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The Structure of Foot-and-Mouth Disease Virus

E. E. Fry[1] · D. I. Stuart[1] (✉) · D. J. Rowlands[2]

[1] Division of Structural Biology, Wellcome Trust Centre for Human Genetics, Roosevelt Drive, Oxford, OX3 7BN, UK
enquiries@strubi.ox.ac.uk
[2] School of Biochemistry and Microbiology, University of Leeds, Woodhouse Lane, Leeds, LS2 9JT, UK

Abstract Structural studies of foot-and-mouth disease virus (FMDV) have largely focused on the mature viral particle, providing atomic resolution images of the spherical protein capsid for a number of sero- and sub-types, structures of the highly immunogenic surface loop, Fab and GAG receptor complexes. Additionally, structures are available for a few non-structural proteins. The chapter reviews our current structural knowledge and its impact on our understanding of the virus life cycle proceeding from the mature virus through immune evasion/inactivation, cell-receptor binding and replication and alludes to future structural targets.

1 Introduction

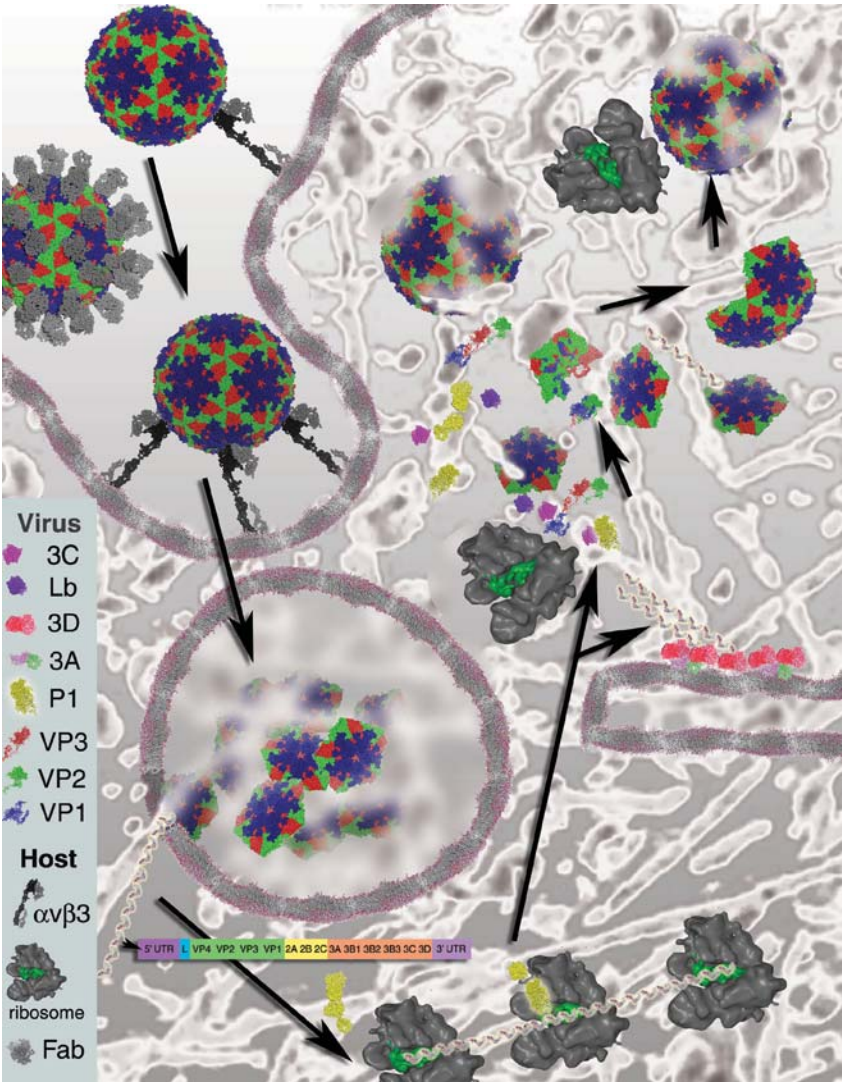
The ultimate goal of structural studies of foot-and-mouth disease virus (FMDV) is to visualize the virus, its sub-components and its interactions with the host cell at a molecular level. The progress to date is summarised in Fig. 1. In essence, FMDV targets epithelial cells and is internalised after interaction with an integrin receptor. Within the endosome, a reduction in pH induces disruption of the capsid and ejection of the RNA genome into the cytoplasm, where it is translated as a single open reading frame. The resulting polypeptide undergoes a series of cleavages to produce in excess of 12 mature polyprotein and partial cleavage intermediates.

Many of the non-structural proteins and precursors, as well as the RNA genome, make up the ribonucleoprotein replication complex which forms on the surface of membranous vesicles present in infected cells. This complex copies the positive-strand viral RNA into negative-strand complementary RNA which then functions as template for the production of multiple positive RNA strands to be used for translation, replication and packaging into viral progeny.

This chapter describes the work behind the structures depicted in Fig. 1, introduced from a historical perspective and proceeding from the mature virus through immune evasion/inactivation, cell-receptor binding and on to replication, alluding to future structural targets along the way.

FMDV was the first animal virus to be identified as a filterable agent (Loeffler and Frosch 1897), although at the time it could not be visual-

Fig. 1 A structural view of the viral life cycle. The background tomography of a cell is taken from Ohad et al. (2002). Membrane sections are taken from the X-ray crystallographic structure of the PRD1 bacteriophage (Cockburn et al., in preparation). The RNA is constructed from coordinates deposited as 157D (Berman et al. 2000). Host components are *grey*, and viral components are coloured. The figure shows the all-atom structure of reduced O serotype virus [structure determined in the presence of DTT (Logan et al. 1993)]. Atoms are depicted as *solid spheres* with radii corresponding to their size (*CPK*): VP1 is *blue*, VP2 *green* and VP3 *red* (VP4 is internal and not visible in these views). Individual viral proteins are depicted as above. The integrin $\alpha v\beta 3$ cellular receptor (1L5G, Berman et al. 2000) is depicted in *dark* and *mid-grey*. An inactivated C-serotype virus complexed with Fab is shown colour coded as above with the Fab in *grey* (1QGC, Berman et al. 2000). Cryo-EM representa-



tions of an active ribosome are depicted in *grey* with difference density interpreted as a pseudoknot and tRNAs shown in *green* (Spanchak et al., in preparation). Nascent proteins are depicted in *yellow*. Poliovirus 3D polymerase (1RDR, Berman et al. 2000) is depicted in *rose*. The poliovirus 3C proteinase (1L1N, Berman et al. 2000) is depicted in *magenta* and the FMDV leader proteinase dimer in *purple* and *orange*. The dimeric N-terminal fragment of poliovirus 3A (1NG7, Berman et al. 2000) is shown in *pink* and *green*. All molecular renditions were prepared with Bobscript (Esnouf 1997) and Raster3D (Merritt and Murphy 1994)

