an altered form of pentamers with characteristics that resemble pentamers from the virus disassembly pathway.

6.1.1 The picornavirus maturation cleavage of VP0 into VP4 and VP2

Picornavirus assembly proceeds via the multimerisation of five molecules of P1 into pentameric structures (reviewed in Jiang et al., 2014 and demonstrated in chapter 4). The process of pentamer assembly in FMDV may also be chaperoned by heat shock proteins (as demonstrated in chapter 5). The assembly of P1 into pentamers which contain intact VP0 is a required intermediate step in the encapsidation of RNA (Verlinden et al., 2000). Twelve pentamers and RNA assemble into a provirion which contains uncleaved VP0 and sediments identically to the corresponding polio (Fernandez-Tomas and Baltimore, 1973, Guttman and Baltimore, 1977, Ansardi and Morrow, 1995) and FMD viruses (Knipe et al., 1997). The “maturation cleavage” is the proteolytic cleavage of VP0 into VP4 and VP2, and occurs during, or after encapsidation of RNA. As such, in picornavirus structures VP2 and VP4 are found as separate entities e.g. FMDV (Acharya et al., 1989), although in PV, one or two copies of VP0 are thought to remain uncleaved in the structure (Jacobson and Baltimore, 1968a).

The VP4/2 cleavage site is buried on the inside of the capsid and is inaccessible to exogenous cellular or viral enzymes and as such is considered an autocatalytic event (Arnold et al., 1987). All picornaviruses that undergo the maturation cleavage (apart from HAV) have the tri-peptide proline-histidine-glutamine motif in their VP2 protein (Palmenberg, (1989), and data obtained from the NCBI “Protein” database). In PV, the proline and histidine amino acids are at positions 194 and 195 (P194 and H195) of VP2 respectively and two water molecules are in the vicinity of the VP0 scissile bond between the last amino acid of VP4 and the first of VP2 (Basavappa et al., 1994). The imidazole ring of H195 is in an appropriate position to activate these water molecules due to a bend in the amino acid chain caused by P194 at this position. Basavappa and colleagues, (1994) have proposed that

catalysis of the peptide bond occurs due to the docking of RNA into a depression provided by the carbonyl oxygens of the scissile bond and the following amino acid, which polarises the scissile bond rendering it susceptible to attack by the activated water molecules. Support for this model comes from the high similarity of a structure at this site in bean pod mottle virus that binds viral RNA (Chen et al., 1989). In FMDV, the tri-peptide motif is found in VP2 at amino acid positions 144-146 and the H145 is exposed to the scissile bond suggesting that it conforms to the proposed model of maturation cleavage in PV (Curry et al., 1997).

The model described above is widely accepted to be the method by which the maturation cleavage of the scissile bond between VP4 and VP2 occurs, however, there appear to be several exceptions to the rule. Natural and recombinant FMDV empty capsids that lack RNA are largely expected to have an intact VP0 (Rowlands et al., 1975), but several A serotype (Curry et al., 1995, Curry et al., 1997, Gullberg et al., 2013a) and O1 Manisa (Gullberg et al., 2013a) empty capsids have been identified that show a high degree of maturation cleavage in their protein composition. One explanation for this finding is that these capsids have undergone abortive attempts at encapsidating RNA. However, the presence of cleaved VP0 has also been reported in recombinant empty capsids, produced in a vaccinia virus expression system, in the absence of replicating RNA (Gullberg et al., 2013a). There may also be additional VP2 residues in PV (Compton et al., 1990), assistance from the viral protein 3CD (Franco et al., 2005), a requirement for an acidic compartment in PV (Richards and Jackson, 2012) or entry compartment in HAV (Bishop, 1999) for the maturation cleavage to occur. Interestingly, Vazquez-Calvo and colleagues (2014) have identified an acid and temperature resistant FMDV mutant that has a tyrosine mutation mapping to VP2 H145 (which is at an inter-protomer interface) and still fully undergoes the maturation cleavage. These results show that FMDV can forgo the requirement for the conserved histidine in the tri-peptide motif to perform the maturation cleavage, which contrasts with the lethal phenotype induced by threonine, arginine, glycine and aspartic acid substitutions in the PV H195 residue (Hindiyeh et al., 1999). The FMDV VP0 protein

therefore appears to have a less stringent set of requirements for the maturation cleavage than enteroviruses, and it appears that this might be linked to its acid sensitivity.

6.1.2 The maturation cleavage confers an increased stability to the virion and is required for cell entry

The structural changes resulting from the maturation cleavage confer important properties on the virus. The cleavage of the VP4/2 peptide bond releases the termini of the two proteins allowing them to participate in the interactions at the 3-fold axis of symmetry, reinforcing the strength of the inter-pentamer interactions (Acharya et al., 1990, Basavappa et al., 1994, Filman et al., 1989) which allows the virus to adopt a “meta-stable” state for the infection of new cells (reviewed in Hogle, 2002).

Provirions are considered non-infectious with any infectiousness expected to be caused by contamination of virions in provirion preparations (Bishop and Anderson, 1993, Compton et al., 1990, Knipe et al., 1997). This difference is possibly caused by interactions made by the protein termini and endosomal membranes during the cell entry process that have been demonstrated in PV (Strauss et al., 2013), EV71 (Shingler et al., 2013) and HRV (Panjwani et al., 2014). Several intermediate capsid states during virus entry are thought to be facilitated by the maturation cleavage and therefore confer infectiousness upon the virions. In PV, VP4 and the N-terminus of VP1 are normally located on the inside of the capsids, however, at 37°C, VP4 and the N-terminus of VP1 are reversibly externalised (Li et al., 1994) in a process known as ‘breathing’ and are locked in to an externalised state upon receptor binding (Fricks and Hogle, 1990). Capsids locked into this form are known as ‘A particles’, sediment at 135S and are less infectious than virus. Many enteroviruses have been shown to undergo breathing e.g. PV (Li et al., 1994) and HRV (Lewis et al., 1998) as well as several viruses from different families e.g. Flock House virus (Bothner et al., 1998) and tomato bushy stunt virus (Jaegle et al., 1988). The PV 135S, A particle entry intermediate (reviewed in Hogle, 2002) is thought to be the form of the capsid that facilitates interaction with the endosomal membrane

(Strauss et al., 2013) and A particles and 80S capsid disassembly intermediates have also been identified in the *Aphthovirus*, ERAV (Tuthill et al., 2009, Bakker et al., 2014). The presence of VP4 is important for receptor binding in FMDV (Goodwin et al., 2009) and preliminary evidence by researchers at the Pirbright institute demonstrates the presence of an “A” particle in FMDV (Gold et al., unpublished findings) suggesting that the maturation cleavage in FMDV allows for the same membrane interactions as described for other picornaviruses.

6.1.3 Picornaviruses without the maturation cleavage

Whilst the maturation cleavage occurs in numerous picornaviruses including the many virus species classified into the aphtho-, cardio-, hepato- and enterovirus genera, there are a significant number of virus genera which contain viruses that have an intact VP0 in the infectious virion. These include the viruses of the kobu, parecho and avihepatoviruses (Knowles et al., 2012, Knowles, 2014) which do not contain the P-H-Q tripeptide motif conserved in the other members of the *Picornaviridae* (HAV is the exception) which is proposed to facilitate the maturation cleavage (amino acid sequence search performed using the NCBI “Protein” database). Although HAV undergoes the maturation cleavage (Ross and Anderson, 1991), it does not contain this tripeptide motif and may achieve the cleavage by a different mechanism in an acidic compartment (Bishop, 1999) during virus exit (Feng et al., 2013). How the genomes of the *Kobu*-, *Parecho*- and *Avihepatovirus* genera (which contain an intact VP0 protein) gain entry into host cells or achieve capsid stability is unknown.

6.1.4 Particles with and without VP4 and their antigenic properties

During entry into host cells, capsids of PV remain intact after the loss of RNA and VP4, and sediment at 80S (Fricks and Hogle, 1990). In contrast, FMDV particles rapidly disassemble into 12S particles when the pH of endosomes becomes more acidic (<pH6.8) (Brown and Cartwright, 1961, Burroughs et al., 1971), although, as described above, the disassembly of ERAV proceeds via an icosahedral capsid intermediate (Tuthill et al., 2009) and preliminary data suggests the same for FMDV (Gold et

al., unpublished findings). It has been proposed that the propensity of FMDV to fall apart into pentamers under acidic pH or elevated temperatures can be explained by the protonation of two histidine residues near the 2-fold pentamer interface causing electrostatic repulsion between two pentameric subunits in increasingly acidic conditions (Curry et al., 1995, van Vlijmen et al., 1998, Ellard et al., 1999).

