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Picornaviruses

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Abstract

The picornavirus family consists of a large number of small RNA viruses, many of which are significant pathogens of humans and livestock. They are amongst the simplest of vertebrate viruses comprising a single stranded positive sense RNA genome within a $T = 1$ (quasi $T = 3$) icosahedral protein capsid of approximately 30 nm diameter. The structures of a number of picornaviruses have been determined at close to atomic resolution by X-ray crystallography. The structures of cell entry intermediate particles and complexes of virus particles with receptor molecules or antibodies have also been obtained by X-ray crystallography or at a lower resolution by cryo-electron microscopy. Many of the receptors used by different picornaviruses have been identified, and it is becoming increasingly apparent that many use co-receptors and alternative receptors to bind to and infect cells. However, the mechanisms by which these viruses release their genomes and transport them across a cellular membrane to gain access to the cytoplasm are still poorly understood. Indeed, detailed studies of cell entry mechanisms have been made only on a few members of the family, and it is yet to be established how broadly the results of these are applicable across the full spectrum of picornaviruses. Working models of the cell entry process are being developed for the best studied picornaviruses, the enteroviruses. These viruses maintain particle integrity throughout the infection process and function as genome delivery modules. However, there is currently no model to explain how viruses such as cardio- and aphthoviruses that appear to simply dissociate into subunits during uncoating deliver their genomes into the cytoplasm.

1 Introduction to Picornaviruses

The *Picornaviridae* is a large family of RNA viruses and currently comprises nine genera distinguished by a range of biological, biophysical, and genetic characteristics. The cell entry characteristics of the best studied picornaviruses are presented in Table 1. The family

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includes agents that are responsible for a variety of human and animal diseases, for example, poliomyelitis, the common cold, hepatitis A, foot-and-mouth disease, and many more. Foot-and-mouth disease virus (FMDV) was the first animal pathogen to be identified as a virus (on the basis of passing through bacteria-retaining filters), and vaccines against FMD and poliomyelitis were amongst the earliest developed against viral diseases.

Picornaviruses have single-stranded positive sense RNA genomes of approximately 7,000–8,500 nucleotides with similar but not identical organizations across the family (Fig. 1; Racaniello 2007). The 5′ end of the genome is linked to a small peptide (VPg), and the 3′ end terminates with a poly(A) tract. There is a single open reading frame flanked by untranslated regions (UTRs) at both ends. The 5′ UTR is especially long (~600–1,200 nts) and contains a number of important replication and translation control elements, including an internal ribosome entry site that is directly involved in the initiation of protein translation. The genome is translated into a single polyprotein, which is subsequently cleaved into mature protein products by virally encoded proteases. The structural proteins are located within the N terminal one third of the polyprotein, while the remainder includes proteins involved in modifying the cellular environment to optimize virus replication and the proteins directly responsible for replication. In some picornaviruses (e.g., the enteroviruses), the structural precursor protein is situated directly at the N terminus of the polyprotein, while in others (e.g., aphthoviruses and cardioviruses), the structural precursor is preceded by nonstructural protein sequence. In the majority of picornaviruses, the N terminus of the structural precursor protein (P1) is modified by the covalent addition of a myristic acid residue, which is thought to have important roles both in particle assembly and in the cell entry process. P1 is typically about 90 kD and is further proteolytically processed into the mature viral proteins (VP1–4 or P1 A–D) found in the viral capsid. The VP designation system was first used to distinguish the structural proteins according to their apparent molecular weights, while the P1 system describes their order on the viral genome. Accordingly, P1A is equivalent to VP4, P1B to VP2, P1C to VP3, and P1D to VP1.

2 Structure of Picornavirus Particles

VP1–3 together form the icosohedral shell of the virion (Fig. 2), while VP4 is distributed on the inner surface of the particle (Racaniello 2007). Although the cleavage of P1 into its constituent parts is necessary for virion assembly and maturation, the products never become separated. Consequently, the structural protomer is a single processed P1 monomer of a T = 1 icosahedron that may be described as quasi T = 3 if the mature proteolytic products are considered.

VP1–3 each has a similar basic structure consisting of a wedge-shaped eight stranded β-barrel, the β strands being linked by loops of varying lengths. The lengths and compositions of the connecting loops, together with the tilts of the β-barrels determine the surface topology of the virus particle and are responsible for receptor binding specificities and antigenic characteristics. Proteolytically processed monomers assemble into pentameric subunits, 12 of which go on to form the complete icosohedral shell of the virus. VP1 components are clustered around the fivefold axes of symmetry while VP2 and 3 alternate around the threefold axes. In viruses of the enterovirus genus, there is a deep depression encircling the fivefold axes below VP1 (Fig. 2). This so-called “canyon” separates the surface-oriented protrusions of VP1 from those of VPs 2 and 3 and in many cases is the site of engagement with cellular receptors (Colonno et al. 1988;Olson et al. 1993). The canyon is partially filled-in in the cardioviruses to leave a series of depressions that span the twofold axes (sometimes referred to as “pits”), which again are involved in receptor binding (Grant et al. 1994;Hertzler et al. 2000;Luo et al. 1987;Toth et al. 1993). FMDVs, on the other hand, have a much smoother surface, and the cellular receptor binds to a flexible loop projecting

from the particle surface rather than into depressions in the structure (Fig. 2;Acharya et al. 1989;Logan et al. 1993).

In the paradigm of picornavirus assembly, proteolytic processing of the viral polyprotein results in the cleavage of P1 into VP0, VP1, and VP3, although, as stated earlier, these proteins do not physically separate. The cleavages precede and are necessary for assembly into pentameric subunits, which in turn go on to form the icosohedral “pro-virion” in association with a copy of the viral RNA genome (Guttman and Baltimore 1977; Hoey and Martin 1974; Lee et al. 1993). The mechanism underlying the incorporation of the viral RNA and the features that confer specificity on this process are still not well understood. The final event in virus maturation is associated with cleavage of VP0 into VP2 and VP4 (Curry et al. 1997; Hindiyeh et al. 1999). This is usually coincident with the incorporation of RNA into the immature particle, and it was initially proposed that a conserved serine in VP2 played a catalytic role in the cleavage process (Arnold et al. 1987). However, the X-ray structure of an empty capsid assembly intermediate of poliovirus (PV) (Basavappa et al. 1994) showed that a stretch of peptide containing the scissile bond of VP0 lies at the top of each blade of a trefoil-shaped outward depression on the inner surface surrounding the threefold axes. This depression is analogous to the binding site for icosahedrally ordered segments of the genomic RNA in beanpod mottle virus (Chen et al. 1989), and the RNA from this structure docks very nicely in the empty capsid structures. In the empty capsid structures, immediately below the scissile bond, there is a water molecule bound to a histidine corresponding to His 195 from VP2, and the carbonyl oxygen of the scissile bond and a neighboring residue point outward toward the “RNA binding site.” Based on these observations, Basavappa et al. (1994) proposed a mechanism for cleavage in which the RNA either directly (by donating hydrogen bonds from nucleotide bases) or indirectly (through coordinating divalent cations) polarizes the carbonyl oxygen, creating positive charge on the carbonyl carbon, and the histidine side chain and its water move up to engage the carbonyl carbon. This cleavage mechanism is analogous to metalloprotease mechanisms. A subsequent study by Hindiyeh et al. (1999) confirmed that mutations in His 195 inhibited VP0 cleavage, resulting in the accumulation of noninfectious virions. Structural studies have also implicated the equivalent histidine residue in the cleavage of VP0 of FMDV (Curry et al. 1997).

It has been known for some time that hepatitis A virus (HAV) does not fit with this general scheme as the VP4 moiety is vestigial in size and is not myristoylated (Tesar et al. 1993). More recently, a number of newly discovered picornaviruses have been shown to lack the VP0 maturation cleavage (Stanway et al. 2000) or, like HAV, to lack the consensus myristoylation signal at the N terminus of VP0 (Johansson et al. 2002). It will be interesting to see whether the assembly and entry processes adopted by these viruses will fit with the paradigms being developed for the better studied members of the family.

3 Picornavirus Receptors

Receptors play a fundamental role in the entry of most viruses. In the case of picornaviruses, they are involved in cell attachment, signaling and endocytosis (Sect. 4), and the triggering of capsid alterations required for infectious entry (Sect. 5). Virus–receptor interactions have been extensively characterized for several viruses in the more established genera. However, there remains a large number of viruses, especially those in more recently identified genera, for which receptors have not yet been identified. Receptors used by different picornaviruses include members of the immunoglobulin-like family, the low density lipoprotein receptor (LDLR) family, the complement control family, the integrin family of cell adhesion molecules, and the T cell immunoglobulin domain mucin-like domain receptors.

In this section, we will review the diverse types of receptors used, the nature of virus–receptor interactions, the plasticity of receptor usage, and the role for receptors in determining host and tissue tropism and pathogenesis.

3.1 Immunoglobulin Superfamily Receptors: VCAM-1, ICAM-1, PVR, CAR

This group of molecules includes several well characterized receptors for viruses of the enterovirus genus: Intercellular adhesion molecule-1 (ICAM-1) is the receptor for major group human rhinoviruses (HRVs) (Greve et al. 1989; Staunton et al. 1989; Tomassini et al. 1989). CD155 or nectin-like molecule-5 (NECL-5) is the PV receptor (PVR) (Mendelsohn et al. 1988), a cell adhesion molecule with roles in embryonic development and cancer progression (Gromeier et al. 2000; Takai et al. 2008). The Coxsackie and adenovirus receptor (CAR) is a component of the tight junction between cells in intact epithelium (Coyne and Bergelson 2005; Freimuth et al. 2008). In addition, murine vascular cell adhesion molecule-1 (VCAM-1) has been identified as a receptor for the cardiovirus encephalomyocarditis virus (EMCV) (Huber 1994). These molecules are all type 1 membrane glycoproteins comprising seven (VCAM-1), five (ICAM-1), three (PVR) or two (CAR) extracellular immunoglobulin-like (Ig-like) domains, a transmembrane domain, and a cytoplasmic domain. For the enterovirus receptors, ICAM-1, PVR, and CAR, genetic manipulation of the molecules has shown that the first (membrane distal) Ig-like domain is responsible for virus binding. The interactions of these receptors with virus capsids have been further studied by cryo-electron microscopy, which has shown that each inserts into the “canyon,” the surface depression that encircles the fivefold axis of entero- and rhinoviruses. The resulting high surface area of contact between receptor and canyon increases the energy of this interaction such that receptor binding can act as a trigger for the initiation of capsid structural transitions involved in genome release and membrane penetration (see Sect. 5). Thus, these Ig-like receptors can facilitate binding of the virus at the cell surface, targeting it into a specific endocytic pathway and initiating the process of genome delivery to the cytoplasm.

The interaction between VCAM-1 and EMCV has not been extensively studied. However, the fact that the EMCV capsid does not contain a canyon indicates that this feature is not necessarily a requirement for binding to Ig-like receptors. There is also no evidence that receptor binding initiates conformational changes in viruses of the cardiovirus genus.