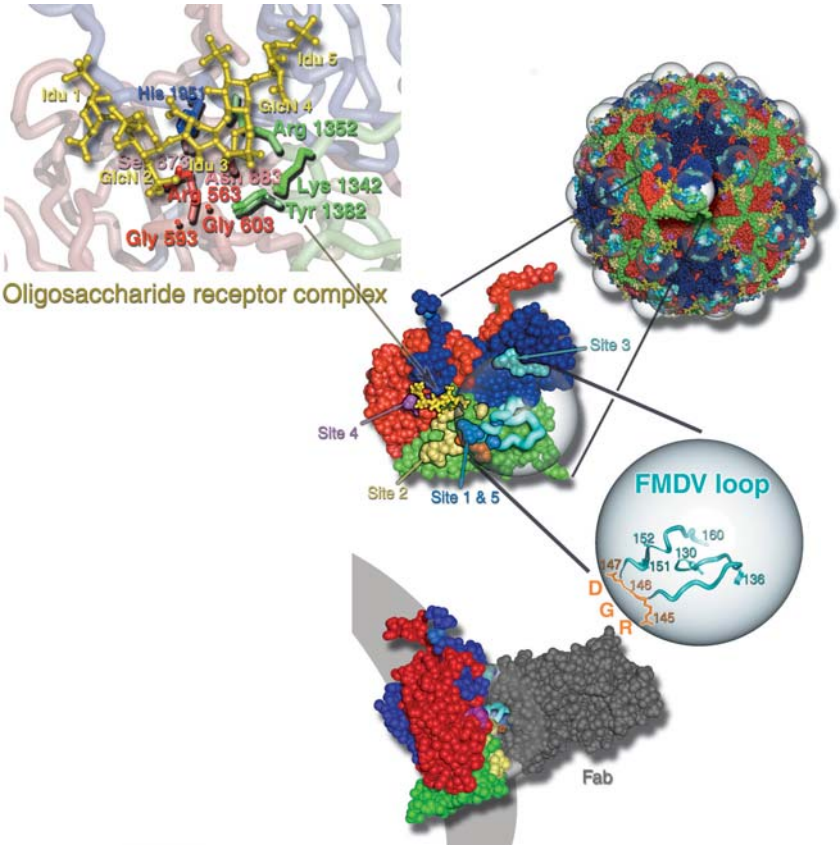


Fig. 2 Virus interactions. The figure shows the all-atom structure of reduced O serotype virus (as in Fig. 1). The VP1 GH loop residues 130–160 (FMDV loop) is shown as a 'worm' in *cyan* with the Arg-Gly-Asp residues in *orange* CPK (speres corresponding to atomic radii). Antigenic residues are colour-coded according to their classification into sites (Crowther et al. 1993; Kitson et al. 1990): sites 1 and 5 (*mid-blue*), site 2 (*pale yellow*), site 3 (*light blue*), site 4 (*magenta*). The potential occupancy of the VP1 GH loop [which appears to behave as a mobile domain hinged at its base (Parry et al. 1990)] is modelled by a *transparent sphere* centred at the mid-point between the two ends of the loop. The heparin motif which has been visualized in complex with the virus (Fry et al. 1999) is drawn in *yellow ball-and-stick*. A protomeric subunit (the smallest repeating unit, representing 1/60th of the capsid) is outlined and enlarged. The FMDV loop is further enlarged with residues that are conserved (or virtually conserved) across all strains drawn in full. In this depiction of the loop sequence variability between strains is proportional to the thickness of the tube; thus the most variable regions are thicker and the most conserved regions thinner. The residue numbers are shown for the conserved residues with the residue type being indicated for the proposed integrin binding RGD motif (*orange*). All resi-

ised. Elucidation of its general morphology was not possible until the advent of the electron microscope, when negative-stained images to a resolution of 40–50 Å revealed smooth, round particles of 30-nm diameter (Wild et al. 1969). A detailed understanding of its structure came two decades later when advances in technology and the production of crystals of the virus (Fox et al. 1987) permitted the application of X-ray crystallography. The X-ray diffraction data, together with knowledge of the complete protein sequence and structural information from related picornaviruses, led to the atomic resolution structure for the protein shell of an O serotype virus (Acharya et al. 1989) (Figs. 1, 2). Subsequent studies permitted comparisons with other serotypes and subtypes, e.g. the European A, O and C serotypes (Fig. 3) (Acharya et al. 1989; Curry et al. 1996; Lea et al. 1995; Lea et al. 1994), and monoclonal antibody-resistant mutants (MAR mutants) (Parry et al. 1990). A striking feature of all of these virus particle structures was that a particularly interesting sequence within the VP1 protein, which had been shown to be of major antigenic and immunogenic importance, was not visible because of its disordered state relative to the remainder of the particle surface. Contemporaneously with the structural work this poorly visualised surface loop was also identified as being important in interactions with host cell receptors. In addition, the MAR mutant results suggested, unexpectedly, that a single mutation might produce significant structural rearrangements in this sequence. Often referred to as the FMDV loop, this structure has since been more clearly visualised (Logan et al. 1993) (Fig. 2) and its role in antibody and receptor recognition delineated with some precision. It appears from these studies that neutralisation of FMDV occurs by blocking of receptor attachment. Indeed, complexes of the virus with large fragments of monoclonal antibodies (Fabs) have been visual-

dues are numbered such that the least significant digit defines the protein chain, e.g. 1382 is residue 138 of VP2. The heparin binding site is enlarged to show the protein side chains which act as ligands for the five sugar residues IDU1–IDU5. The protein backbone is shown in the background colour-coded as above and the side-chains interacting with the heparin are in ball-and-stick and coloured as the protein backbone; His195 of VP1, Lys134, Arg135 and Tyr138 of VP2 and Arg56, Gly59, Gly60, Ser87 and Asn88 of VP3. A Fab fragment interacting with an FMDV C serotype protomeric subunit is viewed from the side. This model was produced from a cryo-EM reconstruction (1QGC, Berman et al. 2000). The virus is depicted as above with theVP1 GH loop in *cyan*, the RGD motif in *orange ball-and-stick* and antigenic residues highlighted. The Cαs of the Fab fragment are depicted in *grey* CPK