Vaccine preparations of FMDV and PV containing inactivated intact virus, live-attenuated virus or recombinant assembled empty capsids are unstable for long periods or when the cold chain is not maintained (reviewed in Doel, 2003, Rodriguez and Grubman, 2009). This loss in stability refers to the alteration of PV virions into 135S “A” particles and 80S empty capsids, or the disassembly of FMDV virions into 12S pentamers (Brown and Newman, 1963, Everaert et al., 1989, Rowlands et al., 1975, Rweyemamu et al., 1979). This disassembly correlates with a loss in vaccine efficacy because the 135S, 80S and 12S disassembly pathway particles have antigenic characteristics that differ from virions in both PV (Hummeler et al., 1962 and reviewed in Hogle, 2002) and FMDV (Doel and Chong, 1982, Cartwright, 1962, Cartwright et al., 1980, Rowlands et al., 1975) and therefore no longer antigenically resemble viruses. Empty capsids (with intact VP0) from the assembly pathway, are antigenically similar to virus and therefore can be used as vaccines (e.g. Porta et al., 2013a, Gullberg et al., 2013a). Interestingly, FMDV empty capsids can be dissociated into pentamers and still produce a virus neutralising antibody response, in contrast to pentamers dissociated from viruses (Doel and Chong, 1982). The difference between these two types of pentamers appears to be that pentamers derived from the disassembly of viruses have undergone the maturation cleavage and lost VP4, whereas pentamers derived from empty capsids still have the VP4 polypeptide attached in the intact form of VP0. Goodwin et al., (2009) also showed that the recombinant FMDV P1 and P1-2A lacking VP4 is recognised less well by antibodies specific for the G-H loop in the mature virion. Although virions have undergone the maturation cleavage, VP4 is still found associated with the inside of the structure. The structural changes conferred during cell entry and virion disassembly results in the

loss of VP4 from the structure. This loss of VP4 is hypothesised to be an “antigenic switch” which controls how the antigenic epitopes in the other regions of the capsid are recognised and is the basis for the investigation in this chapter.

6.2 Results

6.2.1 A Precision Protease recognition sequence inserted into VP0 mimics the maturation cleavage

The construction of plasmids which encode mutations in the maturation cleavage site are described in chapter 3. The site for both FMDV serotypes A and O contains the aa sequence FGALLA/DKKT (where “/” represents the junction between the VP4 and VP2 proteins). This sequence encoding this site was mutated to encode the sequence LEVLFQ/GPKT which is recognised by the 3C^{pro} from HRV which is commercially available and known as Precision Protease. This cleavage site will be referred to as Pre and the Protease as Pre^{pro}. Constructs engineered to contain the Pre cleavage site are referred to as “mat” (for maturation cleavage site).

In the FMDV structure, the VP4/2 site is inaccessible to external proteases, which is the reason for the generation of a hypothesis that this cleavage is performed by an RNA-induced auto-catalytic event (Hindiyeh et al., 1999). There is some conservation of the cleavage recognition sequences by FMDV 3C^{pro} and HRV 3C^{pro} (Blom et al., 1996), but they were considered to be different enough to allow their differentiation.

To determine whether the VP4/2 cleavage site in recombinant FMDV P1-2AΔ was accessible to Pre^{pro}, and if processing and maturation could be controlled independently, processing assays were performed in the RRL system described in chapter 4. The *wt* and *mat* mutant plasmids used in these experiments are described in chapter 3. Firstly, RRLs were programmed with either pA22P12Awt or pO1MP12Awt with the resulting translation products subjected to various combinations of protease treatment, and then analysed by SDS-PAGE and fluorography (methods sections 2.9 and 2.10) as

presented in figure 6.1A. When the lysates were mock treated or treated with 3C^{pro} alone (the first two lanes from the left on each gel), proteins of the expected sizes were seen for P1-2AΔ and the individual capsid proteins (VP0, VP1 and VP3) respectively. Upon treatment with Pre^{pro} either; after 3C^{pro} treatment, alone, before 3C^{pro} treatment or at the same as it (3rd-6th lanes from the left on each gel) no difference was observed in the migration of the band compared to the mock treatment. This demonstrated that Pre^{pro} had no effect against the 3C^{pro} cleavage sites. The RRLs were then programmed with either pA22P12*Amat* or pO1MP12*Amat* with the resulting translated products subjected to various combinations of protease treatment, and then analysed by SDS-PAGE and fluorography as presented in figure 6.1B. When the products expressed from these constructs were either mock treated or treated with 3C^{pro} alone (the 1st two lanes from the left on each gel in figure 6.1B), proteins of the expected sizes were seen for P1-2AΔ and the individual capsid proteins (VP0, VP1 and VP3) respectively. This demonstrated that 3C^{pro} was unable to cleave the Pre^{pro} site. However, when this 3C^{pro} treated sample was subjected to Pre^{pro} treatment, the band of the expected size for VP0 disappeared consistent with its cleavage. A concurrent increase in the signal for the band of the expected size for VP3 (and VP1 in pA22P12*Amat* due to VP1 and VP3 co-migrating in this serotype) was more intense (the 3rd lane from the left on each gel in figure 6.1B) consistent with VP2 now co-migrating in this position. VP4 (approx. 7kDa) was not expected to be seen in these samples due to it being outside of the resolving range for these 12% gels. This confirmed that the Pre^{pro} cleavage site was accessible to the enzyme after cleavage with 3C^{pro} for one hour which were the conditions that were used for standard pentamer formation. This demonstrated that VP4/2 junction was accessible to exogenous enzymes in the pentamer structure. Additional samples were also treated with Pre^{pro} alone and Pre^{pro} followed by 3C^{pro} (the 4th and 5th lanes from the left on each gel in figure 6.1B). In the first case, the band in the expected position for P1-2AΔ was seen to reduce in size by approximately 10kDa, corresponding with the loss of 7kDa VP4. In the second case, the same cleavage profile was seen as when these sequential cleavages were performed in reverse i.e. resulting in no VP0 and VP2 co-migrating with VP3. This demonstrated that