3.2 Decay Accelerating Factor

Decay accelerating factor (DAF, or CD55) is a receptor for a variety of enteroviruses. It is a member of the complement control family of receptors, which are often used as receptors for microbes (Lea 2002;Lindahl et al. 2000) and comprises four extracellular short consensus repeat modules attached to the membrane by a glycosylphosphatidyl inositol (GPI) anchor. In contrast to the virus–receptor interactions seen with Ig-like receptors, the mode of DAF binding is not conserved and different viruses have diverse binding sites on both capsid and receptor (Lea 2002;Powell et al. 1999). Binding does not necessarily involve the enterovirus “canyon” and does not trigger structural alterations in the capsid. Instead, DAF functions as a primary receptor, recruiting virus to the cell surface, after which interaction with a second or co-receptor (Sect. 3.8) is required for triggering infectious entry.

3.3 LDLR Family

The LDLR, LDLR-related protein (LRP), and very low density lipoprotein receptor (VLDLR) have been identified as receptors for minor group HRVs by their ability to block virus binding to cultured cells (Hofer et al. 1994; Marlovits et al. 1998a, b, c). These receptors comprise an extracellular domain of 7 (LDLR), 8 (VLDLR), or 31 (LRP) ligand-binding repeats, a transmembrane domain, and a cytoplasmic domain. The multiple

imperfect repeats may contribute to the ability of these molecules to bind efficiently to multiple virus serotypes. In contrast to the major group HRV/ICAM-1 interaction, LDLR binding by minor group viruses does not involve the canyon and does not induce structural alterations in the capsid. Instead, five copies of the ligand-binding repeats are thought to bind around the fivefold axis of symmetry to encircle the fivefold vertex so that multiple low affinity events combine to provide high avidity (Hewat et al. 2000; Konecsni et al. 2004; Querol-Audi et al. 2009; Rankl et al. 2008; Wruss et al. 2007). As a natural consequence of symmetry in the particle, picornaviruses are also able to bind to multiple copies of a receptor to achieve high avidity binding.

The 99 conventional HRV serotypes can be grouped by phylogenetic analysis into two clades: HRV-A and HRV-B (Palmenberg et al. 2009). Although the majority of the 12 minor group serotypes reside in two clusters within HRV-A, it is interesting to note that the conservation of receptor usage within these clusters is not absolute. For example, major group HRV39 clusters with four minor group HRVs, while minor group HRV1 clusters with three major group HRVs. Analysis of capsid sequences has revealed that all minor group viruses have a conserved lysine in VP1. Some major group serotypes were also found to contain this feature, and after experimental testing, two of these, HRV23 and HRV25, were reclassified as minor group viruses (Vlasak et al. 2005).

3.4 Integrins

Integrins are cell adhesion receptors (Akiyama 1996) consisting of a noncovalent, heterodimeric complex between α and β subunits. Each subunit is a glycoprotein with a large globular extracellular domain, a transmembrane domain, and a small cytoplasmic domain. A classical feature of many integrin receptors is their recognition of the Arg-Gly-Asp (RGD) tripeptide, and the presence of this motif in virus capsids has become synonymous with integrin receptor usage. Integrins $\alpha_v\beta_1$(Jackson et al. 2002), $\alpha_v\beta_3$ (Duque et al. 2004), $\alpha_v\beta_6$ (Jackson et al. 2000), and $\alpha_v\beta_8$ (Jackson et al. 2004) can all function as receptors for entry of the aphthovirus FMDV, although only a restricted set of these molecules are used by the virus in natural infections. The presence of integrin $\alpha_v\beta_6$ on bovine epithelia correlates with those sites where lesions develop during natural infection, and it has been proposed as the natural receptor for FMDV (Monaghan et al. 2005). Integrin binding involves an RGD triplet, together with adjacent motifs, displayed by a flexible, exposed loop (the VP-1 G-H loop) on the capsid surface (Dicara et al. 2008; Logan et al. 1993). Receptor binding does not trigger structural alterations but simply serves to tether the virus at the cell surface and facilitate its internalization by endocytosis.

Integrins are also receptors for cell attachment of the enteroviruses, echovirus 1 (Bergelson et al. 1992; Triantafilou et al. 2001), echovirus 9 (Nelsen-Salz et al. 1999), and CAV9 (Heikkila et al. 2009; Roivainen et al. 1994; Triantafilou et al. 1999; Williams et al. 2004), and the parechovirus, human parechovirus 1 (Joki-Korpela et al. 2001; Triantafilou et al. 2000, 2001). Most of these viruses bind integrins via an RGD motif near the C-terminus of VP1, which is an exposed, flexible site (Hendry et al. 1999). However, echovirus 1 is exceptional in binding the RGD-independent integrin $\alpha_2\beta_1$ via interaction with the canyon (Xing et al. 2004). In all cases, integrin binding does not trigger capsid alterations and, in parallel with the situation for DAF-binding enteroviruses, additional receptors or factors are required for infectious entry (Sects. 3.8 and 5).

3.5 HAV Cellular Receptor (TIM-1)

The primate cellular receptor for HAV (HAVcr-1) (Kaplan et al. 1996) and the human analog (huHAVcr-1) (Feigelstock et al. 1998) were identified as novel class I integral membrane glycoproteins with an extracellular domain comprising an N-terminal membrane-

distal Ig-like region and a mucin-like region (Thompson et al. 1998). Soluble forms of HAVcr-1 are reported to neutralize HAV (Silberstein et al. 2001) and induce HAV particle alterations (Silberstein et al. 2003). IgA is also a natural ligand for HAVcr-1 and enhances HAV–receptor interactions (Tami et al. 2007).

huHAVcr-1 was subsequently classified as prototype member of the novel T cell immunoglobulin domain, mucin-like domain (TIM) gene family, or TIM-1. An intriguing study has suggested that childhood infection with HAV may stimulate TIM-1 and protect against allergy in later life, leading to the proposal that the decreasing incidence of HAV in western countries is responsible for the corresponding increasing incidence of allergy and asthma (McIntire et al. 2004).

3.6 EV71 Receptors

EV71 is an enterovirus that causes hand, foot and mouth disease, normally with relatively mild and self-limiting symptoms. However, infection with EV71 is also associated with severe and sometimes fatal neurological disease, and large out-breaks of this virus have occurred recently in Asia. Two receptors for EV71 have recently been identified: human P-selectin glycoprotein ligand-1 is a mucin-like protein that serves as a receptor for EV71 infection of leukocytes (Nishimura et al. 2009) and scavenger receptor class B member 2 (Yamayoshi et al. 2009) is a receptor for endocytosis of high density lipoprotein. Both molecules are significantly different from existing enterovirus receptors. It is hoped that further work may reveal whether these receptors contribute to the pathogenesis and neurotropism of EV71 (Patel and Bergelson 2009).

3.7 Sialic Acid

Sialic acid residues are found on the oligosaccharide chains, which decorate a variety of cell surface glycoproteins. Persistent strains of the cardiovirus Theiler’s murine encephalomyelitis virus (TMEV) recognize sialic acid as a receptor moiety (Lipton et al. 2006), and the structure of virus complexed with sialic acid has been determined crystallographically (Zhou et al. 2000). Another cardiovirus, EMCV, binds via a sialic acid moiety to glycophorin A, which is a receptor molecule on virus nonpermissive mammalian erythrocytes (Tavakkol and Burness 1990). Recent studies have also shown that EMCV can use sialic acid-mediated entry after adaptation in cell culture (Guy et al. 2009). The aphthovirus equine rhinitis A virus (ERAV) also attaches to cells via an interaction with sialic acid (Stevenson et al. 2004; Warner et al. 2001), and the structure of virus complexed with sialic acid has been obtained by X-ray crystallography (Fry et al. unpublished).

3.8 Co-Receptors

A considerable number of DAF-binding enteroviruses have been shown to require co-receptors to affect the capsid structural alterations necessary for productive infection. Well documented examples are the Coxsackieviruses, CAV21 and CBV3, which use the Ig-like molecules ICAM-1 and CAR, respectively, as co-receptors (Shafren et al. 1997a, b, c). In contrast to the primary receptor, DAF, these co-receptors bind into the “canyon” and can trigger capsid alterations. Studies in cultured cells have shown that these viruses can infect via their co-receptors in the absence of DAF (Shafren et al. 1997a); and therefore prompting the following question: what is the role of DAF? This question has been recently answered by studies of CBV3 infection of polarized epithelial cells. In these cells, CAR is found only in its natural location, hidden in the tight junctions between cells and is therefore inaccessible to virus. However, binding of DAF triggers signaling-dependent transport of the receptor–virus complex to the tight junctions so that interaction with CAR and cell-entry can occur (Coyne and Bergelson 2006). This study emphasizes the importance of using in vitro

cell culture systems (e.g., polarized cells), which more closely mimic the morphology of tissues in vivo.

Studies with the DAF-binding enterovirus echovirus 7 have shown that entry in DAF-negative cells can occur (Powell et al. 1998), and that the complement control protein CD59 (Goodfellow et al. 2000; Ward et al. 1998) and the MHC class I component, β_2 microglobulin (Ward et al. 1998), may be co-receptors required for entry.

Like the DAF-binding enteroviruses, the integrin-binding enteroviruses also require co-receptors for entry. Studies with CAV9 have indicated multiple requirements for β_2 microglobulin (Triantafilou et al. 1999), the MHC-I-associated protein GRP78, and MHC-1 itself (Triantafilou et al. 2002). Also with similarity to some DAF-binding viruses (Sect. 3.2), the interaction with the primary receptor has been shown to be dispensable in cultured cells (Hughes et al. 1995).

3.9 Adaptation, Alternative Receptors, Tropism, and Pathogenicity

3.9.1 FMDV and Other Integrin-Binding Viruses—Field strains of FMDV use integrin receptors, but certain virus serotypes can be adapted by passage in cell culture to use the cell-surface sulfated glycan, heparan sulfate (HS) (Jackson et al. 1996; Martinez et al. 1997; Sa-Carvalho et al. 1997). Experimentally selected viruses have also been shown to infect cells via a third, unidentified route, independent of HS and RGD–integrin interactions (Baranowski et al. 2000). Cell culture adapted viruses are nonpathogenic in vivo but rapidly revert to an integrin binding and pathogenic phenotype upon passage (Sa-Carvalho et al. 1997). The sites of virus replication during natural infection also appear to correlate with integrin expression (Monaghan et al. 2005), suggesting that both tissue tropism and pathogenesis are influenced by receptor usage.

The RGD containing enterovirus CAV9 also uses integrin binding to enter cells but can still infect cells after the RGD motif is deleted or when cells are devoid of integrin (Hughes et al. 1995). In contrast, human parechovirus 1 appears more strictly dependent on integrins for entry: after mutation of the RGD, the virus particles were essentially noninfectious and only viruses in which the RGD sequence had been restored by reversion were recovered after passage (Boonyakiat et al. 2001).

3.9.2 Major Group Human Rhinoviruses and Ig-Like Receptor-Binding Viruses —Major group serotypes of HRV normally use ICAM-1 as a receptor. However, upon serial passage in cells bearing low levels of ICAM-1, the major group virus HRV89 acquired the ability to enter ICAM-1 negative cells using HS as a receptor (Reischl et al. 2001; Vlasak et al. 2005). Furthermore, major group HRV54 is able to enter cells (without adaptation) by using ICAM-1 or HS. In contrast, the closely related enterovirus PV appears to have an absolute dependence on its cognate receptor PVR.