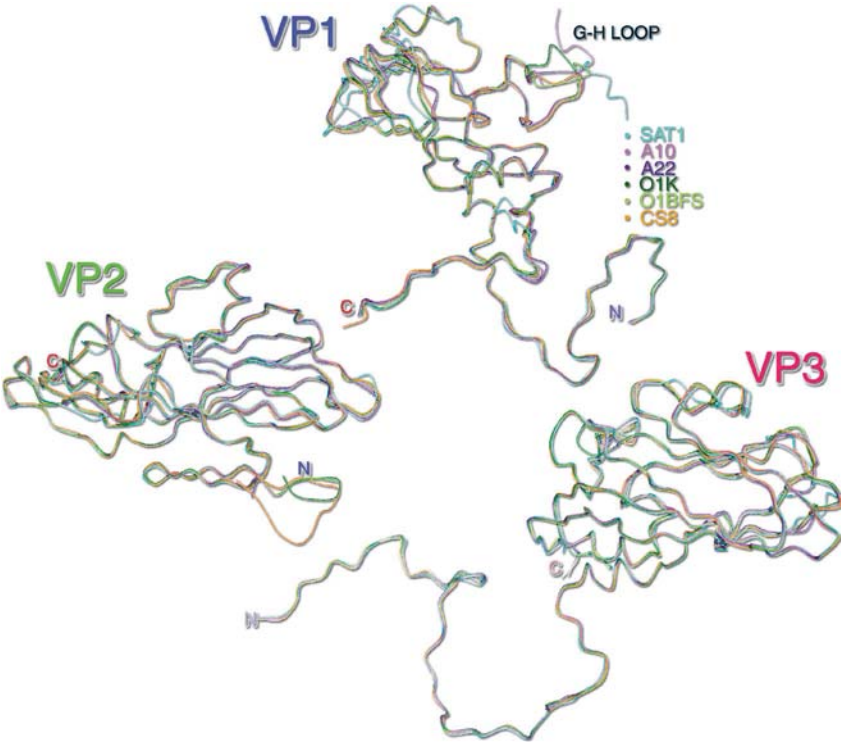


Fig. 3 Structural comparison of serotypes. A superimposition of the VP1, 2 and 3 proteins of FMDVs: A10 (E.E. Fry. et al., unpublished data), A22 (Curry et al. 1996), O1BFS (Acharya et. 1989), O1K (Lea et al. 1995), CS8 (Lea et al. 1994) and SAT1 (Adams et al., unpublished data). Each is drawn as a thin worm coloured according to the key. The O serotype structures depicted are not reduced; hence the VP1 G-H loop is not shown

ised by cryoelectron microscopy (Figs. 1, 2) (Hewat et al. 1997) and synthetic peptides representing antigenic portions of the FMDV loop have been imaged complexed with Fabs by X-ray crystallography (Verdaguer et al. 1994, 1999).

The identification of cellular receptors has also progressed greatly since the proposition that a conserved RGD motif in the FMDV loop binds the virus to cells via an integrin (Fox et al. 1989; Pfaff et al. 1988; Surovoi et al. 1988). Two classes of receptors are now recognised: integrins and heparan sulphate proteoglycans (Berinstein et al. 1995; Jackson et al. 2000b, 1996, 2002; Neff et al. 2000). Interactions with the latter have been detailed via structural studies of two virus-heparan sul-

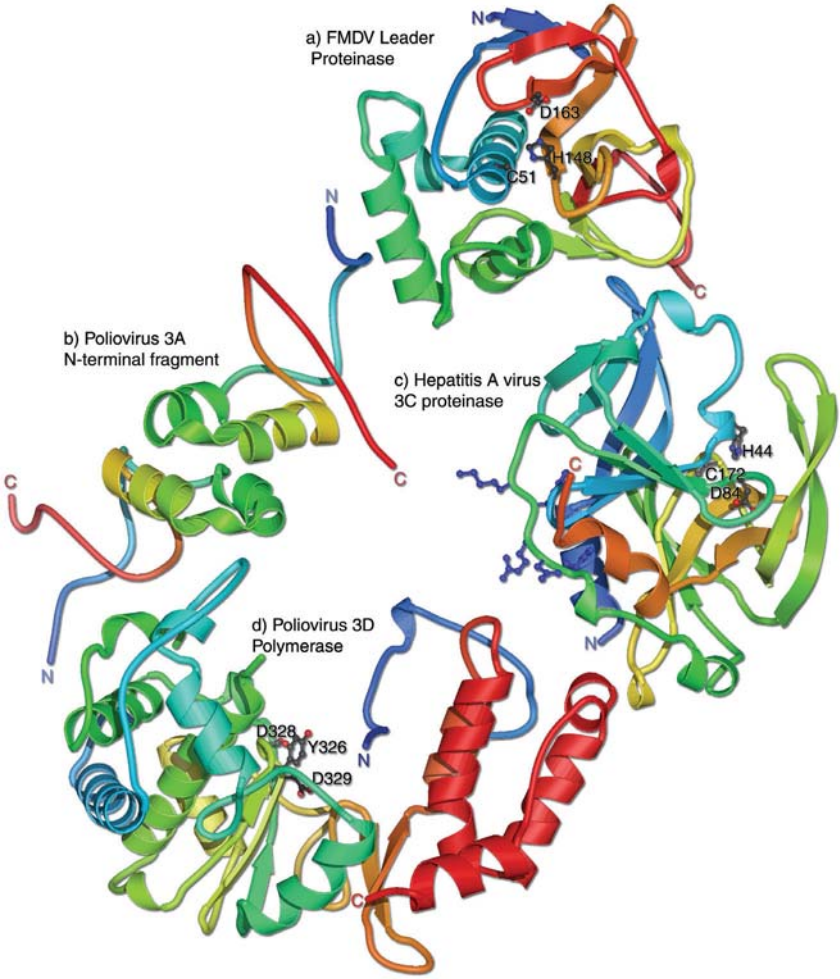


Fig. 4 Non-structural proteins. **a** The FMDV leader proteinase (Guarné et al. 2000) (a papain-like cysteine proteinase) is drawn as a *ribbon*, colour-ramped from *blue* to *red* from the N- to C-terminus. The secondary structure elements were defined with RASMOL. The catalytic residues [His148 and Cys51 (mutated to an Ala) and the proposed third member of the catalytic triad (Asp163)] are depicted in *ball-and-stick* with standard atom colouring. **b** The dimeric N-terminal fragment of polio 3A (Strauss et al. 2003) drawn as **a**. **c** Hepatitis A virus 3C proteinase (Bergmann et al. 1997) drawn as **a**. The nucleophile Cys A172 and the acid-base catalyst His A44 are depicted in *ball-and-stick* with standard atom colouring together with Asp84 (originally thought to be the third member of the catalytic triad). Residues 95–99 KFRDI (the RNA recognition sequence) are depicted in *blue ball-and-stick*. **d** The poliovirus 3D polymerase (Hansen et al. 1997) drawn as **a**. The conserved metal binding motif 4xDD (residues 326–329) is depicted in *ball-and-stick* with standard atom colouring

phate receptor complexes (Fry et al. 1999) (Fig. 2). The molecular processes that follow receptor recognition remain unclear, but the capsid structure of FMDV has provided an explanation for the observation that FMDVs share with the cardioviruses a mechanism of dissociation distinct from rhino- and enteroviruses.

The capsid proteins constitute approximately one-third of the proteins encoded by the virus genome. Of the remaining proteins which regulate viral replication, protein processing and modifications of the host, structural information is available for the leader proteinase of FMDV (Guarné et al. 2000), the 3C proteinases of related picornaviruses (Matthews et al. 1994; Allaire et al. 1994; Mosimann et al. 1997), the 3D polymerase of poliovirus (Hansen et al. 1997), the soluble domain of 3A (Strauss et al. 2003) of poliovirus and the 2A proteinase of rhinovirus (Petersen et al. 1999) (Fig. 4). Knowledge from all of these structures will be drawn upon, bearing in mind that, although many aspects of protein replication will be similar for related picornaviruses, studies of the capsid proteins and comparison of the genomes reveal significant differences between the genera.