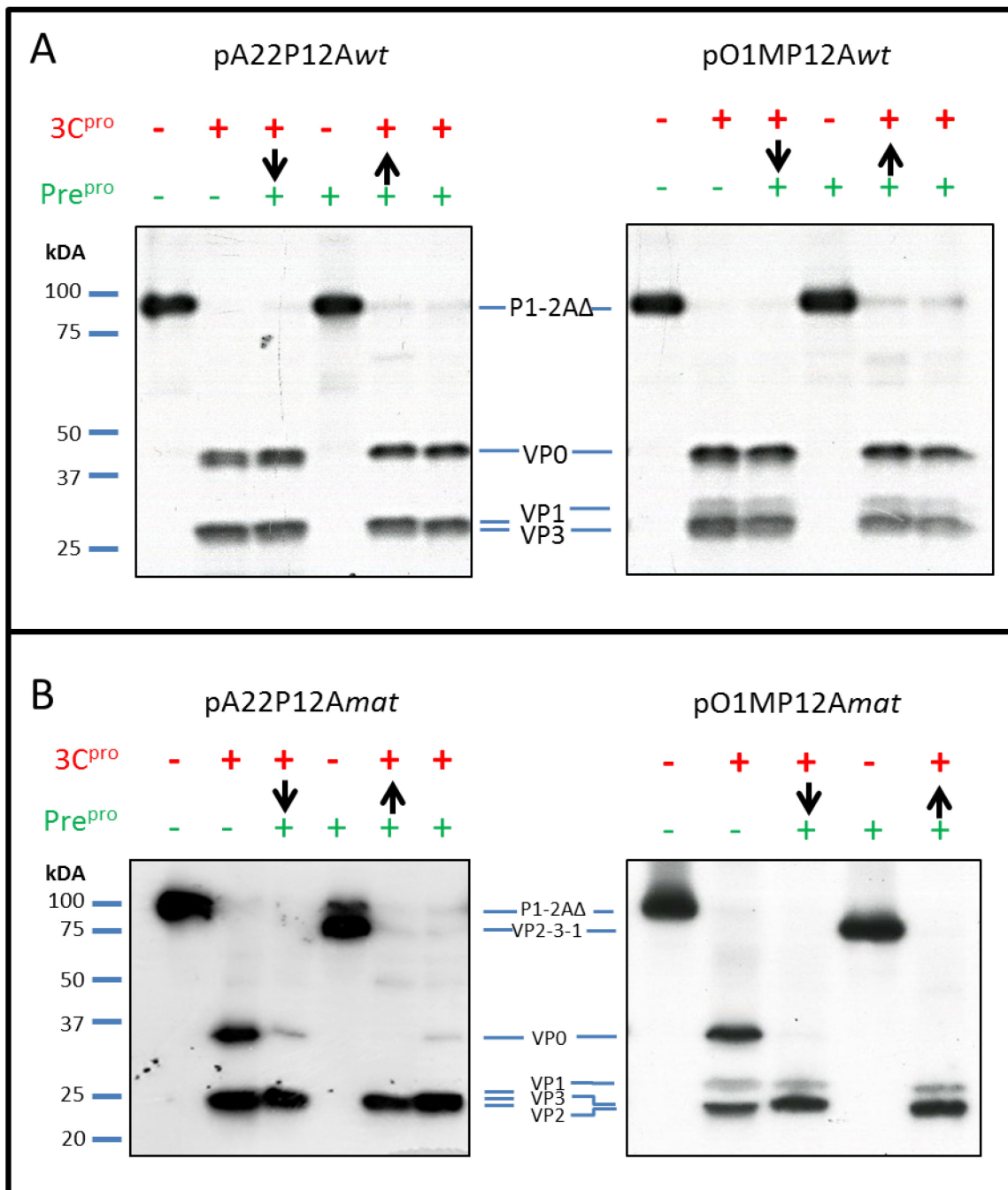


Figure 6.1 VP4/2 maturation mutant processing assay. TnT lysates were programmed with; pA22P12Awt or pO1MP12Awt (A), pA22P12A_{mat} or pO1MP12A_{mat} (B) and incubated for 1.5 hours at 30°C. After completion of the TnT, the lysate was divided into several tubes which were then mock processed, or processed with combinations of FMDV 3C^{pro} (1μM) and Precision Protease (Pre^{pro}; 0.2U/μl) indicated by “+” and “+” respectively, or were treated sequentially with one protease before the other (indicated by “→”). The reactions were incubated for a one hour at 37°C apart from the sequential protease reactions that were for one hour in each protease. All reactions were then separated on 12% SDS-PAGE gels, soaked in 1M sodium salicylate and exposed to X-ray film at -80°C. Where protein names are separated with a hyphen (-), they are uncleaved.

as well as being accessible in the pentamer, the Pre^{pro} site was also accessible in unprocessed P1-2AΔ, and that its cleavage did not prevent subsequent 3C^{pro} processing.

These results taken together demonstrated that cleavage of the substituted maturation cleavage site could be controlled independently of normal P1-2AΔ processing in what were expected to be P1-2AΔ and pentameric structures. Gradients were performed in the next section to determine the assembly phenotypes of these samples.

6.2.2 The maturation cleavage in protomers and pentamers allows their differentiation from uncleaved samples by sedimentation

The maturation cleavage normally occurs during the RNA encapsidation process in provirions as described in the introduction to this chapter. The structural consequences of the maturation cleavage event in P1 and pentamers is not known, but 12S pentamers, lacking VP4, from the FMDV disassembly pathway are found which suggests that pentamers can accommodate this cleavage and remain as an intact structure. To investigate the effect of structural changes on the sedimentation of P1 and pentamers, sucrose density gradients were performed. Initially, pO1MP12Awt programmed lysates were mock treated or treated sequentially with 3C^{pro} then Pre^{pro} or Pre^{pro} then 3C^{pro} (figure 6.2A; blue, red and green lines respectively). The processing profile established that Pre^{pro} was having no visible effect on wt P1-2AΔ or its 3C^{pro} processed products. The corresponding gradient profiles showed that while pentamers were still able to form in Pre^{pro} and 3C^{pro} treated samples, there appeared to be fewer pentamers formed if P1-2AΔ was treated with Pre^{pro} before 3C^{pro} than the other way round. The next set of gradients used the pO1MP12Awt as mock treated and 3C^{pro} treated as control gradient profiles (figure 6.2B; blue and red lines respectively) for the maturation mutant samples. The pO1MP12A_{mat} mutant was programmed into the RRL and split between conditions of mock treatment, or treatment sequentially with 3C^{pro} and Pre^{pro}. After the first protease treatment in the sequentially treated lysates, samples were taken for gradient analysis before treatment with the second protease and further gradient analysis. The mock treated sample

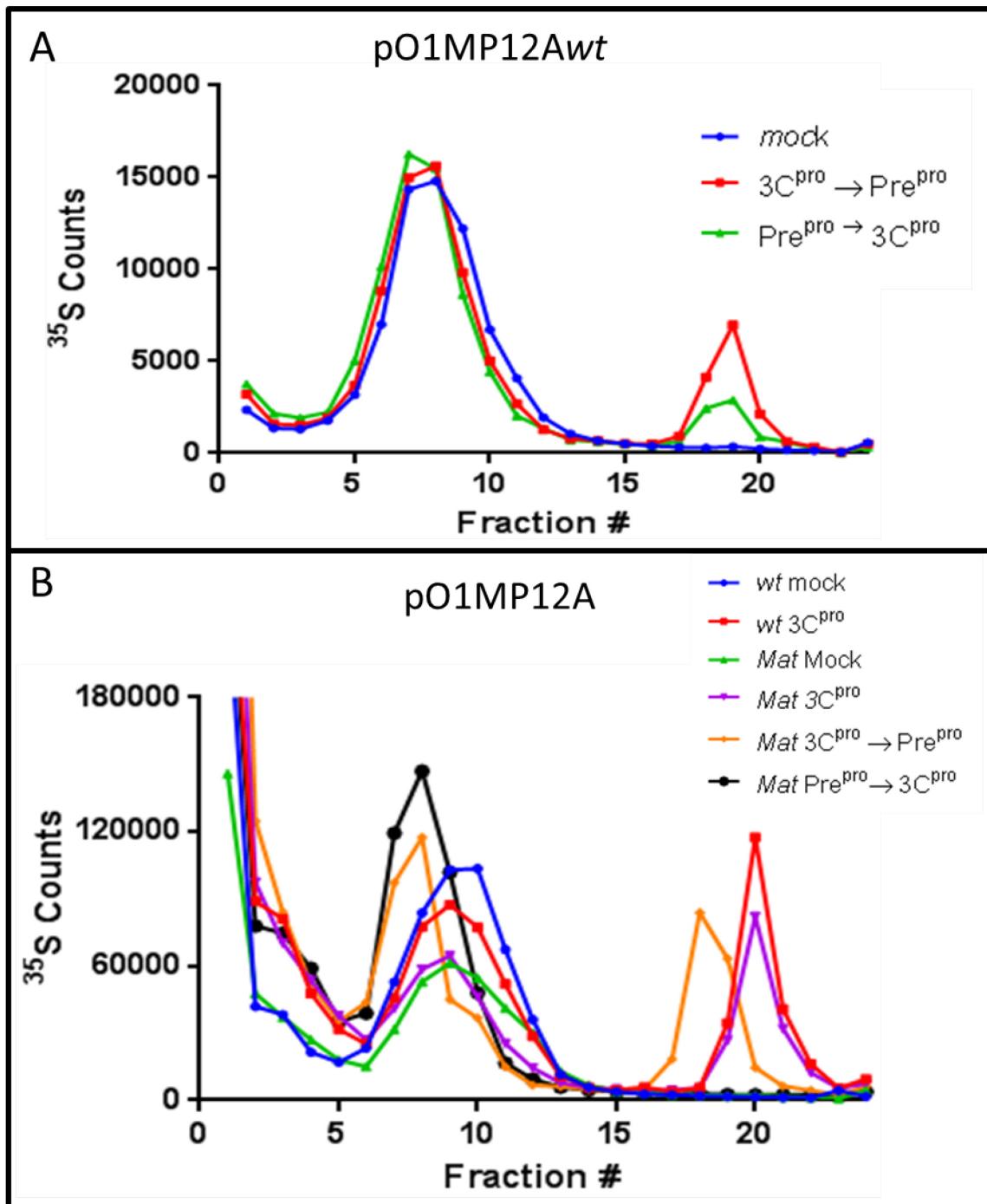


Figure 6.2. Cleavage of VP4 from assembled O1M pentamers retards their sedimentation in sucrose density gradients. TnT lysates were programmed with; pO1MP12Awt (A) and pO1MP12Awt or O1MP12A_{mat} (B) and incubated for 1.5 hours at 30°C. After completion of the TnT, the lysates were divided into several tubes which were then mock processed, or processed with combinations of FMDV 3C^{pro} (1μM) and Precision Protease (Pre^{pro}; 0.2U/μl) or were treated sequentially with one protease before the other (indicated by “→”). The reactions were then dialysed, and ultracentrifuged through a 5-30% SDG for 6 hours at 55000 rpm in an SW55Ti rotor at 10°C. The gradient was fractionated into 24 equal fractions using a Biocomp fractionator into 1mL scintillation fluid in scintillation vials. These were then counted on a scintillation counter and the counts plotted using Graphpad Prism software.