3.9.3 TMEV Pathogenesis—TMEV strains can be grouped according to disease pathogenesis in the mouse. Neurovirulent strains cause acute, fatal encephalitis. In contrast, persistent strains cause mild encephalitis followed by a chronic demyelinating disease of the central nervous system (CNS), which has become important as a model for multiple sclerosis. There is a variety of evidence linking these alternate disease outcomes with receptor usage and tissue/cell tropism (Lipton et al. 2006). Acute and persistent phases of the disease are associated with specific patterns of infection in the CNS, suggesting a link between cellular tropism and pathogenesis. Genetic chimeras between neurovirulent and persistent viruses have identified capsid regions and specific residues (thought to be involved in receptor binding) as determinants of persistence. Persistent strains bind to cell surface sialic acid, and cell attachment is completely prevented by competition with the

sialic acid bearing sugar sialyllactose. In contrast, neurovirulent strains are not neutralized by sialyllactose and instead bind to cells via HS. Interestingly, soluble HS only partially blocks cell attachment, suggesting that a second unidentified receptor may be involved in cell entry. HS and sialic acid may influence cell tropism but remain insufficient for infectious entry, a situation that would parallel the one found with the DAF binding enteroviruses.

3.9.4 CD155/PVR as a Factor in Poliovirus Pathogenesis—PV pathogenesis has been the subject of intensive study and tropism, and pathogenesis are clearly linked to virus entry (Nathanson 2008). CD155 has no known homologs outside of humans and primates and is responsible for the restricted range of susceptible species. One of the three PV serotypes (PV2) could be adapted to intra-cerebral infection of wild-type mice, and the mouse-adapted neurovirulent viruses contained capsid alterations, suggesting that adaptation allowed use of a murine receptor that remains unidentified (Nathanson 2008). However, the generation of PVR transgenic mice allowed intra-cerebral and intra-muscular infection by wild-type viruses with pathogenesis similar to the classical poliomyelitis seen in humans and primates (Nathanson 2008). Despite the utility of these models, the natural route of infection via the gut is not supported unless the interferon response is disabled, in which case pathogenesis no longer resembles classical poliomyelitis (Nathanson 2008). Furthermore, in normally susceptible species, levels of CD155 mRNA appear high in both infected and noninfected organs. It would therefore seem that PVR is necessary but not sufficient to completely explain tropism and pathogenesis of this disease.

4 Endocytosis and Sites of Uncoating

As we have seen, picornaviruses utilize a variety of receptors to facilitate binding to cell surfaces, but entry to the cytoplasm occurs from within endocytosed vesicles. There has been debate as to whether some viruses, including PV, can enter directly through the plasma membrane, but recent studies suggest that this route is not normally used (Berka et al. 2009; Brabec et al. 2003; Brandenburg et al. 2007). However, some viruses can be “forced” to penetrate the plasma membrane by manipulation of the infection conditions (Berka et al. 2009; Brabec et al. 2003). Several endocytic pathways have been identified; one of the best characterized being clathrin-mediated endocytosis in which ligands (including viruses) enter via clathrin-coated pits that are internalized to form clathrin-coated vesicles, which are then uncoated and are subsequently delivered to the early endosome. Other entry routes such as caveolin-mediated endocytosis are dependent on the presence of lipid rafts and cholesterol. Viruses that enter cells via caveolin-mediated endocytosis are sorted to the caveosome. However, alternative sorting of caveolin-1 vesicles to the endosome has been documented, and there is increasing evidence for “cross-talk” between the various endocytic pathways (Pelkmans et al. 2004). Routes of entry that require neither clathrin nor caveolin nor lipid rafts have been reported (Pelkmans and Helenius 2003). In general, the nature of the receptor determines the pathway of entry. For examples, picornaviruses that use integrins with the α_v subunit enter the cell via clathrin-mediated endocytosis, while echovirus 1, which uses integrin $\alpha_2\beta_1$, enters via caveolae. The link between the receptor and the entry route becomes apparent when viruses are forced to use alternative receptors, and the entry route changes accordingly. However, viruses may enter via different routes even when using the same receptor (Brandenburg et al. 2007; Coyne and Bergelson 2006).

A variety of methods have been used to examine the routes of entry by viruses. These include confocal microscopy to demonstrate co-localization of virus particles with specific components of the endocytosis machinery and inhibition of specific pathways by dominant negative mutants, siRNAs, or pharmacologically active compounds. Many of these methods can have by-stander effects on cell function, and it is important to corroborate results

obtained with any of these techniques. In addition, the (very) high particle/pfu ratios typical of many picornaviruses make the distinction of productive and nonproductive routes of entry difficult and can complicate the interpretation of results.

4.1 Clathrin-Mediated Endocytosis

4.1.1 Aphthoviruses—FMDVs bind to a range of RGD motif-dependent integrins but $\alpha_v\beta_6$ appears to be the major receptor in cattle (Berryman et al. 2005; Jackson et al. 2000), and a recent study showed that $\alpha_v\beta_8$ is used to infect cells of porcine origin (Johns et al. 2009). Several reports support clathrin-mediated endocytosis as the route of entry for FMDV. Berryman et al. (2005) analyzed the entry of serotype O virus into SW480 (human colon carcinoma cells) and CHO (Chinese hamster ovary) cells, transfected to express $\alpha_v\beta_6$, using a combination of methods including pathway-specific inhibitors. Sucrose, which causes clathrin-coated pits to disappear and induces abnormal clathrin polymerization into empty cages (Hansen et al. 1993; Heuser and Anderson 1989), inhibited infection by FMDV in a dose-dependent manner, suggesting that entry is clathrin-dependent. It is important to note that a low moi (~0.3) was used in this study, thus avoiding problems associated with high moi, which can saturate the normal entry route and cause infection to proceed via unusual pathways. FMDV entry was also inhibited in cells transfected with a dominant negative form of the clathrin coat assembly protein 180 (AP180), which is required for clathrin cage assembly. The C-terminus of AP180 binds to clathrin, while the N-terminus binds to the inositol polyphosphate in the plasma membrane, and when the C-terminus is over-expressed, it acts as a dominant negative inhibitor of clathrin-mediated endocytosis (Ford et al. 2001; Hao et al. 1997; Ye et al. 1995). In addition, immunofluorescence (IF) analysis showed that co-localization of the virus with markers of clathrin-dependent endocytosis, specifically the early endosomal marker 1 (EEA1) and the transferrin receptor, was inhibited in AP180 dominant negative transfected cells.

FMDV serotypes O and A were also showed by confocal microscopy to enter cells via a clathrin-dependent mechanism (O'Donnell et al. 2005). Although a high moi (10–100) was used in this study, the virus co-localized with markers of the clathrin-dependent entry route (clathrin, EEA1). Chlorpromazine, which causes loss of coated pits from the plasma membrane and induces clathrin-coated cages to assemble on endosomal membranes (Wang et al. 1993), confirmed that the virus enters the cell via the clathrin-mediated route.

Another study, using FMDV serotype C, also showed that entry of the virus is clathrin-dependent but, in addition, suggested a requirement for cholesterol in the plasma membrane (Martin-Acebes et al. 2007). Although nystatin, which selectively disrupts lipid rafts, including those required for caveolae-dependent entry, did not affect virus entry into cells, the cholesterol depleting activity of MβCD inhibited virus internalization (Martin-Acebes et al. 2007, 2009). This contrasts with reports that FMDV serotypes A and O are not inhibited by nystatin or MβCD (Berryman et al. 2005; O'Donnell et al. 2005). However, MβCD has also been shown to reduce clathrin-dependent entry of transferrin receptor and HRV (Rodal et al. 1999; Snyers et al. 2003; Subtil et al. 1999). Although differences in the susceptibility of the cell lines to cholesterol depletion and the concentration of MβCD could be responsible for these discrepancies, it has been proposed that cholesterol is necessary for clathrin-induced curvature and budding of the plasma membrane (Rodal et al. 1999; Snyers et al. 2003; Subtil et al. 1999).

ERAV is also classified in the genus *Aphthoviridae*, but unlike FMDV it attaches to cells via sialic acid residues on an unknown receptor (Stevenson et al 2004; Fry et al. submitted). However, recent studies have shown that it too enters via clathrin-mediated endocytosis (Groppelli et al. submitted). Productive infection was inhibited in the presence of sucrose or chlorpromazine and by the dominant negative form of Eps15 (which is involved in clathrin-

mediated endocytosis). In addition, IF showed co-localization of ERAV with EEA1 at early times post infection

4.1.2 Minor Group Human Rhinoviruses—The minor group HRVs use members of the LDLR family for cell entry (Hofer et al. 1994; Marlovits et al. 1998c). The C-terminal cytoplasmic domains of LDLRs contain tyrosine- and di-leucine-based internalization signals and are responsible for the clustering of LDLRs in clathrin-coated pits (Chen et al. 1990; Glickman et al. 1989; Pearse 1988). Clathrin-dependent entry of HRV2 was implied from IF studies of virus entry into HeLa cells exposed to hypotonic medium followed by potassium depletion, which results in dissociation of clathrin-coated pits from the plasma membrane (Hansen et al. 1993). Under these conditions, the virus was detected mainly at the cell surface, suggesting that entry was inhibited. In addition, dominant negative inhibitors of the clathrin-mediated entry route, including dynamin (K44A), AP180 (C-terminal domain), and Rab5 (S34N), substantially reduced HRV2 internalization, confirming that entry is clathrin-dependent (Bayer et al. 2001; Snyers et al. 2003). Cholesterol depletion by MβCD also significantly inhibited HRV2 entry (Snyers et al. 2003). However, as in the case of FMDV, cholesterol might be required for membrane curvature and is not necessarily an indication of lipid rafts- or caveolae-dependent endocytosis (Subtil et al. 1999). Ceramide, another component of lipid rafts, is also required for HRV2 entry but has been shown to play a role at the receptor-binding step (clustering) rather than in lipid raft-dependent endocytosis (Grassme et al. 2005).

4.1.3 Major Group Human Rhinoviruses—Most HRVs (91 serotypes, with HRV14 as prototype member) belong to the major receptor group and use ICAM-1 as receptor (Greve et al. 1989; see Sect. 3.1). In addition to its role in intercellular adhesion and immune response, it has been proposed that ICAM-1 can function as receptor and direct endocytosis of ligands via a novel pathway (Muro et al. 2003). However, this study did not investigate the internalization of natural ligands such as LFA-1 or HRVs and so did not mimic the natural situation. Also, co-localization of pathway-specific markers (clathrin, caveolin) by IF was not confirmed with other established methods, such as path-way-specific inhibitors (e.g., sucrose, chlorpromazine, nystatin, and MβCD) or dominant negatives (i.e., AP180, Eps15; Muro et al. 2003). Therefore, the putative novel endocytosis pathway directed by ICAM-1 needs further investigation.