2 Structural Overview

In the mature virus particle (Fig. 1), 60 copies of each of the four structural proteins VP1–4 associate to form the icosahedral shell or capsid. The virion has a pseudo T=3 icosahedral architecture, made possible by the broadly similar structures of VP1, VP2 and VP3. The arrangement is similar to that observed in other picornaviruses where the VP1 proteins are located around the icosahedral five-fold axes, VP2 and 3 alternate around the three-fold axes and the shorter VP4 is located entirely at the inner surface of the capsid. VP1–3 adopt a fold almost ubiquitous in RNA viruses [hence often referred to as the RNA virus fold (RVF)], that of a wedge-shaped eight-stranded β-barrel. According to convention the strands of the two sheets are labeled alphabetically proceeding from the N- to the C-termini of the amino-acid sequence, namely CHEF and BIDG. These β-wedges form a well-fitting mosaic that comprises the bulk of the capsid. Loops connecting the strands form the exterior surfaces and are identified by the strands they join; thus the hypervariable sequence known as the FMDV loop, spanning residues 140–160 of VP1, is also known as the GH loop (Fig. 2). For FMDV, the major structural proteins, VP1–3 are smaller than their counterparts in other picor-

naviruses, especially so VP1, each having a molecular weight of approximately 24,000 (Fig. 3). This reduction in size is achieved by the truncation of surface loops rendering the capsid both thinner (excluding VP4 the average capsid thickness is 33 Å as compared to 42.5 Å for human rhinovirus 14) and smoother, with none of the remarkable surface features such as the pits and canyons described for other picornaviruses (Hogle et al. 1985; Rossmann et al. 1985; Luo et al. 1987). In fact, for FMDV the C-terminus of VP1 traverses the biological protomer in a clockwise direction finishing adjacent to the VP1 GH loop of the 5-fold related protomer and in doing so fills the depression which would be analogous to the canyon/pits.

FMDV serotypes have on average 86% sequence identity to each other, although VP1 is substantially more variable. Whereas most structural differences between subtypes are confined to side chains and resemble the changes observed in MAR mutants (Parry et al. 1990; Lea et al. 1995), a comparison of serotypes (Acharya et al. 1989; Lea et al. 1994, 1995; Curry et al., 1996) reveals more significant main-chain differences, providing some structural basis for serotype and subtype differentiation (Fig. 3).

3
Capsid Stability and Permeability

Of the major capsid proteins, VP1 contributes most to the accessible surface of the virus whereas VP3 contributes most towards capsid stability (Fig. 2). The N-termini of VP3 associate to form a β-annulus at the icosahedral 5-fold axes, knitting the protomeric subunits (VP1–4) into pentamers and leaving a small pore or channel along the 5-fold axes which is accessible from the capsid exterior. In common with nearly all other picornaviruses, FMDV possesses a myristoyl group (Chow et al. 1987; Belsham et al. 1991) covalently linked to the N-terminus of VP4. This is not visible in the X-ray structures of FMDVs, but, by analogy with other picornavirus structures (Hendry et al. 1999), it is likely to form a cluster at the base of the icosahedral 5-fold axes, possibly stabilizing the N-terminal strand of the VP3 β-barrel. Further stability is provided by a feature unique to FMDV, a ring of disulphide bonds around the 5-fold axes towards the virus exterior, linking the VP3 N-termini and constricting the channel. VP1-VP2 covalently associated dimers are also seen in serotype O viruses, where they have a special role in modulating the mobility of the VP1 GH loop (Logan et al. 1993). The capsid

as a whole is fairly permeable; FMDV particles have the highest buoyant density of any picornavirus in CsCl and permit the entry of proflavin, the dimensions of which are commensurate with its entry via the 5-fold channel (Acharya et al. 1989).

Capsid integrity is vulnerable to low pH; below pH 6.8, the capsids dissociate to 12S pentameric assemblies (Brown and Cartwright 1961) and this property probably influences virus targeting, pathogenicity and spread (Fig. 1). It has been proposed that the pH instability of FMDV is governed by the switch of the protonation state of a cluster of histidines within 15 Å of the pentamer boundary (van Vlijmen et al. 1998), in particular the interactions of His142 and His145 of VP3 with polar residues across the inter-pentamer boundary. His142 was proposed (Curry et al. 1995) to mediate a histidine-α-helix charge-dipole interaction, a role verified by mutational analysis (Ellard et al. 1999), although electrostatic calculations suggest that this interaction contributes only a small proportion of the destabilisation energy. An extended β-sheet of six strands straddles the pentamer boundaries, composed of four strands from VP3 (pentamer 1) and two (β-A1 and β-A2) from VP2 (pentamer 2). Together with cation ligation on the three-fold axis this maintains capsid integrity. To date, no structural information is available for dissociated pentamers. In rhino- and enteroviruses; the trans-pentamer β-sheet structure is strengthened by a seventh strand contributed by the N-terminal extension of VP1 of pentamer 1 forming a sandwich around the strands contributed by pentamer 2 (Acharya et al. 1990; Fry et al. 1991, 1990). The rhino- and enteroviruses do not readily dissociate into pentameric subunits but rather uncoat via A-particle intermediates (Rueckert 1996) in which VP4 and the N-terminus of VP1 are externalized. A final major, and biologically important, structural distinction divides the FMDVs and cardioviruses from rhino- and enteroviruses. In the latter viruses the β-barrel of VP1 contains a long, hydrophobic pocket that accommodates a fatty acid molecule, often referred to as the pocket factor (Filman et al. 1989; Lewis et al. 1998). The release of this molecule on receptor binding triggers viral uncoating in the rhino- and enteroviruses, whereas FMDVs and cardioviruses dissociate via a different mechanism and have no trace of, nor room for, a pocket factor (Fry et al. 1991).

4
Genome Penetration

A common problem for all picornaviruses is that of transposing their RNA genomes across the cell membrane to initiate the infection process in the cytoplasm. For poliovirus and rhinoviruses conformational changes in the virus capsids, which are induced by receptor binding and/or internalisation into endosomes, result in the loss of the RNA and VP4 without disruption of the particles. There is increasing evidence that these conformational changes result in the insertion of VP4 and the N-terminus of VP1 into the cellular membranes to produce channels through which the genomic RNA might traverse the membrane (Hogle 2002). The infection process by FMDV appears to be markedly different, and there is no model at present as to how the genome enters the cell. All of the available evidence suggests that the process is triggered simply by the acidic environment of the endosome. Acid treatment results in the dissociation of the virus into pentameric subunits, VP4 and the genome, and available evidence suggests that the capsid proteins remain in endosomes while the RNA penetrates into the cytoplasm. Acidification of the virus in vitro produces an insoluble aggregate of VP4 while the pentmeric 12S subunits remain soluble. It is possible, therefore, that VP4 may associate with the endosomal membrane to produce channels into the cytoplasm, but at present this is purely speculative.