gave a peak in the expected position for P1-2AΔ (peak at fractions 9-10) corresponding to the P1-2AΔ peak in the *wt* mock treated sample (figure 6.2B; green and blue lines respectively) showing that inclusion of the mutant sequence for the Pre^{pro} cleavage site was not visibly affecting the sedimentation of P1-2AΔ. When this mutant was treated with 3C^{pro}, a second peak was seen in the expected position for pentamers (peak at fraction 20) corresponding with the pentamer peak in the *wt* sample treated with 3C^{pro} (figure 6.2B; purple and red lines respectively). This demonstrated that the maturation mutant was also able to assemble into pentamers indistinguishable from the *wt* sample. However, when this sample was subsequently treated with Pre^{pro} (figure 6.2B; orange lines), the peak for pentamers was 2 fractions (fraction 18) less far into the gradient than in the *wt* or 3C^{pro} treated samples. This finding was representative of several repeats of this experiment. Interestingly, this peak was in the position calculated in chapter 4 (figure 4.3) for 12S pentamers (fraction 18), whereas the pentamers with the faster sedimentation seen in all other experiments were in the position calculated for 14S poliovirus-like assembly pentamers (fraction 20). The peak for P1 was also one fraction less far (fraction 8) into the gradient than P1-2AΔ in the mock or 3C^{pro} only treated samples (fraction 9-10). The final lysate in this set was treated with Pre^{pro} before 3C^{pro} (black line). In this instance only one peak was observed, which was in the position of the slower sedimenting P1-2AΔ and no pentamers were seen either in the usual position or in the position of VP4-less pentamers. This demonstrated that P1-2AΔ lacking VP4 was unable to assemble into pentamers after processing. This also demonstrated that the cleavage of VP4 from P1-2AΔ or pentamers resulted in changes to their sedimentation characteristics and the reasons and implications of these findings will be analysed in the discussion to this chapter.

These experiments were repeated with pA22P12Awt and pA22P12A*mat*. Identical findings were observed as for the O-serotype P1-2AΔ proteins (albeit the *wt* pentamer formation was very low in this data set) (figure 6.3). This demonstrated that the loss of VP4 from assembled pentamers or P1-2AΔ retarded their sedimentation through sucrose density gradients and that the effect of VP4

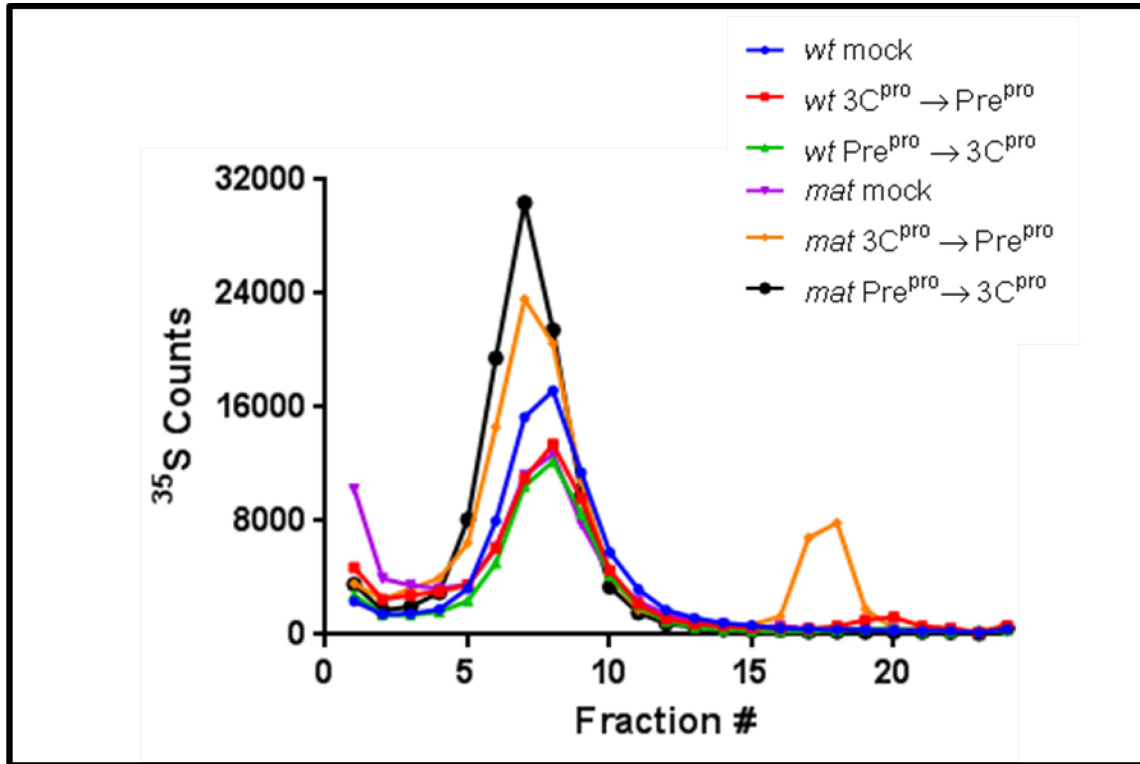


Figure 6.3. Cleavage of VP4 from assembled A22 pentamers retards their sedimentation in sucrose density gradients. TnT lysates were programmed with pA22P12Awt or A22P12A*mat* and incubated for 1.5 hours at 30°C. After completion of the TnT, the lysates were divided into several tubes which were then mock processed, or processed with combinations of FMDV 3C^{pro} (1μM) and Precision Protease (Pre^{pro}; 0.2U/μl) or were treated sequentially with one protease before the other (indicated by “→”). The reactions were then dialysed, and ultracentrifuged through a 5-30% SDG for 6 hours at 55000 rpm in an SW55Ti rotor at 10°C. The gradient was fractionated into 24 equal fractions using a Biocomp fractionator into 1mL scintillation fluid in scintillation vials. These were then counted on a scintillation counter and the counts plotted using Graphpad Prism software.

removal was the same in P1-2AΔ sequences representing an A and O serotype virus of FMDV. A further finding that was observed in both sets of gradients was that the signal for P1-2AΔ was greater for samples where P1-2AΔ was predominantly lacking VP4 due to the cleavage introduced at the VP4/2 junction (figures 6.2B and figure 6.3; orange and black lines) than P1-2AΔ with VP4. The reasons for this observation and the others are speculated upon in the discussion to this chapter.

6.2.3 The antigenicity of protomers and pentamers with different properties can be probed using an antibody panel

The sucrose density gradients in the previous section demonstrated that two populations of protomers and pentamers either containing intact VP0, or having undergone a mimicked maturation cleavage to cleave off VP4, could be differentiated by their sedimentation in sucrose density gradients. FMDV 12S pentamers that lack VP4 no longer possess the same antigenic characteristics as 14S particles on the assembly pathway that have an intact VP0 protein (Doel and Chong, 1982, Rowlands et al., 1975, Saiz et al., 1994, Goodwin et al., 2009). A modified immuno-precipitation assay was performed to investigate whether the loss of VP4, and the subsequent structural changes which resulted in the altered sedimentation observed, were responsible for conferring an altered antigenic phenotype on these P1-2AΔ and pentameric particles. The antibodies used for this assay were:

1. Guinea pig sera raised against A22 Iraq and O1 Manisa vaccine antigens. These antisera are used in indirect sandwich ELISAs for FMDV detection by the World Reference Laboratory at Pirbright and were expected to recognise immunodominant epitopes on the capsid structure (from the world reference laboratories at Pirbright).
2. Two single-domain antibody fragments raised in Llamas that bind specifically and can differentiate between the antigenic structures on 146S intact virions and 12S pentamers from the dissociation pathway of O-serotype viruses. These are named M170 and M3 respectively (Harmsen et al., 2011) (from Eva Perez at the Pirbright institute).