Although the cellular role of ICAM-1-mediated endocytosis is still poorly understood, there is evidence that ICAM-1-bound viruses are internalized via clathrin-dependent endocytosis. HRV14 was shown by transmission electron microscopy of infected HeLa cells to localize in clathrin-coated pits/vesicles at 5 min post entry (Grunert et al. 1997). BHK cells, which lack ICAM-1 and do not bind HRV14, did so when transfected with a construct expressing human ICAM-1. Although virus was internalized, it was associated with abnormal membrane structures of distinct morphology (channels in membrane and HRV14 lined up as pearls on a string) and the cells did not support HRV14 replication. This suggests that HRV14 infection was inhibited after internalization, probably during or shortly after uncoating (Grunert et al. 1997). By contrast, mouse epithelial cells transfected with a human/murine chimeric ICAM-1 not only bound and internalized HRV16 but were also able to support replication (Tuthill et al. 2003). A dominant negative inhibitor of dynamin (K44A) was also shown to inhibit HRV14 infection (DeTulleo and Kirchhausen 1998). However, it is worth noting that dynamin also participates in nonclathrin endocytosis routes.

Analysis of the signaling pathways activated by HRV16 upon ICAM-1 binding in primary airway epithelial cells revealed two links with the endocytosis machinery. The cytoplasmic tail of ICAM-1 was found to interact with actin through an adaptor protein, ezrin (Wang et al. 2006). Interestingly, ezrin functions also as adaptor protein between ICAM-1 and Syk,

resulting in activation of Syk. Syk is a tyrosine kinase that has important regulatory roles in the adaptive immune response (Turner et al. 2000; Ulanova et al. 2005). Activation of Syk upon HRV16/ICAM-1 binding has a twofold effect. It initiates a signaling cascade involving p38 MAPK, which results in up-regulation of ICAM-1, thus facilitating HRV16 infection by providing a positive feedback loop (see Sect. 3; Wang et al. 2006). In addition, HRV16/ICAM-1 binding results in cytosolic Syk being recruited to the plasma membrane in association with clathrin and PI3K. HRV16 internalization is dependent on the presence of a functional Syk and on Syk-mediated activation of PI3K (Lau et al. 2008).

4.1.4 Other Picornaviruses—Other picornaviruses have been shown to use a clathrin-dependent pathway, and these include the enterovirus swine vesicular disease virus (SVDV), which is a porcine variant of human Coxsackievirus B5 (Martin-Acebes et al. 2009) and HAV. Entry of HAV appears to be clathrin-dependent because chlorpromazine inhibits infection (Bishop 1998). There is also indication that inhibition of cytoskeleton organization (actin and microtubules) does not affect HAV (Superti et al. 1987, 1989; Widell et al. 1986). However, it would be useful to reanalyze HAV entry using the wide range of methods currently available.

4.1.5 Role of Endosomal pH—Viruses internalized via clathrin-dependent endocytosis are delivered to the early endosome where they encounter an acid pH, and for many viruses exposure to an acidic environment is necessary to complete the process of cell entry. This is demonstrated by the decrease in infectivity caused by compounds that inhibit endosomal acidification. Concanamycin A and the structurally related bafilomycin A are selective inhibitors of the vacuolar type H^+ ATPase. V-ATPases couple the energy of ATP hydrolysis to transport protons across endosomal membranes, thus establishing a pH gradient. Inhibition of V-ATPase results in an increase in endosomal pH (Drose and Altendorf 1997). Monensin, nigericin, and X537A are carboxylic ionophores that intercalate in membranes and mediate exchange of monovalent cations. When present in the endosomal membrane, they exchange cytoplasmic K^+ for protons, thereby increasing endosomal pH. Ammonium chloride, chloroquine, and methylamine are relatively lipophilic in their unprotonated form and are membrane permeable. When they reach the endosome, they become protonated and accumulate, resulting in increased pH (Mellman et al. 1986).

From the early endosome, internalized cargos and membrane proteins are either trafficked back to the plasma membrane via the recycling endosome or they progress to the late endosome and lysosome for degradation (Gould and Lippincott-Schwartz 2009). In the latter case, the luminal pH progressively decreases from around 6.5 in the early endosome to 5.5 in the late endosome. The maturation from early to late endosome is dependent on microtubules and a set of membrane GTPases of the Rab family, in addition to V-ATPases. Inhibition of the maturation process at different stages is used to identify the pH necessary for a productive infection, for example, pH 6.5 of the early endosome or pH 5.5 of the late endosome. Early-to-late endosome maturation can be inhibited by drug-induced depolimerization of microtubules (e.g., nocodazole treatment; Aniento et al. 1993), dominant negative Rabs (i.e., Rab 5, Rab7, Rab11; Rink et al. 2005), and inhibition of PI3K signaling (i.e., wortmannin; Wipf and Halter 2005).

The requirement for an acidic pH for FMDV infection has been extensively analyzed. Concanamycin A, monensin, and ammonium chloride all inhibit infection suggesting that the low pH of the early endosome is required (Baxt 1987; Berryman et al. 2005; O'Donnell et al. 2005; Wachsman et al. 1998). Inhibition of different stages of the endosomal pathway with dominant negatives of the Rab proteins (Rab 4, 5, 7, and 11) also showed that FMDV entry occurs predominantly from the early endosome and does not require trafficking to the late endosome and to lysosomes (Johns et al. 2009).

Interestingly, FMDV can be forced to enter the cell via alternative receptors and pathways. However, the infection is productive only if an acidic pH is encountered. Growth of FMDV in vitro frequently selects for virus variants that utilizes HS as receptor, which has been shown to result in entry via a caveolae-dependent route (O'Donnell et al. 2008). However, regardless of the receptor and endocytosis mechanism, the virus retains the requirement for an acidic pH, and it has been suggested that FMDV-containing caveolae are sorted to the early endosome where a low pH is encountered (O'Donnell et al. 2008). It is not clear if FMDV binding directs sorting of caveolae to the endosome or if it makes use of the small percentage of caveolae that transiently traffic to the early endosome (Parton 2004; Pelkmans et al. 2004).

FMDV complexed with IgG is endocytosed by and can infect cells expressing Fc receptors, providing further evidence that entry is dependent on acid pH but not on a specific receptor. In contrast, PV entry via FcR did not result in a productive infection, suggesting that PV interaction with the receptor is involved not only in docking but also in uncoating (Mason et al. 1993; see Sect. 5).

HRVs also require a low endosomal pH for cell entry. HRV2 (minor group HRV) infection is strictly dependent on reaching pH 5.5 in the late endosome and is inhibited by monensin and bafilomycin A1 (Bayer et al. 1998; Neubauer et al. 1987; Prchla et al. 1994). Nocodazole and wortmannin treatments also inhibited infection, suggesting that microtubule- and PI3K-dependent early-to-late endosome maturation is required for HRV2 infection (Berka et al. 2009; Brabec et al. 2006). HRV 14 (major group HRV) is also bafilomycin and monensin sensitive, suggesting that it too requires an acidic endosomal experience (Grunert et al. 1997; Perez and Carrasco 1993; Tartakoff 1983).

Surprisingly, cell entry of enterovirus swine vesicular disease virus (SVDV) appears to be pH-dependent on the basis of inhibition by concanamycin A and ammonium chloride. However, the use of nocodazole and wortmannin to determine the requirement for early-to-late endosome maturation gave conflicting results. While nocodazole inhibited infection, wortmannin did not. This might indicate that SVDV entry vesicles are trafficked in a microtubule-dependent manner to the early endosome but there is no requirement to reach the late endosome, or that microtubules are involved in SVDV trafficking to an organelle other than the late endosome (Martin-Acebes et al. 2009). The acid pH requirement for entry by SVDV is unexpected because SVDV particles, like other enteroviruses, are stable between pH 2.5 and 12 (Fry et al. 2003).

HAV is also acid stable, yet infection is affected by monensin (Bishop 1998; Superti et al. 1987, 1989). However, in these studies the drug was left in contact with the cells for many hours and may have affected HAV replication post entry. It would be interesting to repeat HAV entry studies in the presence of concanamycin A and other inhibitors of endosomal acidification to confirm the requirement for an acid pH during the cell entry process.

4.2 Caveolin-Mediated Endocytosis: Coxsackie and Echoviruses

4.2.1 Echovirus 1—Although EV1 entry appears to involve caveolin-mediated endocytosis, there are significant differences with the classical caveolin-mediated entry originally elucidated for simian virus 40 (SV40). EV1 entry has been analyzed in a human osteosarcoma cell line (SAOS) stably transfected to express the receptor for EV1, integrin $\alpha_2\beta_1$, and in the green monkey kidney cell line, CV-1. Although $\alpha_2\beta_1$ is the main receptor, β_2 microglobulin is also required for EV1 entry, which is inhibited by antibodies to β_2 microglobulin. However, the precise role of β_2 micro-globulin in EV1 attachment and entry remains unclear. Integrin $\alpha_2\beta_1$ is a collagen receptor that mediates the interaction between epithelial and mesenchymal cells and the extracellular matrix (Bergelson et al. 1992), and

normally resides in raft-like membrane domains that are negative for caveolin-1. Binding of EV1 to $\alpha_2\beta_1$ induces receptor clustering and initiates redistribution of $\alpha_2\beta_1$ into membrane invaginations reminiscent of caveolae (Upla et al. 2004). It is important to note that, at this stage of entry, there is little co-localization of $\alpha_2\beta_1$ and EV1 with caveolin-1 at the plasma membrane, and dominant negative caveolin-1 has no effect of EV1 internalization. Interestingly, it appears that these invaginations do not require dynamin to pinch off from the plasma membrane. However, some components of the macropinocytosis pathway (including PKC, Pak1, and Rac1) are involved. This is supported by the fact that markers for fluid-phase uptake (10 KDa dextran) are co-internalized with EV1 (Karjalainen et al. 2008; Liberali et al. 2008; Pietiainen et al. 2004; Upla et al. 2004). This suggests that the early events in EV1 entry possibly involve components of different endocytosis mechanisms.

Although EV1 does not co-localize with caveolin-1 at the plasma membrane, IF showed that co-localization increases between 15 min and 2 h and electro micrographs taken at 30 min pi showed the virus in vesicular structures with the morphology typical of caveolae (Marjomaki et al. 2002). Interestingly, $\alpha_2\beta_1$ is internalized alongside EV1 in caveolae and the co-localization is still evident 2 h pi in vesicular structures (caveosomes) in the perinuclear region. Caveosomes seems to be the final target of EV1 because it cannot be detected in other organelles (ER or Golgi) at later time points. This is also supported by the resistance of EV1 infection to the microtubule depolymerizing agent nocodazole (Pietiainen et al. 2004). In addition, the virus could not be localized in endosomes and was not exposedm to acidic pH asassessed by co-internalization with pH sensitive dextrans.

In summary, it appears that the early events in EV1 entry are dependent on the presence of cholesterol and lipid rafts at the plasma membrane (MβCD and nystatin methods) but are not dependent on caveolin-1. EV1 entry is also characterized by faster kinetics than canonical caveolin-1-dependent entry of SV40, and signaling analysis suggests a macropinocytosis-like pathway. However, once EV1 is internalized, it migrates in caveolin-1 positive vesicles that are trafficked to the perinuclear region and fuse with caveosomes (Karjalainen et al. 2008). This is reminiscent of the entry scenario of SV40 in caveolin-1 knock-out mouse cells. Unexpectedly, SV40 is able to enter these cells in a caveolin-1 independent manner, but it is transported to caveosome-like structures with faster kinetics than in canonical SV40 entry (Damm et al. 2005).