5
Immunogenicity/Receptor Binding

Multiple antigenic sites have been identified for the majority of picornaviruses studied (Boege et al. 1991; Hogle and Filman 1989; Sherry et al. 1986). In general, the entire accessible surface of a virus would be expected to be antigenic, although antigenic variation is likely to be more apparent in residues which are not essential for capsid structure or function. For type O1 FMDVs, up to five antigenic sites have been described (Barnett et al. 1989; Crowther et al. 1993; Kitson et al. 1990) involving all three major capsid proteins (Fig. 2). For C serotype viruses, the identification of sites is similar, with a major discontinuous antigenic site located near to the three-fold axis involving residues of VP1, VP2 and VP3 (Lea et al. 1994). Two major antigenic sites have been located on VP1 and VP3 of A10 (Thomas et al. 1988) and corresponding sites identified on A12 (Baxt et al. 1989). There is an underlying structural similarity

between the antigenic sites of the different serotypes, revealed by early studies which demonstrated the key importance of mobile, exposed regions on a single FMDV capsid protein, VP1, for the biological properties of the virus. In all FMDVs the C-terminus and the GH loop of VP1 are highly exposed regions which are central to both antigenicity and receptor binding (Barnett et al. 1989; Baxt and Becker 1990; Crowther et al. 1993; Fox et al. 1989; Jackson et al. 1997, 2000a, 2000b, 2002; Kitson et al. 1990; Liebermann et al. 1991; Mason et al. 1994; McCahon et al. 1989; Parry et al. 1989, 1985; Stave et al. 1988; Surovoi et al. 1988; Xie et al. 1987). In type O FMDV, for example, trypsin cleavage of VP1 within the GH loop and at the C-terminus does not affect the integrity of the virus particle but abolishes the ability of the virus to attach to cells and greatly reduces immunogenicity.

The VP1 GH loop has been found to be structurally disordered in all serotypes studied to date (Acharya et al. 1989; Curry et al. 1996; Lea et al. 1994) (Fig. 3). The mobility of this loop presumably presents more possibilities for interaction with antibodies, leading to its extreme antigenicity. Nevertheless, for certain variant viruses which escape neutralisation by a monoclonal antibody which bound simultaneously to the GH loop and to the C-terminus of VP1, escape substitutions were, surprisingly, found in a quite different part of the virus, the VP1 BC loop (Parry et al. 1990). Crystallographic studies of these mutants and comparisons with the parent virus revealed that single amino acid changes in the VP1 BC loop were responsible for switching the conformation of the VP1 GH loop. The electron density profiles suggested that the VP1 GH loop exists in two predominant conformations, "down" and "up", and that the conformational restriction imposed by the disulphide tethering one of the ends of the loop in O1 BFS (C134 of VP1-C130 of VP2) was responsible for driving it towards the less intrinsically stable "up" conformation in the parent virus. Thus the substitutions in the VP1 BC loop underlying this "up" conformation were able to destroy the integrity of the VP1 GH loop epitope by switching it to a predominantly "down" conformation. On the basis of these data, the VP1 GH loop was proposed to act as a hinged domain (Parry et al. 1990). This hypothesis was confirmed when the VP1 GH loop structure was visualised in a well-ordered "down" conformation by the crystallographic analysis of DTT-reduced O1BFS virus (Logan et al. 1993), in which the destabilizing Cys134 VP1–Cys130 VP2 disulfide was deliberately broken. In the reduced virus structure, the VP1 GH loop lies predominantly over the surface of VP2 (the C_α for Cys134 of VP1 moves some 12 Å). The N-terminal portion of this loop (residues 130–160) traverses the biological pro-

tomer in a clockwise direction, coming into close proximity with the VP3 GH loop (residues 173–180) from a five-fold related biological protomer, resulting in a rearrangement of that loop. Thus observed conformational differences in this VP3 GH loop between serotypes are more likely to be attributable to the flexibility of the VP1 GH loop than to genuine serotype specific changes (Curry et al. 1996). The VP1 GH loop then forms a strand of β-sheet adjacent to strand C of VP2. The RGD motif (residues 145–147) occupies a turn prior to a 3_{10} helix and is in an extended conformation (Fig. 2), similar to that observed for the same motif in γ-crystallin, which is known to bind integrin (Wistow et al. 1983).

Although in native viruses the VP1 GH loop is disordered, there is evidence that the internal structure seen in reduced viruses is preserved in different loop positions. Thus the crystal structure of a synthetic peptide corresponding to the sequence of the loop for a serotype C virus in complex with an Fab fragment from a neutralising monoclonal antibody revealed a loop structure similar to that seen in crystals of reduced O1BFS (Verdaguer et al. 1995). Each of these loop structures was also found to be maintained after multiple amino acid substitutions (Lea et al. 1995; Ochoa et al. 2000). Such conservation suggests that the conformation as well as the chemical properties of these residues is important in integrin recognition.

Because FMDV replicates in the reducing environment of the cytoplasm, O1 strains are initially produced in the reduced state, and so presumably possess a VP1 GH loop that is ordered. Oxidation to the mature, disordered form occurs only after release from the host cell. Indeed, studies of the re-oxidation of reduced virus showed that the disulphide bond reforms. The ability to form the disulphide bond despite the large deviation in the position of residue 134 of VP1 emphasises the residual mobility of the reduced loop as evidenced by the high crystallographic B-factors (measures of thermal motion) and the estimated 85% occupancy compared to the rest of the structure. Not surprisingly, the infectivity of reduced virus with an 'ordered loop' is markedly lowered, unless the virus can use an alternative receptor (A. King, unpublished data). The release of virus initially unable to bind receptor might be important for pathogenesis, facilitating viral spread.

Whereas the reduced virus structure confirmed the "down" position for the loop proposed by Parry et al.(1990), an Fab-C-serotype virus complex (Hewat et al. 1997) determined by cryoelectron microscopy provided direct evidence for the "up" position, because this accounted best for the observed position of the bound Fab (Fig. 2). Analysis of a

further C serotype virus-Fab complex and the corresponding Fab-peptide complex (Verdaguer et al. 1999) showed the VP1 GH loop in a similar conformation but disposed differently on the virus surface, a difference explicable by a simple rotation hinged about the base of the loop (Hewat et al. 1997; Verdaguer et al. 1999). Together these results verify the hypothesis that the loop exists as a self-contained, biologically important folding unit which can occupy at least two radically different positions on the virus surface (Acharya et al. 1989; Parry et al. 1990) (Fig. 2). The combination of flexibility and rigidity in the structure of the VP1 GH loop for native viruses facilitates receptor binding via the conserved RGD integrin-binding motif within it.

The strong antigenicity of this receptor binding site is in complete contrast to what would be expected from the canyon hypothesis for receptor binding proposed for other picornaviruses (Luo et al. 1987; Rossmann et al. 1985), in which the residues involved in cell attachment are located at the base of surface depressions whose dimensions render them accessible to cell receptors but not antibodies. In contrast, the mode of neutralisation for anti-FMDV antibodies recognising this site is clearly by direct blocking of receptor attachment (Verdaguer et al. 1995) and all the antigenic sites flank the RGD triplet [if we consider both of the predominant conformations (Fig. 2)]. Taking into account the large footprint of an antibody (typically 700 $Å^2$), we would expect that variation around the conserved attachment site would be sufficient to evade immune surveillance. In fact, more subtle effects also operate, as we have seen, such that a single mutation in the structure underlying the VP1 GH loop (e.g. residue 82 of VP2 or residue 43 or 59 of VP1) can perturb its structure, resulting in an amplified propagation of structural change (Curry et al. 1996; Parry et al. 1990).