3. The monoclonal antibodies B2 and D9 that are characterised to be specific for the immunodominant G-H loop of VP1 in FMDV O1 Lausanne (Crowther et al., 1993, Kitson et al., 1990) but, which are cross-reactive against O1 Manisa (Mahapatra et al., 2008) (from Terry Jackson at Pirbright).

High-bind ELISA plates were coated in triplicate with each antibody at a dilution determined from existing protocols and published data (in the above references with each antibody description) to be suitable for the detection of FMDV antigens by direct or indirect ELISA. The plates were subsequently blocked with BSA. The P1-2AΔ peak fractions from the pO1MP12A gradient in figure 6.2B were analysed. These samples were *wt* unprocessed P1-2AΔ and 3C^{pro} processed P1 (*wt* mock and *wt* + 3C^{pro} respectively), and maturation mutant unprocessed P1-2AΔ, 3C^{pro} processed P1 and 3C^{pro} followed by Pre^{pro} processed P1 (*mat* mock, *mat* + 3C^{pro} and *mat* + 3C^{pro} → Pre^{pro} respectively). These pooled fractions were diluted in a BSA containing buffer and added in triplicate to each antibody condition and left to adsorb before the wells were washed to remove unbound material. The bound proteins were removed with SDS solution and the ³⁵S counts were analysed. Each sample was also analysed to obtain a value for the total amount of counts that had been in each sample prior to the immuno-precipitation. Each bound sample was then represented graphically as a percentage of the total counts to determine how much of each sample had bound to each antibody (figure 6.4A). It appeared that at most only about 30% of the input counts had bound to any of antibodies and the counts obtained for most antibodies were around the same as the block only control. Guinea pig sera raised against O1 Manisa should have provided a positive control for the assay but only a negligible increase in counts was observed when it was used. The one antibody that showed a higher signal in any of the samples was M3. M3 is the antibody that was expected to be specific for 12S dissociated pentamers (Harmsen et al., 2011). While all of the P1-2AΔ samples had roughly the same levels of binding to M3, P1 that lacked VP4 (figure 6.4A; *mat* + 3C^{pro} → Pre^{pro}) had slightly more counts than the other samples.

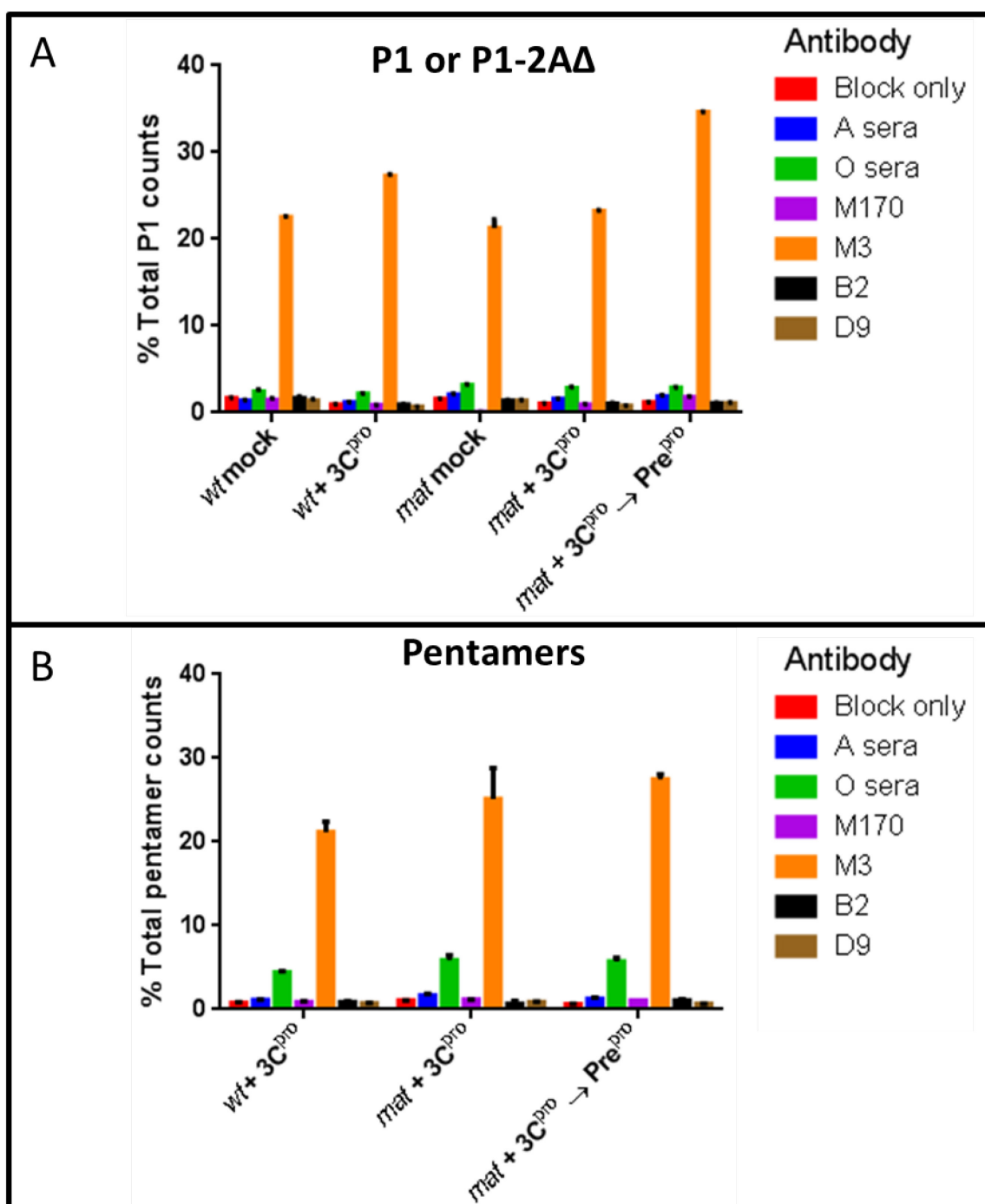


Figure 6.4 Antigenic characteristics of gradient samples. A BSA block, or the antibodies indicated in the figure legend were diluted in a coating buffer, added to high bind ELISA plates in triplicate and left to adsorb overnight at 4°C. The antibodies were then washed off and the plates were blocked. Samples of the peak and shoulder fractions from the observed P1 or P1-2AΔ (A) and pentamers (B) in figure 6.2B were pooled, diluted 1:2, counted by scintillation counting, and added in triplicate to the blocked plates. After 1 hour incubation at room temperature, the samples were washed away and 2% SDS solution was added to each well to remove all proteins which were then counted by scintillation counting and the counts plotted using Graphpad Prism software. Error bars indicating standard error of the mean are shown for the triplicate samples.

Peak fractions were collected in the same way for the two samples from figure 6.2B containing pentamers sedimenting in the regular “14S” position (figure 6.2B; fractions 19-21 of gradients plotted with the red and purple lines), as well as the sample with pentamers that had sedimented in the slower “12S” position (figure 6.2B; fraction 17-19 of the gradient plotted with the orange line). The modified immunoprecipitation assay was performed in the same way as for the P1-2AΔ samples and is presented in figure 6.4B. The assay encountered the same problems as seen in figure 6.4A in that very little sample had bound to any antibody apart from M3, although the O-sera showed a slightly stronger signal than was seen in the P1-2AΔ samples. The counts for the M3 antibody condition were between 20-30% of the input material in every sample. Slightly more input sample from the slower sedimenting pentamers which lack VP4 than from the pentamers with intact VP0 was retained in the M3 coated wells, again suggesting that the M3 antibody has a higher affinity for P1 and pentamers without VP4. Why so little material could be precipitated with the other antibodies during this assay is unknown but it was thought to be a technical problem with the assay rather than a true effect of the antibodies not being specific for the samples and some hypotheses are speculated upon in the discussion.

6.3 Discussion

6.3.1 Summary

This chapter has presented data to show that an artificial protease cleavage site, that is recognised by the enzyme Precision Protease (Pre^{pro}), can be inserted into the VP4/VP2 junction of VP0. This site could be successfully cleaved which resulted in a reduction in the sedimentation of both P1-2AΔ and pentamers derived from sequences of FMDV A22 Iraq and O1 Manisa viruses. Attempts were made to use an immuno-precipitation assay to differentiate P1 or P1-2AΔ and pentamer species containing VP4 from those where it had been cleaved off but further assay optimisation appeared to be required in order confirm if this is possible.