4.2.2 Coxsackie B3: Role of Co-Receptors—CVB3 strain RD entry in polarized epithelial cells is dependent on both DAF and CAR. DAF is a GPI-anchored protein that resides in lipid raft domains in the apical surface of polarized cells (Brown and London 1998), but CVB3 (RD) induces DAF clustering and translocation of DAF/CVB3 complexes to the tight junction (TJ). Infection is prevented by the lipid raft destabilization agent MβCD, which inhibits DAF clustering and translocation of the DAF/CVB3 complex to the TJ. It was originally thought that translocation of CVB3 to the TJ promotes TJ disassembly which, in turn, facilitates CVB3 binding to CAR (Coyne and Bergelson 2006). However, it appears that CVB3 does not induce significant reorganization of the TJ, but unexpectedly induces internalization of occludin (Coyne et al. 2007b). Occludin is a transmembrane protein that is part of the TJ complex. Although occludin does not interact directly with CVB3, its depletion inhibits virus entry and infection. Both CVB3 and occludin are internalized in a caveolin-1-dependent manner. CVB3 localizes in caveolin-1 positive vesicles (caveolae and caveosomes) within 60 min pi, but in cells transfected with a dominant-negative caveolin-1 mutant, the virus is still detected in the TJ at 90 min pi. Interestingly, it appears that caveolin-1 phosphorylation is necessary for CVB3 internalization. Although tyrosine kinase activity has been associated with caveolin-1-dependent endocytosis, specific phosphorylation of caveolin-1 had not previously been linked to virus entry (Pelkmans et al. 2005). Therefore, phosphorylation of caveolin-1 might

be a specific requirement of CVB3, but its precise function needs elucidation. Although CVB3 and occludin require caveolin-1 entry, it appears that they do not require all components of the canonical caveolin-1-dependent endocytosis. In fact, dynamin is not involved in CVB3 and occludin entry, as shown by the lack of inhibition by a dominant negative dynamin (K44A). Therefore, although the requirement for caveolin-1 and the slow entry kinetics would suggest a canonical caveolin-1 mediated entry for CVB3, the lack of dynamin requirement and the involvement of occludin suggest that other entry mechanisms are involved in CVB3 internalization. Indeed, when macropinocytosis was inhibited (with an amiloride analogue and rottlerin), CVB3 and occludin internalization was also inhibited (Coyne et al. 2007b). Micropinocytosis involvement is further supported by the use of dominant negatives and siRNA against Rab34, a GTPase implicated in micropinosome formation.

Although occludin clearly participates in CVB3 entry, its precise role is not clear. Occludin does not bind to CVB3 or CAR, but interacts with a number of signaling molecules and also with caveolin-1 (Nusrat et al. 2000a, b). Therefore, a potential mechanism for CVB3 entry involves engagement of CAR to trigger uncoating and the presence of occludin to provide a scaffold protein to recruit signaling and regulatory molecules.

In contrast to the entry of CVB3 (RD), CVB3 H3 strain entry has been shown to be mediated by clathrin and to require dynamin and endosomal acidification. It is important to note that this study was carried out in nonpolarized epithelial cells (HeLa) (Chung et al. 2005) and, therefore, might be relevant specifically to nonpolarized nonenterocytic cells. In enterovirus studies, HeLa cells represent a more artificial system than polarized CaCo2 cells, which are a model for the small intestine and mimic more closely the natural situation for an enterovirus (Coyne et al. 2007b; Engle et al. 1998). Therefore, the difference in entry route between the strains RD and H3 and the unexpected requirement of H3 for an acidic pH might be caused by the cell type and might not represent a difference between the two virus strains. It would be certainly helpful to analyze both strains side-by-side in polarized and nonpolarized cells.

4.3 Noncaveolin Nonclathrin Mediated Endocytosis: Poliovirus

To further characterize the PV entry pathway, Brandenburg et al. (2007) used live cell microscopy of HeLa cells infected with virus labeled with separate fluorescence dyes bound to the capsid protein and the viral genome. They were thus able to follow the kinetic of RNA release and to probe the effects of inhibitors of cell trafficking pathways on PV entry at the single particle level at a low multiplicity of infection. In parallel, they used a neutral red assay as a surrogate assay for RNA release whose readout is infection. The study showed that the kinetics of RNA release and the effects of a variety of small molecule inhibitors and siRNAs were indistinguishable in the fluorescence assay and the biological assay, providing confidence that the RNA release that is observed in the fluorescence assay is productive. The study showed that internalization of the virus required receptor-mediated conversion to the 135S or A particle, that RNA release is rapid ($t_{1/2}$ ~ 20 min) and efficient (and is therefore not a significant factor in the high particle to pfu ratio of the virus), and that RNA release occurs within 100–200 nm of the cell surface. The study further showed that PV infection of HeLa cells was dependent on actin, ATP, and an as yet unidentified tyrosine kinase, but was independent of clathrin, caveolin, flotillin, microtubules, and pinocytosis. Curiously, after (and apparently only after) RNA is released, vesicles containing empty virus particles are rapidly transported to the perinuclear region in a microtubule-dependent pathway. This may represent a scavenging pathway for degradation and recycling of the empty capsids. In a follow-up study, the authors demonstrated that early infection virus particles (apparently in vesicles) undergo very rapid actin-dependent movement, reaching

speeds higher than known myosin motors (Vaughan et al. 2009). The role of this movement is as yet unclear.

In another study, Coyne et al. (2007a) characterized the entry pathway of PV in Human Brain Microvascular Endothelial Cells (HBME), a highly polarized cultured cell line considered to be a model for the blood–brain barrier (Coyne et al. 2007a). In contrast to earlier studies showing that PV infection of HeLa cells is fast and independent of both dynamin (Brandenburg et al. 2007; DeTulleo and Kirchhausen 1998) and caveolin (Brandenburg et al. 2007), they showed that PV infection of HBME is very slow and utilizes dynamin-dependent caveolar endocytosis. They also showed that entry requires tyrosine kinase and Rho GTPase activation induced by virus binding to PVR, that virus binding induced tyrosine phosphorylation of the cytoplasmic tail of PVR, that this phosphorylation results in recruitment and activation of SHP-2, and that activation of SHP-2 is required for entry and infection. The differences between these studies highlight the flexibility of these viruses in adapting to multiple routes of entry, and re-emphasize the fact that cell entry pathways can vary by cell type.

4.4 Concluding Remarks on Picornavirus Endocytosis

The great majority and possibly all picornaviruses gain entry to the cytoplasm via an endocytic vesicle. However, the complexity and variety of endocytic mechanisms has become increasingly apparent in recent years. Moreover, several picornaviruses have now been shown to require complex receptor/co-receptor interactions for delivery to appropriate sites on the cell surface for the induction of endocytosis and to initiate uncoating. It has been shown that some viruses are adapted to enter the cell via specialized sites, such as tight junctions, that may not be present in normal cell culture systems, emphasizing the need for caution in interpreting data obtained from artificial systems.

There is still a lot to be learned about the biochemistry and cell biology of endosomal trafficking and their relationships with virus uncoating. In addition, it has become clear that picornaviruses can tolerate and adapt to use alternative routes of entry into cells. It is clear that the dominant mechanism of entry for any virus may be the most efficient, but as long as viruses can be endocytosed by whatever route many can infect cells, albeit at low efficiency.

5 Capsid Alterations During Uncoating

Although the trigger for genome release differs for different picornaviruses, the final result is the externalization of the RNA genome. For a productive infection, this must occur in a manner that facilitates its delivery to the cytoplasm of the host cell in an intact and infectious form. The process of genome release has been studied in depth for relatively few picornaviruses, and we still do not have a detailed understanding of the mechanisms involved for any. However, the viruses for which information is available can be divided into two groups based on the final products of the uncoating process. In the enteroviruses (including HRVs), the icosohedral structure is maintained throughout and an empty particle remains after the RNA and VP4 have been externalized (Belnap et al. 2000b; Chow et al. 1987; De Sena and Mandel 1977; Levy et al. 2010). On the other hand, the aphthoviruses and cardioviruses dissociate in acidic conditions into pentameric subunits with release of the RNA and VP4 (Dubra et al. 1982; Mak et al. 1970). It has been supposed that this is the mechanism of genome release during infection as endosomal acidification to pH values concomitant with capsid disassembly appears to be essential for triggering the cell entry process (Berryman et al. 2005; Johns et al. 2009). These differences are sufficient to warrant separate consideration of the capsid alterations associated with genome release in the two groups of viruses.

5.1 Enteroviruses

In early studies of the infection process, it was observed that a proportion (50–90%) of rhinovirus or enterovirus particles that had attached to cells at low temperatures were released into the medium as modified forms, termed A (altered) particles, on warming the cultures to physiological temperatures (Crowell and Philipson 1971; Joklik and Darnell 1961). Eluted particles are generally thought to be no longer infectious (but see below) and sediment in sucrose gradients more slowly than native virus particles (Lonberg-Holm et al. 1975). Mature entero- or rhinovirus particles sediment at ~ 160S, while the A particles sediment at about 135S, and are often referred to as 135S ~ particles. These and subsequent studies have defined a catalogue of changes that accompany the conversion from virions to A particles which, in general, are common to all entero- and rhinoviruses. A particles lack some or all of their content of VP4 (Fricks and Hogle 1990; Greve et al. 1991; Hewat and Blaas 2004) and have externalized most of the amino-terminal extension of VP1 (which is normally on the inside surface of the virus), but retain the full complement of genomic RNA (De Sena and Mandel 1977). While mature virus particles bind their cognate receptors on host cells, A particles have lost this function, hence their post-binding elution into the culture medium. In contrast to the generally hydrophilic characteristics of mature virions, A particles are hydrophobic in nature and can directly bind to membranes in the form of artificial liposomes or associate with detergent micelles (Danthi et al. 2003; Everaert et al. 1989; Fricks et al. 1985; Lonberg-Holm and Yin 1973). In addition, A particles are antigenically distinct from mature virions and have different protease sensitivities (Danthi et al. 2003; Everaert et al. 1989; Fricks et al. 1985; Lonberg-Holm and Yin 1973).

Although the eluted A particles as originally observed can take no part in the infection process, since they have been shed from cell surfaces prior to entry and have no appreciable affinity for the receptor, apparently identical particles can be isolated from within cells after attachment and entry (Fricks and Hogle 1990). This led to the proposal that they are important intermediates in the cell entry process (Curry et al. 1996; Huang et al. 2000). This proposal was based on the observation that the 135S particle can induce infection in a receptor-independent infection (albeit with a specific infectivity four orders of magnitude lower than virus; Curry et al. 1996) and that the specific infectivity of 135S particles can be brought to within an order of magnitude of that of virus by binding a non-neutralizing antibody to the A particles and initiating infection in Fc expressing cells (Huang et al. 2000; Mason et al. 1993). This suggestion is also supported by the observation that neutralization of infectivity induced by a number of capsid binding compounds parallels their inhibition of the conformational changes, resulting in A particle formation (Andries et al. 1990; Cox et al. 1996; Smith et al. 1986). The intracellular conversion of native virions into A particles is followed by their further conversion into empty particles, which sediment at 80S and have shed their genomic RNA (Fricks and Hogle 1990). There is now wide acceptance that A particles and empty particles represent a relatively stable intermediate structure and the final product of the entry process, respectively. Although study of these particles can provide clues as to the mechanisms of genome delivery into the cell, it will be necessary to trap less stable intermediate structures in the process of transferring their cargo of RNA to fully understand the process.