Because the receptor attachment site is at a peripheral 'flexible' location, receptor binding is unlikely to mediate any capsid dissociation by imposing mechanical strain on the capsid [as is thought to occur in other picornaviruses (Rueckert 1996)], hence the requirement for an additional factor, acid lability, to promote disassembly (Acharya et al. 1989).

6 Receptor Interactions

FMDV targets epithelial cells, and much progress has been made in identifying cellular receptors for FMDV (Baranowski et al. 2000; Berinstein et al. 1995; Jackson et al. 1996, 2000b, 2002; Neff et al. 2000).Two

classes of receptors have been recognised, integrins and heparan sulphate proteoglycans. The most favoured integrin isoforms are $\alpha v\beta 3$, $\alpha v\beta 6$ and $\alpha v\beta 1$ (Jackson et al. 1997, 2000b, 2002). It has also been shown that antibody-bound FMDV can infect cells via Fc receptor-mediated adsorption (Mason et al. 1993) and via an engineered artificial receptor consisting of a single-chain anti-FMDV monoclonal antibody fused to ICAM-1 even with RGD-deleted viruses, suggesting that regions of the virion other than the G-H loop of VP1 might bind to and infect cells in culture.

7
Heparan Sulphate Proteoglycan Receptors

Heparan sulphates (HSs) are polymers of disaccharide repeats of L-iduronic acid and D-glucosamine, which carry random patterns of sulfation and are hence negatively charged (Bernfield et al. 1999; Kjellen and Lindahl 1991). They are found as the carbohydrate component of many proteins called heparan sulphate proteoglycans (HSPGs). They are expressed on the surface of virtually all cell types, either as integral membrane proteins or as components of the extracellular matrix.

HS was originally identified as a potential enhancer (or co-receptor) of cell entry by certain strains of type O FMDV (Jackson et al. 1996). Subsequently, viruses representing several other serotypes, including serotypes A (Fry et al. 1999), C (Baranowski et al. 1998, 2000; Escarmis et al. 1998), Asia-1 and SAT-1 (T. Jackson, unpublished data) have been shown to bind HS and/or use HSPGs as cellular receptors. It was at one time thought that HS acts as the virus' first point of contact with the cell, en route to its integrin receptor (Jackson et al. 1996). However, a functional relationship between HS and integrins was not established, and it is now clear that propagation of certain strains of FMDV in cultured cells selects for virus variants that have a high affinity for HS (Sa-Carvalho et al. 1997). Such viruses can then completely dispense with their RGD integrin-binding site and use HSPGs as alternative receptors without the mediation of integrins (Baranowski et al. 2000; Martinez et al. 1997; Neff et al. 1998).

Viruses that bind to HS arise rapidly in cell culture and are characterised by having a small plaque phenotype and an increased virulence and expanded host range for cultured cells typified by an acquired ability to infect CHO and K562 cells (Baranowski et al. 2000; Jackson et al. 1996; Neff et al. 1998; Sa-Carvalho et al. 1997). Remarkably, the ability of

FMDV to bind HS arises from only one or two residue changes on the outer capsid surface which result in a net gain in positive charge (Fry et al. 1999; Sa-Carvalho et al. 1997). Subtype O strains, for example, have a histidine residue at VP3-56 which changes to arginine on passage in tissue culture, and this single change was identified as important for HS binding by genetic studies using a cloned infectious copy of the genome (Sa-Carvalho et al. 1997). For a single substitution to confer such a dramatic change in HS affinity suggests that the icosahedral virion, with 60 binding sites, interacts with HS polymers at multiple sites, so amplifying the effect of any sequence changes, although direct crystallographic evidence demonstrates that monovalent interactions can be significant (see below).

The question arises as to whether the ability of FMDV to switch rapidly between its integrin and HS receptors plays any role in FMDV pathogenesis. Despite conferring a clear advantage for growth in cultured cells, viruses that have a high affinity for HS are attenuated for cattle (Sa-Carvalho et al. 1997). Furthermore, infection of cattle by an HS-binding strain gave rise to virulent revertant viruses which had lost the ability to bind HS. These observations imply that a high affinity for HS is a disadvantage for FMDV in an animal. It remains to be established whether low-affinity interactions between FMDV and HS occur in vivo, or whether the ability of FMDV to switch rapidly from integrin to HS receptors plays any role in the latter stages of FMDV pathogenesis, once an infection has been established (Fry et al. 1999).

8
The Structural Basis of Heparan Sulphate Recognition

The crystal structures of two tissue culture-adapted strains of FMDV, O1BFS (Fry et al. 1999) and A10 (E.E. Fry, unpublished data), complexed with HS have been determined. The HS binding site in both complexes is formed by a shallow depression in the centre of the biological protomer similar to the putative receptor-binding 'pit' of cardioviruses (Luo et al. 1987). The models reveal a segment up to five sugar residues binding in a position, orientation and conformation which are almost identical for the two serotypes (Fig. 2). The bound sugar motif is fully sulphated and makes contact with approximately nine amino acid residues derived from all three major structural proteins (VP1–VP3). A comparison with the unliganded virus structures shows that virtually no conformational changes occur in either virus on HS binding. Arg56 of

VP3 occupies a critical position in both complexes, making ionic interactions with two sulphate groups. This residue switches to Arg from the wild-type His when O1 viruses adapt to tissue culture (Sa-Carvalho et al., 1997). A second contact residue, Arg 135 of VP2, which is also conserved between O1BFS and A10, plays a subsidiary role in interacting via water molecules with one of the HS disaccharides. Most other contact residues are not conserved between these two viruses (E.E. Fry, unpublished data). In contrast, studies with type C FMDV (Baranowski et al. 2000; Escarmis et al. 1998) have identified residues involved in HS adaption at widely spaced locations on the capsid, leading the authors to suggest that there may be more than one potential binding site in this serotype.

The C-terminal residues of VP1 (residues 201–211) have also been implicated in cell attachment because selective removal of these residues by treatment with the lysine-specific endoproteinase Lys-C results in virus particles which are no longer capable of binding to cells (Fox et al. 1989). It is unclear whether this region is involved in binding to HS or to integrin receptors. The sequence of this region is similar to the heparin-binding site of vitronectin, leading to the suggestion that there is a direct interaction with HS. However, the crystal structure of the virus-HS complex demonstrated that this is not the case (Fry et al. 1999). Nevertheless, the nearby residues, His 195 and Lys 193 of VP1 of O1BFS and A10, respectively, do make contact with HS and it is possible that the C-terminus of VP1 may serve to stabilise these residues in a suitable position for HS binding. Interestingly, the HS binding site is also one of the five antigenic sites (site 4; Fig. 2). As the antibody that mapped to this site was raised against the wild-type (i.e. integrin dependent) form of the virus, its mechanism of neutralisation may not be via receptor blocking.