A concern raised in the design of the plasmid constructs to mutate the sequence around the FMDV VP4/2 junction to the Pre^{pro} recognition sequence was that the recognition sequences of Pre^{pro} and 3C^{pro} may overlap because Pre^{pro} is the 3C protease from the picornavirus HRV. The FMDV 3C^{pro} cleavage site shows more promiscuity in its cleavage site requirements than Pre^{pro} which has fairly stringent requirements for glutamine-glycine residues in the P1 and P1' sites (Cordingley et al., 1990, Blom et al., 1996). FMDV 3C^{pro} cleaves at sites where glutamine and glutamic acid are in the P1 position, and glycine, threonine and leucine in the P1' position. This is especially true of the cleavage sites between the P1-2A structural proteins of A and O serotype viruses (Carrillo et al., 2005) but the protease also has strong requirements for appropriate residues in the P2, P4, P2' P4' sites relative to the cleavage junction (Gullberg et al., 2014, Birtley et al., 2005, Zunszain et al., 2010, Curry et al., 2007). It was thought to be this requirement for recognition sequence either side of the cleavage junction that meant that 3C^{pro} was unable to cleave the artificial input site introduced into the VP4/2 junction, and conversely that Pre^{pro} was unable to cleave the VP0/3 and VP3/VP1 sites in FMDV P1-2AΔ. It is possible that Pre^{pro} was able to cleave the VP1/2A junction, but this could not be determined easily in our assays and was thought unlikely due to conserved glutamate/leucine and glutamine/threonine sequences present in at the P1-P1' sites in this junction. It was thought that if Pre^{pro} was able to cleave the VP1/2A junction there would be no impact on pentamer formation anyway because 2A has been shown to be dispensable for pentamer formation (Goodwin et al., 2009). The demonstration that VP4 was being released following Pre^{pro} treatment was not possible because of its small size (~7kDa), which would have migrated off of the end of the 12% SDS-PAGE gels that were used. However, upon Pre^{pro} treatment, the band of the expected size for P1-2AΔ reduced in size by approximately 10kDa and the band in the expected position for VP0 disappeared to be replaced with a band of increased signal strength in the expected position of VP2. A future experiment would be to look for the presence of VP4 on a higher percentage Tris-Tricine gel to confirm that the processing phenotypes observed were due to the loss of a VP4 protein that could be visualised.

The maturation cleavage site is buried within the structure of the virion, inaccessible to exogenous proteases, and as such is considered an autocatalytic event (Arnold et al., 1987). The experiments presented in this chapter demonstrate that a minimally altered maturation cleavage site can be accessed by an exogenous protease in the structures of P1-2AΔ and pentamers. This may indicate that while structural change are likely to be occurring in the other parts of the P1 structure after 3C^{pro} processing and subsequent pentamer formation, the structure of the amino acids around the maturation cleavage site remain accessible to the protease.

Having a high cleavage specificity at these sites allowed the control of these events during assembly, and this was explored using sucrose density gradients. The most striking finding from these experiments was that by mimicking the maturation cleavage and therefore removing VP4 from assembled pentamers, the new “pentamers” had a reduction in their sedimentation coefficient. This new sedimentation value was in the expected position for 12S pentamers when compared with the sedimentation markers in chapter 4 (figure 4.3), and the pentamers from which they were derived resembled the position of 14S pentamers reported in other picornaviruses. Other than the *Aphthoviruses*, picornaviruses do not fall apart into pentamers upon entry into cells (Vasquez et al., 1979), but rather form empty capsid shells lacking VP4 and RNA e.g. in PV (Fricks and Hogle, 1990). Therefore the only pentamers that are found in these infected cells are from the assembly pathway and have intact VP0 (reviewed in Jiang et al., 2014, and Ansardi et al., 1996). FMDV pentamers are frequently reported as being 12S (Goodwin et al., 2009, Doel and Chong, 1982) but perhaps the pentamers that are 12S are those without VP4, isolated from the disassembly pathway, and FMDV assembly competent pentamers (with intact VP0) are in fact 14S as well. The sizing of pentamers in these experiments is based upon comparisons with gradient markers. To gain accurate measures of pentamer sedimentation coefficients, it would be necessary to perform analytical ultracentrifugation, however it does appear that the loss of VP4 does retard their sedimentation.

The loss of VP4 also means that these pentamers lack the myristate groups, shown in chapter 4 and by others (Abrams et al., 1995, Ansardi et al., 1992) to be necessary for assembly. Given that myristate groups are thought to play a role in compacting the virion structure for stability (Filman et al., 1989), their loss appears to result in “less aerodynamic” pentamers, that sediment more slowly through sucrose. The sedimentation of P1-2AΔ was also reduced without VP4 also suggesting a less compact structure although the reduced mass of these samples due to VP4 loss may also contribute to their reduced sedimentation. The VP4-less P1-2AΔ was no longer competent for assembly after 3C^{pro} processing. This may be as a result of the loss of the myristate group required for assembly or a requirement for VP4 in the assembly process, but the effect of these two factors was not possible to separate due to not being able to myristoylate VP4-less P1 in this study. However, in future studies, the N-terminus of VP2 could be substituted to encode a myristoylation signal and the assembly phenotype of these protomers investigated. In agreement with data in chapter 4, the lack of VP4 again appeared to reduce the adsorption of the samples to plastics as higher gradient counts were obtained in these samples. If the assembly of pentamers without VP4 could be scaled up in the vaccinia virus expression system described in chapter 3, they could be used as a novel means to obtain their crystal structure to determine the structural basis for changes to antigenicity.

Few conclusions could be drawn from the preliminary immuno-precipitation assay to test the antigenicity of assembly components due to the low counts that were obtained with most antibodies. The precise reasons for this are unknown but several alterations were made to the protocol including increasing the antibody concentrations and reducing the stringency of the block and wash steps but to no effect. The assay was performed in ELISA plates due to the number of samples and replicates required but, performing the assay as a standard immuno-precipitation with fewer samples may make the results easier to analyse.

The only antibody that did show a higher level of binding than the others was M3, which appeared to have affinity for all forms of P1 and pentamer whether cleaved with 3C^{pro} or Pre^{pro}. This antibody has been shown to be specific for dissociated pentamers but does not recognise intact virions (Harmsen et al., 2011). From several data replicates using this antibody the working hypothesis is that the epitope for M3 is located on the inside surface of the capsid. This would preclude it from binding to intact virions and empty capsids but would allow it to bind to P1 and pentamers of any form. This hypothesis would need to be tested by having a capsid control in the experiment but M3 does not appear to be useful in understanding the reasons for the change in the antigenicity caused by the loss of VP4 (Rowlands et al., 1975). M3 did appear to bind slightly more strongly to pentamers and P1 without VP4 than with though, so perhaps VP4 slightly obscures the binding site on the inside of the capsid. Of note, the 146S specific M170 antibody was used at the same concentration as M3 and so would be expected to be at the correct titre required to bind if an interaction was available, but this could also be a result of the antibodies having different affinities. Perhaps the reason this antibody is only specific against 146S particles is that its epitope is located at the interface between two pentamers. Having a capsid or virus control would confirm this theory.

Recently a panel of monoclonal antibodies have become available that are specific for the complete range of neutralising epitopes in O-serotype capsids (Asfor et al., 2014). By using these reagents in a modified assay to test antigenicity, the precise contributions of each antigenic site on the O-capsid (Crowther et al., 1993, Kitson et al., 1990) in differentiating between immunogenic and non-immunogenic pentamers can be determined.

Chapter 7: Discussion and future work

Chapter 7: Discussion and future work

Capsid assembly is fundamentally required for viruses to generate a protein coat with which to protect and transport their genomes between host cells. Picornaviruses are no different in this respect and they code for the production of a structural protein in the P1 region of their RNA genome which multimerises first into pentameric structures and then into an icosahedral capsid in which the genome is housed (section 1.7.3). In bacteriophages, and viruses such as HBV and HIV, the mechanisms by which the capsid proteins are able to assemble and produce infectious virions have been fairly well characterised (section 1.7.2). Much of what is known about picornavirus assembly has been inferred by the atomic structures of the virions and findings in enteroviruses. The cellular chaperone Hsp90 has been demonstrated to be required for proteolytic processing of PV P1 (Geller et al., 2007) and glutathione has been demonstrated to stabilise pentamer formation in certain enteroviruses (Ma et al., 2014a, Thibaut et al., 2014). The assembly of enterovirus pentamers into empty capsid shells has been demonstrated to proceed in the presence of high enough concentrations of pentamers (Rombaut et al., 1991, Li et al., 2012) and the packaging of RNA into capsids has been shown to require interactions between the non-structural protein 2C^{ATPase} and the viral capsid protein VP3 (Liu et al., 2010) as well as interactions between pentamers and RNA (Verlinden et al., 2000). The maturation cleavage in poliovirus packaged capsids has been shown to require amino acid residues on the inside of the capsid (Hindiyeh et al., 1999) that are proposed to activate local water molecules in a nucleophilic attack of the scissile bond (Basavappa et al., 1994) and lock capsids into a meta-stable state for infection of new cells (Hogle, 2002). Upon infection of new cells, and in vaccine preparations that lack a sufficient cold chain, PV virions undergo conformational changes and form empty capsids (De Sena and Mandel, 1977). For FMDV the equivalent process leads to dissociation of the capsid into pentamers (Mak et al., 1970). In both PV and FMDV the resulting structures lack VP4 and are no longer immunogenic as vaccines (reviewed in Hogle, (2002) and, Rodriguez and Grubman, (2009)).