A particles were first identified as the result of aborted infections, but subsequently it was found that, for some entero- and rhinoviruses, the formation of A particles could be induced with complete, solubilized receptor molecules (Kaplan et al. 1996) or with truncated receptors from which the membrane anchor domains had been deleted (Casasnovas and Springer 1994). Receptor catalyzed conversion to A particles is temperature-dependent but the optimal temperature at which this occurs varies for different viruses (Gomez Yafal et al. 1993; Hoover-Litty and Greve 1993; Kaplan et al. 1996). A particles can also be produced by heating virus particles to super-physiological temperatures (50°C) in defined ionic

conditions (Curry et al. 1996; Wetz and Kucinski 1991). The properties of these thermally induced particles are indistinguishable from those produced at physiological temperatures in the presence of receptor.

Receptor docking is clearly a trigger for the initiation of profound conformational changes in these viruses, and the mechanisms involved are beginning to emerge from a combination of structural and biochemical studies. In those viruses for which receptor engagement catalyses conversion to A particles, the receptor molecules bind within the “canyon”, the depression encircling the fivefold axis of symmetry. Initial interaction with receptor molecules results in a “loose” binding, which is subsequently converted to “tight” binding following minor conformational changes within the “canyon” (Casasnovas and Springer 1995; Casasnovas et al. 1998; McDermott et al. 2000). A common feature of entero- and rhinoviruses is the presence of a hydrophobic pocket within the core beta barrel of VP1 and lying at the base of the “canyon”. The pocket is often occupied by a fatty acid moiety (pocket factor) in native virus particles (Kim et al. 1989; Oliveira et al. 1993; Smyth et al. 1995; Verdaguer et al. 2000), but a number of synthetic compounds have been shown to displace the natural occupant of the pocket due to higher affinity (Smith et al. 1986). These capsid binding compounds neutralize virus infectivity by two mechanisms. Some distort the conformation of the base of the “canyon” overlying the pocket and interfere with receptor binding (Pevear et al. 1989); others allow receptor binding but are less easily displaced than the molecule(s) normally occupying the pocket (Grant et al. 1994; Smith et al. 1986), and so inhibit the receptor induced production of the A particle. Flexibility here is clearly important for the structural transitions required to convert native particles into A particles, as occupancy of the pocket by such compounds stabilizes them against thermal or receptor-induced conformational change (Diana et al. 1989; Fox et al. 1986; Tsang et al. 2000). Although it was originally thought that drug binding inhibited the production of A particles by making the virus particle more rigid, both computation modeling (Phelps and Post 1999; Speelman et al. 2001) and kinetic studies (Tsang et al. 2000) demonstrate that the observed stabilization of the virus was due to entropic effects rather than enthalpic effects, and suggest that the stabilization is the result of increased compressibility rather than increased rigidity of the drug bound state. Displacement of the pocket factor as a result of receptor engagement was thought to be of major importance in the conformational conversion into A particles, but it has been recently argued that the presence of a deformable space is the important requirement (Katpally and Smith 2007).

In contrast to enteroviruses and major receptor group HRVs, binding of the receptor for minor receptor group HRVs occurs not in the “canyon” but at a surface exposed location around the fivefold axis of symmetry (Hewat et al. 2000; Hofer et al. 1994). These viruses also have a VP1 pocket and can be stabilized by pocket binding drugs, but here the trigger for initiating conversion into the A particle form is provided by the acid pH encountered in endocytic vesicles and not by receptor engagement (Prchla et al. 1994).

Irrespective of whether the trigger for conversion is receptor binding or exposure to acidic conditions, the conformational consequences are broadly similar. These are the ejection of some or all of the internal protein VP4 and the externalization of the N terminal region of VP1 (Fricks and Hogle 1990; Lewis et al. 1998). Interestingly, the native virus particle has been shown to transiently and reversibly externalize both the VP4 and the N-terminus of VP1 at physiological temperatures in a process called “breathing” (Broo et al. 2001; Lewis et al. 1998; Li et al. 1994; Reisdorph et al. 2003). Moreover, kinetic studies probing the rate of virus to A particle conversion in PV as a function of temperature in the presence and absence of receptor have shown that the virus particle is trapped in its native state by a large energy barrier (enthalpy of activation), and that receptor binding significantly lowers this barrier (Tsang et al. 2001). Together these observations lead to a model in which the virus is

metastable, and a combination of physiological temperature (which by itself allows reversible breathing) and receptor binding (enteroviruses) or acidification (rhino-, cardio-, and aphthoviruses) release the particle from its metastable state and catalyze irreversible changes that ultimately facilitate genome delivery.

Receptor binding and acidification can induce significant conformational changes in the virus capsid, but the question remains as to how the N terminus of VP1, VP4, and the RNA are released. A number of $T = 3$ plant viruses whose structures are similar to picornaviruses have been shown to undergo a significant (~10%) expansion upon chelation of divalent cations at neutral or alkaline pH, and in tomato bushy stunt virus (TBSV), this has been shown to result in the externalization of the amino-terminal extensions of the capsid proteins through large openings in the surface of the capsid (Golden and Harrison 1982; Robinson and Harrison 1982). The relatively large change in sedimentation coefficient in the virus to A particle transition (160S–135S) led to the prediction that the picornavirus particle undergoes a similarly large expansion, leading to holes for the previously observed exit of VP4 and the amino-terminal extension of VP1. Based on the observation of partially open solvent containing channels at the fivefold axis of rhinovirus, Rossmann and colleagues (Hadfield et al. 1997) proposed that the release of VP4, RNA, and the amino-terminus of VP1 would occur through expansion of these channels at the fivefold axes. Based on the analogy with the plant viruses (where the fenestrations open at the quasi threefold axes of the $T = 3$ particle) and on genetic data demonstrating that mutations in the corresponding interfaces in PV played key roles in assembly and stability of the virus, others proposed that VP4 and the amino-terminal extension of VP1 would exit at the base on the “canyon” through openings in the inter-protomer interface (Filman et al. 1989).

Low resolution cryo-EM reconstructions of the A particle and 80S empty particles of PV (Belnap et al. 2000a) revealed that the particles had expanded by only 4% and that they lacked the obvious large fenestrations that are observed in the expanded states of TBSV (Robinson and Harrison 1982) and subsequently in cowpea chlorotic mosaic virus (Speir et al. 1995). The lack of obvious exit sites for the peptides in the A particle or for RNA in the 80S empty particles led to the suggestion that there must be additional as yet unidentified and probably transient intermediates in the 160S to A particle transition and the A particle to 80S empty particle transition that were significantly more expanded to create openings for the release of internal components. Although the 135S reconstruction excluded the fivefold axes as the site of RNA release (there was not enough space in the reconstruction to thread five copies of a peptide through the channel at the fivefold), the low resolution reconstruction shed little light on the site of release or the final position of the amino-terminal extension of VP1.

More recently, the cryo-EM structures of the PV A particle and a proteolyzed version of the A particle in which the first 31 amino acids from the amino-terminus of VP1 have been removed by V8 protease have been determined at approximately 10 Å resolution. Both reconstructions suggest that the amino-terminal extension is released at the base of the “canyon”, and bridges the “canyon” between the tips of a star-shaped feature at the fivefold axis (formed by the loops at the narrow end of VP1 and the tips of a propeller-shaped feature surrounding the threefold axes (formed by the EF loops of VP2; Bubeck et al. 2005a). A difference map comparing the density of the intact and proteolyzed A particles suggest that the last well-ordered residues of VP1 located at the tip of the propeller, and that the extreme amino-terminus of VP1 (believed to be an amphipathic helix) is flexibly attached in the A particle. These observations contradict all previous models that predict that regardless of where VP1 is externalized, its extreme amino-terminus would be located at the fivefold axis where it would insert into the membrane to form a fivefold helical channel, which would allow translocation of the viral RNA across the membrane. A recent study showing that a

ser5cys mutation in VP4 from HRV14 forms specific disulfide linked dimers upon breathing has led to the suggestion that VP4 also exits at a side at or near the particle twofold axes, rather than through the channel at the fivefold axes (Katpally et al. 2009).

Higher resolution cryo-EM reconstructions have also been produced for the 80S empty particles of HRV2 (~15 Å; Hewat et al. 2002), rhinovirus 14 (~12 Å), and PV (~9.5 A Å; Levy et al. 2010). Curiously, for both of the higher resolution reconstructions (HR14 and PV), the 80S preparations were shown to contain two different structures, one of which appears to contain more density corresponding to RNA inside than the other. In all structures, there was a notable disruption of the inter-pentamer and inter-protomer interfaces. Although the significance of the two different structures for the 80S particle is not completely clear, the variable levels of RNA suggest that RNA release is sufficiently slow that particles can be trapped midway through the process (the externalized RNA presumably then cut by endogenous contaminating RNases). In the PV 80S preparations, there were also a small number of particles that appear to be literally caught in the act of RNA release with density for RNA inside, crossing, and outside the capsid. Almost all of these were classified in the group of particles that contain more RNA. Asymmetric three-dimensional reconstructions of these particles by cryoEM and cryo-electron tomography (cryoET) confirm the presence of contiguous RNA-like density on the inside and outside surfaces of the particles, and demonstrate that the RNA exits from openings at the base of the canyon at a position analogous to the quasi threefold axes of $T = 3$ particles (the same position as the site of release for the amino-terminal extension of VP1; Bostina et al. submitted). The icosahedral reconstructions for all the 80S structures reveal possible openings at this site, but the openings are rather small, again suggesting the existence of an intermediate that is more expanded and has larger openings.

Although it is clear that the transition from native to A particle precedes the A particle to 80S particle transition in vitro and in vivo, and that the receptor plays a key role in the first transition but not the second, neither the trigger for RNA release nor the mechanism of release are known. It is, however, clear that RNA release must initiate from a single unique site on what is initially an ico-sahedrally symmetric particle. This could be explained by either of two mechanisms: (1) steric mechanism in which there is a unique structural site (perhaps linked to the residual VP0, to contact with the VPg molecule bound to the 5′ end of the RNA, or to some other asymmetric interaction of the RNA and the capsid protein) that uniquely defines the site of RNA egress. Although some special interaction between the virus and a vesicle membrane could also serve as an external cue during infection, the fact that heat mediated production of the 80S particle is efficient in vitro shows that this is not a necessary trigger for initiation or cue for the site of RNA release. (2) A kinetic model that postulates that the initiation of RNA release is difficult, but that once started the propagation of release is either fast or irreversible.