The heparan sulphate receptor binding site is located some 15 Å away from the RGD motif in the reduced conformation. The adaptation to HSPG receptors by O1 viruses appears not to compromise their ability to make use of integrin receptors, implying that the two attachment sites function independently of each other (S. Najjam, unpublished data).

Overall, we feel that the similarities in the crystal structures of the O and A type virus-HS complexes suggest that this binding site has an important role in the survival of the virus, perhaps via persistent infections (Fry et al. 1999).

9
Integrin Receptors

Integrins are heterodimeric proteins composed of two, differing, type 1 transmembrane subunits, α and β, with large extracellular domains and usually a short cytoplasmic tail and are the key determinants of cell entry by FMDV. Most integrins recognise their ligands by binding to short linear peptide sequences, and several members of the integrin family, including $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$ and $\alpha 5\beta 1$, recognize their ligands by binding to the tripeptide RGD. Of these $\alpha v\beta 5$ and $\alpha 5\beta 1$ have not been shown to act as receptors for FMDV (Baranowski et al. 2000; Jackson et al. 2000b; Mason et al. 1993; Neff et al. 1998), and according to the distribution of the others in tissues, $\alpha v\beta 6$ is most likely to correspond to the true cellular receptor (though the distribution of $\alpha v\beta 6$ in bovine tissues remains unclear). FMDV is thought to enter the cell through endosomes where capsid uncoating is triggered by the acidic environment of this compartment (Curry et al. 1995; Ellard et al. 1999; Mason et al. 1993) and infection is inhibited by pre-treatment of cells with reagents that raise endosomal pH (Baxt 1987; Carillio et al. 1984; Miller et al. 2001). Mutant $\alpha v\beta 6$ receptors with deletions in a conserved motif (known to function in other internalisation receptors as a signal which directs membrane proteins into clathrin-coated vesicles) (Chen et al. 1990; Trowbridge et al. 1993) are defective at mediating infection despite retaining the ability to bind FMDV (Miller et al. 2001), suggesting that the $\beta 6$ cytoplasmic domain may contain signals necessary for virus uptake into endosomes.

Several lines of evidence show that the residues following the RGD motif of FMDV, including those at the RGD +1 and +4 positions, are important for receptor recognition (Rieder et al. 1994, Mateau 1996, Jackson et al. 2000a). The similarity between the RGD site of FMDV and LAP-1 and the observation that the DLXXL motif is a ligand for $\alpha v\beta 6$ (Kraft et al. 1999) suggest that conservation of the leucine residues at the RGD +1 and RGD +4 positions is likely to reflect the use of $\alpha v\beta 6$ rather than $\alpha v\beta 3$ as a specific receptor. A model of the virus with the loop in the "up" position can readily be docked with the extended $\alpha v\beta 3$ (Xiong et al. 2001) structure in a fashion analogous to the Fab interaction.

10
Virus-Integrin Complexes

To date no complex of FMDV with integrin has been visualised. Perhaps the closest approximation comes from work with hepatitis B cores expressing the FMDV loop, which have been visualised decorated with $\alpha v\beta 3$ by EM (Sharma et al. 1997).

Nevertheless, the recent report of a crystal structure for the ectodomain of $\alpha v\beta 3$ should, in the future, facilitate interpretation of cryo-EM reconstructions if these can be obtained.

11
Non-structural Proteins

For FMDVs, the product of translation of the single-stranded positive-sense RNA genome is a polyprotein that is subsequently processed by a variety of virus-encoded 'proteases' (Leader, 2A, 3C).

The leader proteinase L^{pro} (201 residues in serotype O) is found at the N-terminus of the polyprotein in aphthoviruses [closely conserved between the distantly related equine rhinovirus A (ERVA) and FMDV (Hinton et al. 2002)]. It is synthesised in two forms, Lab and Lb, the latter predominating in vivo. A papain-like proteinase, it mediates autocatalytic cleavage of itself from P1 and cleaves the host protein eIF4GI, resulting in the shut-off of host cap-dependent mRNA translation. A similar function is performed by protein 2A in human rhino- and enteroviruses, although the cleavage occurs at a different site. L^{pro} appears to play a critical role in pathogenesis. An X-ray crystallographic study of L^{pro} (Guarne et al. 2000) (Fig. 4) confirms that the active site triad comprises Cys51, His148 and Asp163 (the latter in place of the Asn usually found in papain-like proteinases). In addition, it is unusual in that the His-Asp interaction is exposed to solvent and that the transition state analogue is stabilized by an Asp (Guarne et al. 2000). Other notable differences to papain include a high sensitivity to increases in cation concentration and activity limited to a narrow pH range. The structure suggests that these characteristics are due to the presence of Asp49, 163 and 164 close to the active site (not normally present in papain-like enzymes).

There is as yet no structural information for proteins and their intermediates derived from the P2 and P3 regions of the FMDV genome, but some information is available for analogous P3 proteins from rhinovirus

and poliovirus and these may provide a paradigm for picornaviruses. These proteins participate in RNA replication and protein folding and assembly.

In aphtho- and cardioviruses, the primary polyprotein cleavage occurs not between P1 and 2A but at the C-terminus of 2A. The three C-terminal residues of aphtho- and cardiovirus 2A are completely conserved (-NPG-), as is the N-terminal residue (proline) of 2B (Palmenberg et al. 1992). Although FMDV 2A comprises only 18 amino acids, it appears to function as an autoproteinase [as do the 19 C-terminal residues of cardiovirus 2A (Donnelly et al. 1997)]. However, it has been hypothesized for FMDV 2A that it actually acts at the level of the ribosome, using a novel mechanism to modify the translational machinery, allowing the release of 2A whilst permitting the synthesis of the downstream proteins (Donnelly et al. 2001). Thus the structure of the rhinovirus 2A protease (Petersen et al. 1999) is probably irrelevant to FMDV 2A.

Picornaviral 2B (154 residues type O) and 2C (318 residues type O) proteins localise to the ER–derived outer surface vesicles which are the sites of genome replication (Lubroth and Brown, 1995). In poliovirus, 2B appears to enhance membrane permeability and block secretory pathways whereas 2C is required for the initiation of negative-strand RNA synthesis. In all picornaviruses, these proteins contain conserved nucleoside triphosphate-binding and helicase motifs, have ATPase and GTPase activity and are highly conserved.

For the P3 proteins 3A and 3B are anomalous in FMDV. 3A is almost twice as long in FMDV (143 residues) as in poliovirus. A C-terminal truncation produces a bovine-attenuated phenotype which is highly virulent in pigs (Beard and Mason 2000) and adaptation to guinea-pigs is conferred by a single amino acid change in 3A, implicating it in host adaptation. FMDV 3A has been shown to co-localize with the intracellular membrane system where replication occurs (O'Donnell et al. 2001). This correlates with the prediction of a transmembrane helix between residues 59 and 76. 3A also forms stable intermediates 3AB and 3ABB. For poliovirus, 3AB can bind the 5′ cloverleaf structure of the viral genome (Xiang et al. 1995) and can directly bind to and act as a co-factor of 3D (Richards and Ehrenfeld 1998, Xiang et al. 1998). An NMR structure for the soluble N-terminal fragment of poliovirus 3A (Fig. 4) is available (residues 1–59 of 87) (Strauss et al. 2003). This structure consists of a symmetrical amphipathic α-helical hairpin dimer with unstructured termini and a charged surface patch, presumably the site of RNA interaction. The hydrophobic portion may contribute to a second dimeric interface in line with pore formation and its ability to permeabilise membranes.