This thesis has presented work to address some of the gaps in understanding that were felt to be present in the FMDV assembly and disassembly pathways. Recombinant proteins were largely used by these investigations in a reductionist approach to isolate and control the processing of P1 by 3C^{pro} that would not be possible in the virus life cycle as a whole. Our findings in these systems are discussed in the context of the whole virus infection system.

Rabbit reticulocyte lysates have proven useful tools in the study of assembly in picornaviruses with type II IRES elements e.g. FMDV (Clarke and Sangar, 1988) and EMCV (Palmenberg, 1982) and are thought to provide an environment that can be used to express and analyse authentic virus proteins. Rabbit reticulocyte lysates were used to express P1-2AΔ and then develop a P1-2AΔ processing and assembly assay (using exogenous 3C^{pro}) in which the early stages of the assembly process could be analysed. Processing was assessed using SDS-PAGE gels and autoradiography and assembly was analysed by using sucrose density gradients validated with marker proteins (chapter 4). Several interesting findings were obtained during the development of this assay including the apparent requirement for a component that facilitates pentamer assembly but is lost during dialysis. We hypothesised that this could be a requirement of the chaperone machinery (Geller et al., 2007) such as ATP, or a requirement for glutathione (Ma et al., 2014a, Thibaut et al., 2014), however, re-addition of these compounds was unable to return the assembly phenotype (chapter 5). Subsequent information has suggested that the concentration of GSH was approximately 3log₁₀ too low and so this concentration should now be attempted along with including an appropriate concentration of Mg²⁺ ions for the activity of ATP. It is possible that FMDV pentamer assembly requires additional small molecules, but it was also observed that performing a dialysis step before processing resulted in a reduction in the amount of P1 signal. This was investigated further and it was found that pentamer formation is more efficient at higher concentrations. This, along with the requirement for sufficient pentamer concentrations for capsid assembly (Rombaut et al., 1991, Li et al., 2012), supports the mathematical model, described in 1.7.2.1, in which kinetic barriers to assembly are

overcome when subunit components are at a higher concentration. To determine whether other small molecules are involved in assembly, mass spectrometry could be performed on the purified material described in chapter 3 to analyse binding partners that co-purify with P1-2AΔ. A threshold concentration for PV pentamers to self-assemble into capsids *in vitro* was previously determined to be 1.6nM (Rombaut et al., 1991). P1-2AΔ in the assembly reactions used in this study could be quantified to determine if there is also a threshold concentration for pentamer assembly *in vitro*. To quantify P1-2AΔ in RRLs, a radiolabelled P1-2AΔ expression could be performed in vaccinia viruses, the P1-2AΔ protein purified as described in chapter 3 and quantified using a Bradford or BCA assay. A dilution series of this reagent could then be run alongside RRL expressed P1-2AΔ on SDS-PAGE gels or counted by scintillation counting. The radioactive signal from RRL expressed P1-2AΔ could then be quantified by comparison with the corresponding dilution with the same signal strength.

Specific mutations were made to P1-2AΔ plasmid expression constructs to analyse the effect on pentamer formation of preventing P1-2AΔ myristoylation and 3C^{pro} processing. P1-2AΔ with a substitution in the myristoylation signal was processed as efficiently by 3C^{pro} as the wild-type samples but was unable to assemble into pentamers (chapter 4). These findings agree with the work of Ansardi et al. (1992) who showed that this was also true of unmyristoylated PV P1. However, in the study by Ansardi and colleague, unmyristoylated P1 could assemble into a higher order structures when complemented with myristoylated P1 provided by an infecting virus. In PV, myristoylation is thought to stabilise protomer-protomer interactions in pentamers (Moscufo and Chow, 1992). It would be interesting to determine whether FMDV pentamers can also support the incorporation of a proportion of unmyristoylated protomers. These experiments could be performed by making a pool of unmyristoylated radiolabelled protomers and mixing them in a range of molar ratios with unlabelled myristoylated protomers and detect whether any radiolabel is present in the pentamer position.

Previous studies have shown that processing at the VP3/1 junction is required for pentamer assembly (Parks and Palmenberg, 1987). In PV, the VP0/3 junction is cleaved relatively inefficiently by the protease compared with the VP3/1 junction, and increasing the efficiency at VP0/3 is lethal for the virus (Blair et al., 1993). This suggests that there is a requirement for processing to happen in a sequential manner, indicating that either the VP0/3 junction may only become accessible after cleavage of the VP3/1 junctions or that sequential processing allows for structural rearrangements to occur for assembly. The order of cleavage in FMDV has also been demonstrated to be VP3/1 before VP0/3 (Grubman et al., 1995). The data presented in chapter four demonstrate that when a mutation was inserted into the DNA sequence to change the amino acid sequence at the VP3/1 junction in FMDV P1-2AΔ, processing at this junction and the assembly of pentamers was prevented. Changes to the amino acid sequence at the VP0/3 junction almost completely prevented processing at this junction and only a correspondingly small amount of pentamer formation was observed. In both cases P1-2AΔ was still fully processed at the non-substituted sites demonstrating that both sites can be cleaved independently and do not require structural rearrangements in order for the cleavage site to be made accessible to the protease. The small amount of pentamer assembly in the VP0/3 mutant suggests that retarding this cleavage, which is naturally slower in the virus, does not prevent pentamers assembling. Taking all of the existing and new information together, it would be interesting to introduce a cleavage site at VP3/1 that only retards, rather than prevents cleavage entirely and analyse the effects on pentamer formation with the hypothesis being that pentamers cannot form when the junctions are cleaved in the order VP0/3 the VP3/1. The reasons for the requirement of a sequential cleavage are likely to be that structural rearrangement is necessary between cleavages that facilitate assembly. Given the role played by cellular chaperones in protein folding, the function of the Hsp90-P1 interaction (demonstrated in chapter 5) could be to chaperone the structural transitions induced in the P1 structure during processing to facilitate its assembly into pentamers.

Cleavage at VP1/2A is the slowest of the P1-2A cleavages (Grubman et al., 1995). Mutations that are present some distance upstream of this junction and found in cell culture adapted FMDV, can prevent P1-2A cleavage but empty capsids can still assemble with 2A still attached (Gullberg et al., 2014). Only a residual four amino acids of the 2A protein were encoded in our expression constructs and it is not required for FMDV pentamer assembly *in vitro* (Goodwin et al., 2009) but perhaps the cleavage of the P1-2A junction is sufficiently slow that 2A participates in a downstream process such as RNA encapsidation.

Chapter 5 demonstrated the requirement for Hsp90 in the lifecycle of FMDV. Treatment of cells with an Hsp90 inhibitor reduced the growth of virus in culture, without affecting the translation and RNA synthesis of an FMDV sub-genomic replicon. Pentamer assembly in the cell-free system was affected by treatment with the Hsp90 inhibitor but in a processing-independent manner. The canonical model for Hsp90 function is that client proteins are passed from translation complexes and cycled through folding interactions with Hsp70 proteins before being passed to Hsp90 (Röhl et al., 2013). Interestingly PV P1 has been shown to co-immunoprecipitate with Hsp70 as well (Macejak and Sarnow, 1992). In the current study, it is therefore likely that upon completion of the translation reactions, P1-2A Δ is already bound to either Hsp70 or Hsp90 and the interaction must persist through P1-2A Δ processing to facilitate pentamer assembly. Treatment of this complex with the Hsp90 inhibitor, 17DMAG, did not affect processing in the cell-free assay. In a previous study, treatment of PV infected cells with 17DMAG removed the interaction of P1 with Hsp90 (Geller et al., 2007) but in contrast to the current report, P1 processing was inhibited. It is possible that FMDV and PV have evolved different requirements for Hsp90, but, otherwise this discrepancy is difficult to explain without further knowledge of the interactions that P1 has with chaperones. These could be further investigated by using inhibitors of proteins such as Hsp70 and depleting lysates of certain chaperone components such as p23 and the Hsp90 organising protein (HOP). A pulse-chase

experiment in FMDV infected cultures may also be able to determine whether Hsp90 inhibition affects P1-2AΔ processing.