It is also believed that the release of RNA requires unfolding of secondary structure. Indeed this hypothesis is supported by observations by Brandenburg et al. (2007) that the Syto82 dye (which binds to double stranded RNA) is released from the RNA upon its externalization from the capsid during infection, but remains stably bound to isolated RNA in vitro after multiple dilutions. This is not an issue for the 80S transition in vitro, because the production of 80S particles in vitro requires heating at temperatures approaching the melting temperature of the viral RNA. It is more problematic during infection at physiological temperatures, and has raised the question of whether externalization requires the action of a helicase or single strand RNA binding protein. However, the absence of any noncapsid proteins at stoichiometries approaching 1 per virion would appear to rule out the participation of viral proteins (other than the capsid proteins themselves), and any model

postulating a role for a cellular factor would require that RNA release would be able to be initiated to an extent sufficient for the cellular protein to have access to the RNA.

The appearance of the RNA in the cryo-EMs of particles caught in the act of RNA release may shed light on the process of RNA unfolding during egress. In these micrographs, the density for the RNA outside the particle appears to be highly branched, consistent with a model in which the RNA is transiently unfolded as it passes through the shell and then refolds after release (Levy et al. submitted, Bostina et al. unpublished). This refolding could provide a molecular ratchet, which drives RNA release (already favored by the very high concentration of RNA inside the particle and low concentration outside) toward completion, and as more and more structure accumulates outside would make additional release progressively more favorable. Note that this model does not preclude the involvement of cellular proteins in the early stages, indeed a model in which a region at an end of the genome that already is predominantly single-stranded initiates egress (e.g., the polyA tail at the 3′ end) and is bound by a cellular partner (e.g., the polyA binding protein). The model could also explain the presence of variable amounts of RNA in the preparations of the 80S particles of rhinovirus 14 and PV. Thus egress would progress relatively rapidly in regions where the secondary structure of the RNA near the egress site is minimally stable and pause where the secondary structure is more stable.

5.2 Aphthoviruses

A characteristic feature of members of the genus aphthovirus is their extreme sensitivity to inactivation at acid pH. FMDV infectivity is lost when the pH is reduced below 6.8 (Brown and Cartwright 1961), although this is modulated somewhat by ionic strength and factors such as protein content of the suspending medium. In contrast to the more acid labile enteroviruses, such as HRVs, which maintain icosohedral integrity during the inactivation process, aphthoviruses dissociate into pentameric subunits, releasing RNA and VP4 (Burroughs et al. 1971). The pentamer interfaces of all picornaviruses are stabilized by β-sheet interactions, which span the subunit junctions. Acid pH-induced changes in the ionization state of clusters of histidine residues along the pentamer interfaces of FMDV particles are thought to disrupt these β-sheet interactions, resulting in particle disassembly (Curry et al. 1995; van Vlijmen et al. 1998). Extra β-sheet interactions provided by regions of VP1 further stabilize the pentamer interactions in enteroviruses and result in the greater capsid stability of these viruses (Filman et al. 1989). ERAV is another acid labile picornavirus and has also recently been included in the aphthovirus genus (Li et al. 1996). Although slightly more stable at acid pH than FMDV, it also dissociates into pentameric subunits (Tuthill et al. 2009). Again, particle disassembly follows disruption of the β-sheet interactions at the pentamer interfaces, but the driving force for these changes appears to be the rearrangement of loops in the structure of VP2 rather than the ionization of histidine residues at the interfaces (Tuthill et al. 2009).

As we discussed in Sect. 4, the site of uncoating of both FMDV and ERAV during the infection process is the early endosome, and the acidification of this compartment is crucial for the process. However, it is difficult to envision how the RNA is protected within the endosomal lumen following its release, and how its transfer across the endosomal membrane is achieved. Recent studies with ERAV have shown that disassembly into pentameric subunits under acid conditions proceeds via a transiently stable icosohedral empty particle from which the RNA has been ejected (Tuthill et al. 2009). The pH, time of exposure to acid conditions, ionic strength and the presence of protein all influence the proportion of virus present in this form. Furthermore, these empty particles are sufficiently stable if returned to neutral pH to permit physicochemical studies such as analysis of their sedimentation characteristics in sucrose gradients. The optimal conditions for crystal growth for X-ray diffraction studies were at a pH and ionic strength that favored conversion to the empty

form, thus enabling the near atomic structure to be determined. It is possible that this empty particle may be a transiently stable intermediate structure that is intimately involved in the entry process, serving to protect the RNA and direct its transfer across the endosomal membrane. In this context, it is worth noting that earlier studies had described the formation of empty capsid particles lacking both RNA and VP4 from FMDV (Rowlands et al. 1975).

6 Membrane Penetration

As we have seen in previous sections, the endocytic pathways used to enter cultured cells have been determined for several picornaviruses; receptor engagement, low pH, and further unknown factors have been demonstrated to have roles in initiating the uncoating process by inducing alterations to the capsid structure, which facilitate membrane interaction. Beyond this, little is known about the potential mechanism(s) for breaching the membrane. In terms of membrane topology, the endocytosed particle and its genetic cargo are still outside of the cell and the genome is still to be delivered from within the capsid, through/across the membrane, into the cytoplasm. The lumen of the endocytosed vesicle is likely to be a hostile environment for the RNA genome, potentially containing co-endocytosed molecules such as ribonucleases, which exist in serum at high concentrations. We therefore believe that during infection, uncoating is likely to be coordinated with a mechanism for membrane permeabilization such that genome release from the capsid will be concomitant with its safe delivery to the cytoplasm.

Comparative structural analyses of virions, A, and empty particles (reviewed in Sect. 5) have revealed capsid alterations that may be involved in membrane interaction and permeabilization during the entry process of entero- and rhinoviruses. The capsid components with potential for involvement in this process are the small N-terminally myristylated protein VP4 and the N-terminal hydrophobic region of VP1. Both of these are internal to the virion but are transiently exposed during capsid “breathing” at physiological temperature and become irreversibly externalized during the entry process. Here we review the evidence for virus–membrane interactions and the involvement of VP1 and VP4 in membrane permeability.

6.1 Review of Experimental Data

6.1.1 Interaction of Altered Particles with Membranes—Enterovirus (PV and HRV) A particles generated by heating virus particles in vitro have increased hydrophobicity and have acquired the ability to bind to liposome membranes (Fricks and Hogle 1990; Lonberg-Holm et al. 1976). In addition, PV particles bound to receptor-decorated liposomes are converted to A particles at physiological temperature. A proportion of these A particles dissociate from the receptor and instead associate directly with liposomes via the N-terminal extension of VP1 becoming inserted into the membrane (Tuthill et al. 2006).

6.1.2 Virus-Induced Membrane Permeability—PV has been shown to induce the formation of conductance channels in planar model membranes (Danthi et al. 2003; Tosteson and Chow 1997; Tosteson et al. 2004) and to induce permeability in liposome membranes (Tuthill and Rowlands, unpublished), both with temperature-dependent characteristics, suggesting that transient “breathing” or formation of A particles are involved in membrane permeabilization. In contrast, the permeability induced in planar membranes by pre-formed A particles is temperature-independent (Tosteson and Chow 1997), as would be expected if the membrane permeabilizing properties transiently expressed in “breathing” particles became irreversibly fixed in A particles. Perhaps the strongest evidence of virus-induced membrane permeabilization in vivo has been the demonstration of size-selective release of dextrans from the endosomes of cells infected with minor group HRV2 (Brabec et al. 2005; Prchla et al. 1995; Schober et al. 1998). These studies provide evidence that pores

of defined size are formed in otherwise intact endosomes. Interestingly, similar studies with major group HRV14 infected cells suggested that endosomes do not remain intact but are instead completely disrupted (Schober et al. 1998) as is observed during adenovirus entry.

6.1.3 Role of Externalized N-Terminus of VP1—The interaction between PV A particles and membranes is mediated by the hydrophobic N-terminus of VP1, which is externalized in the A particle (Fricks and Hogle 1990; Tuthill et al. 2006). This interaction may also induce membrane permeability: peptides corresponding to VP1 N-terminus of HRV2 are able to permeabilize endosomes (Prchla et al. 1995). Pre-formed PV A particles retain a reduced ability to induce channel formation in planar membranes (Tosteson and Chow 1997) and to infect cultured cells (Curry et al. 1996). If the A particle has lost all of its VP4 (see below), then these properties must be attributed to VP1 membrane interactions alone.

The N-terminal region present in the VP1 proteins of entero- and rhinoviruses is not seen in all viruses. For example, the VP1 protein of aphthoviruses is truncated at the N-terminus relative to PV, such that the hydrophobic region is absent (Tuthill et al. 2009). Aphthovirus uncoating does not appear to involve a “stable” A particle, as seen in the entero- and rhinoviruses, and how these viruses interact with and breach cellular membranes is not known.

6.1.4 Role for Released VP4—PV VP4 released during the conversion to A particles becomes associated with cellular (Danthi et al. 2003) or liposome membranes (Tuthill et al. 2006). In addition, recombinant HRV VP4 can associate with intact liposomes and induce membrane-permeability (Davis et al. 2008). Compelling evidence for the involvement of VP4 in entry has come from electrophysiology experiments with PV particles containing mutant VP4 (Danthi et al. 2003); specific mutations that alter or prevent channel formation in model membranes also delay or prevent functional delivery of viral RNA into the cytoplasm of cells, strongly suggesting that VP4 mediated channel formation is a critical process for PV infection. Interestingly, HAV has only a vestigial VP4, while in parechoviruses VP0 remains uncleaved so that VP4 presumably cannot be released as an independent moiety. How do these closely related viruses overcome the apparently critical requirement for VP4 seen in PV?

6.1.5 How Much VP4 Is Released During Picornavirus Entry?—Several reports state that entero- and rhinovirus A particles lack VP4 (De Sena and Mandel 1977; Goodfellow et al. 2005; Gromeier and Wetz 1990; Korant et al. 1972; Lonberg-Holm and Korant 1972). However, this may be related to issues of sensitivity of detection since Curry et al. (1996) found that a small proportion of VP4 remains associated with PV A particles and variable levels of residual VP4 have been reported in (or associated with) A particles or empty particles of HRV3, 14, and 16 (Greve et al. 1991; Hoover-Litty and Greve 1993). Cryo-EM reconstructions of PV A and empty particles (Belnap et al. 2000a) or HRV2 empty particles (Hewat et al. 2002) do not show density, thought to be due to residual VP4. However, density attributable to VP4 has been reported in reconstructions of HRV14 and 16 (Hewat and Blaas 2004). Because of the averaging involved in such reconstructions, nonsymmetrical information about the location of residual VP4 in these structures cannot be obtained. Consequently, it is not possible to differentiate whether the VP4 molecules are shed in a coordinated way from one or a small number of the fivefold axes of symmetry or randomly from any part of the particle.

6.2 Models for Entero/Rhinovirus Membrane Penetration

Comparative structural analyses of cell entry intermediate particles and biochemical evidence for virus membrane association and permeabilization have led to the development of working models for membrane penetration. Such a model derived from studies with PV has been described in detail (Bubeck et al. 2005b) and is depicted in Fig. 3. In brief, receptor binding triggers capsid rearrangements that result in the externalization of VP4 and the N-terminus of VP1, dissociation of the A particle from the receptor, and its direct interaction with the membrane via the externalized N-terminus of VP1. At the same time, released VP4 also interacts with the membrane. VP1 and/or VP4 form a membrane pore through which the genomic RNA is transported into the cytoplasm. The existing data clearly indicates that VP1 is involved in tethering the particle to the membrane, but whether it also forms the pore (with VP4 in a critical support role) or if VP1 is merely the anchor while VP4 forms the pore remains to be seen.