3B (VPg) is covalently bound to the 5′ terminus of the genome and implicated in priming replication. The first step in poliovirus RNA synthesis is the covalent linkage of UMP to 3B (Paul et al. 2003). FMDV unusually possesses three non-identical copies of 3B in tandem (23–24 amino acids apiece) with a highly conserved Tyr at position 3 which mediates the phosphodiester linkage to the viral RNA (Forss and Schaller 1982). Not all three copies are required for replication (Falk et al. 1992) but they are clearly important to the virus, possibly playing a role in pathogenesis or host-range (Pacheco et al. 2003).

FMDV $3C^{pro}$ (213 residues) can efficiently process all 10 cleavage sites in the polyprotein (Bablanian and Grubman 1993). Whereas in poliovirus, $3CD^{pro}$ has been implicated as the major viral proteinase, responsible for cleavage of the structural protein precursor, in FMDVs levels of 3CD are variable and it appears to be rapidly cleaved. FMDV $3C^{pro}$ shows greater heterogeneity in cleavage sites than poliovirus $3C^{pro}$. It can also cleave the host proteins eIF4A (which is part of the cap-binding complex and functions as an RNA helicase) and eIF4G, late in the infectious cycle and at a different site to L^{pro} (Belsham et al. 2000), and it may inhibit host cell transcription by cleavage of histone H3.

The sequences of $3C^{pro}$s show that they belong to the chymotrypsin-like family of serine proteases, and the high degree of conservation suggests that the three-dimensional structures for rhinovirus, hepatitis A and poliovirus (Matthews et al. 1994; Allaire et al. 1994; Mosimann et al. 1997) should provide reasonable models for other members of the family (Fig. 4). However, there is a difference in the nature of the catalytic site whereby the family divides into two lineages. The entero- and rhinoviruses have a Glu as a member of the serine proteinase-like catalytic triad which interacts with a His, as observed in the HRV14 structure. The Asp which replaces the Glu (conserved in lineage B) was observed in the hepatitis A 3C proteinase structure not to interact with the histidine (Allaire et al. 1994), a water molecule fulfilling this role. Biochemical studies have identified the residues involved in the catalytic triad in FMDV 3Cpro as Cys163, His46 and Asp84 (Grubman 1995). Thus FMDV is a member of the second lineage and we would expect the active site to more closely resemble the hepatitis A 3C proteinase structure (Fig. 4).

$3CD^{pro}$ has been shown to bind to positive-strand RNA (Andino et al. 1990, 1993). Mutations shown to affect RNA-binding have been located on the three-dimensional structure of $3C^{pro}$, revealing the binding site to be on the opposite side to the catalytic site and in close proximity to the N- and C-termini such that RNA binding may regulate protein processing.

FMDV 3D (470 residues) is an RNA-dependent RNA polymerase which, together with other viral proteins and possibly host proteins, carries out viral RNA replication to generate new template, messenger and viral RNAs from the original infecting RNA. This appears to take place within a membranous complex. There is a high degree of conservation among the different FMDV sero- and subtypes for 3D at both the nucleotide and amino acid sequence levels. The structure of poliovirus 3D (Hansen et al. 1997) (Fig. 4) revealed a strong similarity to other classes of polymerases with the characteristic 'palm', 'fingers' and 'thumb' subdomains. The core 'palm' subdomain comprises four of the conserved sequence motifs found in other polymerases. The active site consists of the conserved YGDD sequence and a highly conserved D residue also found in this subdomain. Interestingly, the crystal structure also revealed polymerase-polymerase interactions at two interfaces which appear to be functionally significant (Hobson et al. 2001). This tallies with the high degree of cooperativity in RNA binding and polymerisation activities, suggesting that the polymerase may function as an oligomer (Beckman and Kirkegaard 1998). This could produce multiple interactions with other viral and host cell factors consistent with a recent model of initiation of poliovirus negative-strand RNA synthesis which includes a poly (A)-binding protein (PABP)-3CD complex and a PCBP-3CD complex that interact with each other to form a circular RNP complex (Barton et al. 2001, Herold and Andino 2001).

12 Capsid Assembly

Structural proteins accounting for approximately one-third of the polyprotein are encoded towards the 5′ end of the open reading frame (the P1 region) and appear to fold into a structure which is antigenically very similar to the virus even prior to the cleavages which yield the viral proteins VP0, VP1 and VP3 (also known as 1AB, 1D and 1C, respectively).

The P1/2A cleavage to form the structural protein precursor, P1 (Fig. 1), is performed by the 3C protease. P1 folds into a protomer, five of which associate to form a pentamer after the VP2/3 and VP3/1 cleavages have taken place. We have no structural information for either of these intermediates. Beyond this point the assembly and encapsidation mechanism is unclear; either the pentamers assemble into empty capsids and the RNA [covalently linked to VPg (3B)] is inserted to form the

provirion (the immature particle in which the maturation cleavage of VP0 has yet to occur) or the pentamers interact directly with the RNA/VPg, forming the provirion without an empty capsid intermediate. More recent studies with poliovirus (Verlinden et al. 2000) favour the latter hypothesis whereby empty capsids are either a failed product or serve as a repository of pentamers. The final step in producing the mature virion is the cleavage of 1AB (VP0), which occurs via a presumed autocatalytic mechanism in the presence of viral RNA, converting the N-terminal 85 residues into VP4 and the remaining residues into VP2 (1A and 1B, respectively), although a few copies of 1AB may remain in the intact virion.

Structural analyses of empty capsids of type A22 FMDV (Curry et al. 1997) (which despite lacking RNA possess cleaved VP0) reveal that increased ordering of the N-terminus of VP1 and C-terminus of VP4 in the vicinity of the 3-fold axes is associated with RNA encapsidation and that a further stabilization of the capsid is attributable to VP0 cleavage. A conserved histidine (His145 of VP2) could mediate the autocatalytic VP0 cleavage in FMDV (Curry et al. 1997), as predicted in poliovirus for the analogous His195 of VP2. Naturally occurring FMDV provirions have not been observed; however, noninfectious mutated provirions with uncleaved 1AB have been generated by reverse genetics and these remain acid sensitive.

13 Concluding Remarks

The small size, icosahedral geometry and relative simplicity of FMDV lend it to detailed structural analysis. Thus far, these factors have enabled much progress in understanding in intimate detail how the virus binds its cellular receptors and uncoats during the process of cell entry, and also how neutralising antibodies recognise the particle. It now remains to obtain a clearer picture of the processes involved in virus replication in atomic detail. FMDV was the first animal pathogen to be identified as a virus, and today, more than a century later, it remains at the forefront of structural virology. Future progress towards more humane and cost-effective ways of controlling FMD will benefit from a better understanding of the molecular mechanisms underlying infection and transmission of this most infectious of all viruses.

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