Myristoylation, proteolytic processing of P1-2AΔ and the assistance of the molecular chaperone Hsp90 were shown to be required for assembly of P1-2AΔ into pentamers. This suggests that important structural changes are required in the transition of P1-2AΔ into pentamers. The external structure of bovine enterovirus pentamers has been reconstructed from transmission electron microscopy and can be fitted precisely into the space occupied by pentamers in the structure of the complete virus (Li et al., 2012). This suggests that large-scale structural changes are not required for pentamers to assemble into capsids. To be able to analyse what structural changes in P1-2AΔ are involved in the transitions from P1-2AΔ to pentamer, large amounts of purified, recombinant P1-2AΔ material are required. Previous studies using recombinant P1 and P1-2A expressed in, and purified from *E.coli* (Goodwin et al., 2009), were not able to produce sufficient material to facilitate the analysis of structure. To address this problem, we developed a system to express and purify high levels of P1-2AΔ from recombinant vaccinia virus infection of mammalian cells (chapter 3). P1-2AΔ was seen to express to high levels, and after extensive optimisation, a proportion could be solubilised and purified. Future studies should be performed to scale up of production of this material to produce the quantities required for biophysical studies of assembly, such as dynamic light scattering and X-ray crystallography. Due to the high levels of expression observed in this system, and the role of P1-2AΔ as a client of Hsp90, perhaps over-expression of Hsp90 in the cells would improve P1-2AΔ purification and should be performed in future experiments. Additionally 3C^{pro} is present in infected cells, and is known to perform many roles (section 1.5.2.2), so it perhaps has a role in chaperoning P1-2AΔ along with its canonical role in processing. This could be tested by expressing P1-2AΔ with substituted protease recognition sites in the presence and absence of co-expressed 3C^{pro} in order to isolate such a role for 3C^{pro} from the normal role of processing.

FMDV virions have a propensity to dissociate into pentamers under slightly acidic conditions (and other mild stresses such as increases in temperature) thought to be as a result of electrostatic repulsion between two pentameric subunits in their structure (Curry et al., 1995, Ellard et al., 1999). This poses a problem for the stability of vaccines because upon dissociation these pentamers no longer possess the same antigenic characteristics as the virus (Rowlands et al., 1975). In contrast, pentamers on the assembly pathway are antigenically similar to the virus (Doel and Chong, 1982, Goodwin et al., 2009). The only apparent difference between these pentamers is the lack of VP4. The investigations performed in chapter 6 demonstrated that an artificial cleavage site could be engineered into the VP4/2 junction and used to control a maturation-like event in recombinant proteins. It was demonstrated that P1-2AΔ processed at the VP4/2 site to release VP4 was unable to assemble into pentamers after subsequent processing with 3C^{pro}. Processing as normal with 3C^{pro} first led to the expected assembly pentamers that sedimented in a position of approximately 14S, consistent with the properties of pentamers on the assembly pathway. Subsequent processing of such pentamers to mimic the maturation-like event induced a shift in sedimentation to approximately 12S, consistent with the properties of pentamers dissociated from virus, and presumably due to the release of VP4. Antibodies that recognise virus-like antigenicity (Harmsen et al., 2011) did not appear to bind to either of these reagents suggesting that the epitope that they recognise is at a pentamer interface. Conversely antibodies raised against dissociated virus particles (Harmsen et al., 2011) recognised both of these components as well as unassembled P1-2AΔ molecules which suggested that the epitope for this antibody is on the inside surface of pentamers. Further refinement of this assay was required, but it appeared to be a useful means by which to investigate problems with capsid antigenicity.

The data presented here demonstrate how the assembly and disassembly processes in FMDV can be modelled and explored using a range of recombinant systems. Findings in these recombinant systems can be used to inform and direct the investigation of how these processes work in more

complicated systems. For example, by demonstrating a requirement for Hsp90 in pentamer formation, the interactions of Hsp90 (and closely related chaperones) with components of the assembly pathway can be targeted *in vitro* and *in vivo*. The findings in these experiments have led to a refined model for FMDV assembly and disassembly that can be further investigated in future studies.

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Appendices

Appendix 1: Primers for generation of FMDV capsid precursor

Primer Sequence 5' to 3'	Description
TATATGGATCCGTTTAAACTTTCCACAAC <u>TG</u>	Forward primer to make A22 and O1M P1 constructs with a BamHI site
ATATGGATCCTCAGTGGTGGTGGTGGTGGTGTGC -AGCTTGTTTTGCAGGTGCAATGATC	Reverse primer to make A22 P1 construct with a His tag
ATATGGATCCTCAGTGGTGGTGGTGGTGGTGGTGTGC -AGCGAAGTTCAAAAGTTGTTTTGCAGGTG	Reverse primer to make A22 P1-2A construct with a His tag
ATATGGATCCTCAGTGGTGGTGGTGGTGGTGGTGTGC -AGCCTGTTTCACCGGTGCCACAATC	Reverse primer to make O1M P1 construct with a His tag
ATATGGATCCTCAGTGGTGGTGGTGGTGGTGGTGTGC -AGCAAAATTTAGAAGCTGTTTCACCGGTG	Reverse primer to make A22 P1-2A construct with a His tag

Key:

- **Red** = Insertion of restriction site
- **Purple** = Insertion of His-tag
- **Green** = 2A sequences
- **Blue** = Insertion of spacer sequence
- **Underlined** = Part of primer that binds to template

Appendix 2: Primers for mutagenesis of FMDV Capsid Precursors

Primer Sequence 5' to 3'	Description
GAAAGGTTACCATGGCAGCCGGGCAATCCAG	Forward primer to mutate the myristoylation signal in A22 and O1M constructs
CTGGATTGCCCGGCTGCCATGGTAACCTTTC	Reverse primer to mutate the myristoylation signal in A22 and O1M constructs
GCTCCCCCTCGAAAGACGGGATTGTACCGGTC	Forward primer to mutate the VP2/3 cleavage junction in A22
GACCGGTACAATCCCCTCTTTTCGAGGGGAGC	Reverse primer to mutate the VP2/3 cleavage junction in A22
AGTTCCCTTCCAAAGACGGGATCTTCCCTGTGG	Forward primer to mutate the VP2/3 cleavage junction in O1M
CCACAGGGAAGATCCCCTCTTTGGAAGGGAAC	Reverse primer to mutate the VP2/3 cleavage junction in O1M
TTGACCCCCGCTCAACACCACTACCACCGGG	Forward primer to mutate the VP3/1 cleavage junction in A22
CCCGGTGGTAGTGGTGTGAGCGGGGGTCAA	Reverse primer to mutate the VP3/1 cleavage junction in A22
GTGGATGCTCGCACAAACACTACCTCCGCGGGC	Forward primer to mutate the VP3/1 cleavage junction in O1M
GCCCGCGGAGGTAGTGTGTCGAGCATCCAC	Reverse primer to mutate the VP3/1 cleavage junction in O1M
CTGAGGCTTCTTTAAAGCGCTC	Forward flanking primer for insertion of a cleavage-site into VP4/2 for A22 and O1M
CTGGAAGTTCTGTTCAGGGGCCAAGACCGAGG-AGACCACTCTCCT	Forward primer to insert a cleavage-site into the VP4/VP2 junction for A22
GGGCCCCTGGAACAGAACTTCCAGAAGACCGCTG-AAAGCAGAACTG	Reverse primer to insert a cleavage-site into the VP4/VP2 junction for A22
GTCTCACCGCCGTAGTTTTCAA	Reverse flanking primer for insertion of a cleavage-site into VP4/2 for A22
CTGGAAGTTCTGTTCAGGGGCCAAAACCGAGG-AGACCACTCTTCTG	Forward primer to insert a cleavage-site into the VP4/VP2 junction for O1M
GGGCCCCTGGAACAGAACTTCCAGAAGACCGCTG-AAAGCGGAAC	Reverse primer to insert a cleavage-site into the VP4/VP2 junction for O1M
CTGTGTCTCGCCACCGTAATTCT	Reverse flanking primer for insertion of a cleavage-site into VP4/2 for O1M

Key:

- Red = Mutated or inserted bases