6.3 Membrane Penetration by Other Picornaviruses

The physicochemical properties of many picornaviruses such as those of the aphthovirus and cardiovirus genera appear to be incompatible with the model described above for enterovirus membrane penetration. As we have seen in Sect. 5, for these viruses, acidic conditions encountered during endocytosis are critical for infection and trigger the dissociation of capsids into pentameric subunits. It therefore has been thought that this is a simple and straightforward mechanism by which the virus releases its genome. However, this would release the genomic RNA into the lumen of the endocytosed vesicle, a potentially hostile environment, without an obvious means of reaching the cytoplasm. A strategy for minimizing exposure of the genomic RNA to the contents of the endosome, that is, a mechanism for coordinating genome release with membrane penetration, seems more likely.

Recent work with the aphthovirus ERAV has revealed that acid-induced capsid dissociation proceeds via a transient, intact empty particle (Tuthill et al. 2009). The release of RNA from an intact aphthovirus particle suggests that even viruses that dissociate into subunits may use a mechanism that coordinates genome release and delivery, as suggested by the models for entero- and rhinoviruses. In support of this hypothesis, further recent experiments with ERAV have shown that co-endocytosed ribonuclease has no effect on infectivity, suggesting that the productive virus entry mechanism does indeed protect the genomic RNA from exposure to vesicle contents (Gropelli et al. unpublished).

6.4 Questions Remaining on Membrane Penetration

Is it possible that a general mechanism exists for membrane penetration by all picornaviruses? While we would argue that genome release from an intact particle is now a possibility for all picornaviruses, it remains far from clear exactly how the genome reaches the cytoplasm, and it remains uncertain that all viruses will conform to a single mechanism. For example, what tethers aphthovirus particles to the membrane in the absence of the N terminal extension of VP1? What is the significance of the different proportions of VP4 released from A particles? What about those viruses that contain only vestigial VP4 or that do not even contain VP4 (VP0 remains cleaved)? Is there a mechanism that co-ordinates genome release and membrane penetration, for example, is close/direct contact with the membrane required for triggering genome release in a “polarized” fashion? Alternatively, it is possible, given the high particle/pfu ratios typical of picornaviruses, that most genomes are sacrificed by ejection within the endosomal lumen and are not transported into the cytoplasm.

7 Overall Conclusions

Despite intense investigation over many years, the mechanisms by which picornaviruses initiate infection of cells is still poorly understood. However, recent advances are offering tantalizing glimpses of how these processes may operate. The complexities of receptor usage are becoming clearer and the restrictions imposed by studying the interactions of viruses in simple cell culture systems as opposed to organized tissues are becoming apparent. Viruses can act as ligands capable of inducing cell signaling events and the roles of these interactions for cell entry are only just beginning to be investigated. Picornaviruses undergo profound structural alterations required for membrane penetration and genome release during the entry process. Although important advances have been made in unraveling these events, there is still a lot to be learned before we understand the molecular mechanisms involved. Although picornaviruses present a bewildering array of possibilities for cell entry (Fig. 4), recent studies suggest that the principle features of the process may be more similar across the spectrum of picornaviruses than had hitherto been suspected.

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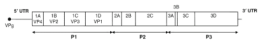

Fig. 1.

Picornavirus genome and polyprotein organization. The *boxed area* represents the single open reading frame (ORF) with untranslated regions (UTR) at each end. Translation and proteolytic processing produce primary products P1, P2, and P3. The P1 polyprotein is the capsid precursor and contains the structural proteins (VP1-4) found in the mature capsid. The P2 and P3 regions contain proteins involved in polyprotein processing, alteration of the host cell environment and replication of the viral RNA genome. The genome shown represents an enterovirus. Other genera contain subtle differences, for example, aphthoviruses contain an additional leader protein directly upstream of VP4, a small 2A protein and three copies of 3B which encode the genome-linked viral protein (VPg)

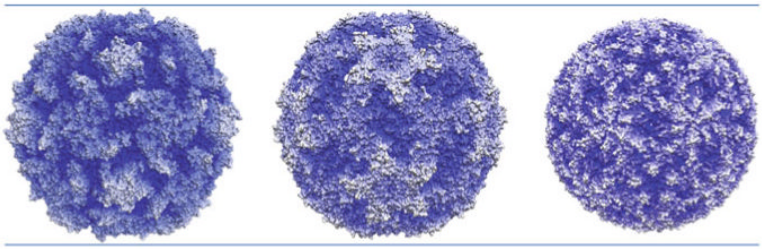

Fig. 2.

Picornavirus capsid structures. Radial depth cued images of picornavirus particles with a color gradient from innermost (*dark blue*) to outermost (*white*) surfaces. From *left* to *right*: poliovirus (enterovirus), 32 nm in diameter with five-pointed *star shape* at the fivefold axes, deep “canyon” surrounding the fivefold axes and three-bladed propeller at the threefold axes; Theiler’s murine encephalomyelitis virus (cardiovirus), 32 nm in diameter with extended star at the fivefold axes and surface depressions or “pits” spanning the twofold axes; foot-and-mouth disease virus (aphthovirus), 30 nm in diameter with relatively smooth surface. Images kindly produced by Hazel Levy

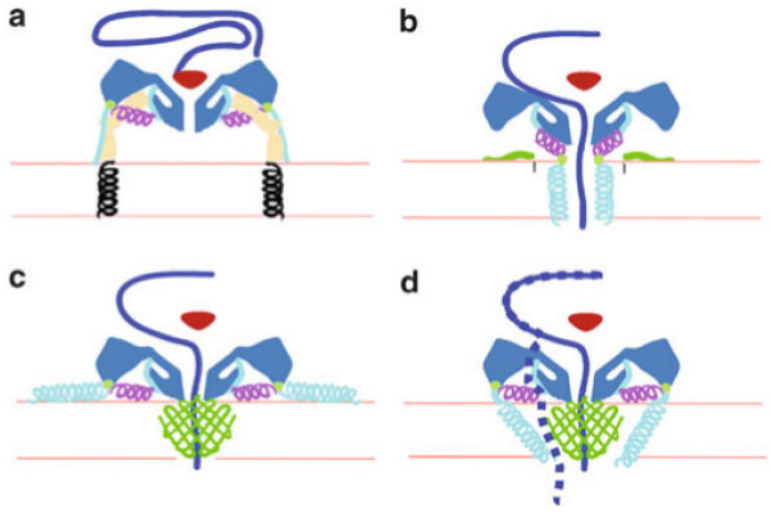

Fig. 3.

Working models for poliovirus entry. A cross section of a portion of the capsid is shown in *dark blue*, VP4 is *green*, and the N terminus of VP1 is *cyan and magenta*. (**a**) Native poliovirus binds its receptor, Pvr (ectodomains 1–3, tan; transmembrane domain, *black helix*), and at physiological temperature undergoes an irreversible change to the 135S particle. The path of egress of the N terminus of VP1 is shown. At this stage, the VP3 β tube (*red*) blocks an otherwise open channel along the fivefold axis. (**b–d**) Alternative models for the direct anchoring of the virus to the membrane via the N terminus of VP1 and formation of a transmembrane pore for RNA translocation. To accommodate the passage of RNA (*purple*), the VP3 β tube has shifted, and the channel has expanded, becoming continuous with a pore through the membrane. (**b**) Amphipathic helices at the N terminus of VP1 (*cyan*) may form a five-helix bundle close to the fivefold axis, which would require the magenta helix to dissociate from the body of the virus. Alternatively, VP4 may play a more central role in pore formation (**c** and **d**). In that case, VP1 may serve as a nonspecific membrane anchor (**c**) or participate directly in forming the pore (**d**). Recent studies have suggested an alternative path for release of the genome, from the base of the “canyon”, as indicated by the *dashed line* (**d**). Adapted from Bubeck et al. (2005a,b) with permission from the American Society for Microbiology

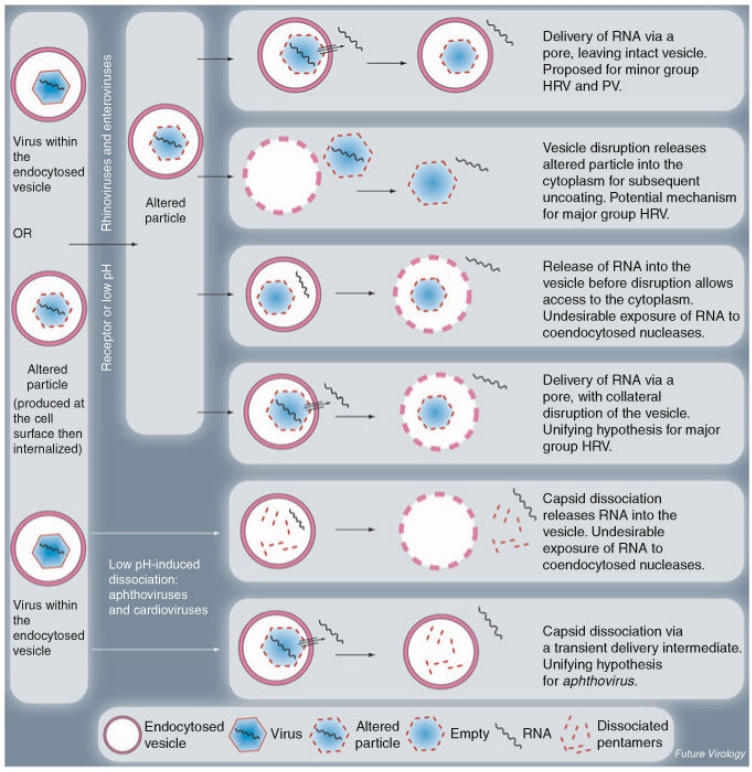

Fig. 4.

Possible scenarios for picornavirus structural changes and genome delivery to the cytosol during endocytosis. Adapted from Tuthill et al. (2007), with permission from Future Medicine

Table 1

Entry characteristics of selected picornaviruses

Genus	Virus	Structural features	Receptor(s)	Endocytosis pathway	Initial trigger	Final product of uncoating	Membrane penetration
Entero	PV	Canyon circling star shaped protrusion at fivefold Pocket/pocket factor	PVR (CD155) binds in canyon	Nonclathrin, noncaveolin	Receptor binding	Intact empty particle	Proposed to form a membrane pore VP4 implicated in channel formation/ genome delivery
	Minor group HRV	Canyon circling star shaped protrusion at fivefold Pocket/pocket factor	LDLr family binds in shallow pits circling fivefold axes	Clathrin mediated	Low pH	Intact empty particle	Bafilomycin blocks entry (prevents progression to late endosome) Size-selective pores formed in endosome membrane
Aphtho	FMDV	Smooth capsid No canyon No pocket	Integrin binds to flexible exposed loop	Clathrin mediated	Low pH	Acid-induced dissociation to pentameric subunits	Dissociation likely to proceed via the formation of a transient intact empty particle
Cardio	TMEV	Protrusion at fivefold Depression at twofold	Sialic acid Heparan sulfate Receptor usage linked to neurotropism and pathogenesis	?	Low pH	Acid-induced dissociation to pentameric subunits	?