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John E. Johnson
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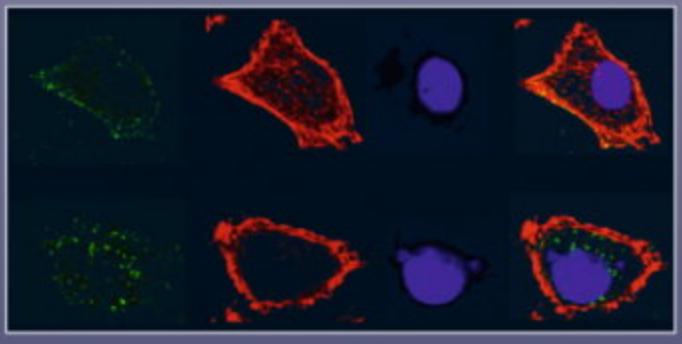
Cell Entry by Non-Enveloped Viruses



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Preface

This volume covers non-enveloped viruses and their cellular entry and describes the most thoroughly investigated members in this category. It is an appropriate time for such a comprehensive review, since structural, biochemical, and cell biological investigations have advanced to a point where strategies for entry, particle transitions, and membranes breached are reasonably well defined for most members. We anticipate that the information presented here will be of lasting relevance. We also hope that this volume will stimulate additional studies that contribute to an elucidation of the molecular details of membrane disruption and genome translocation for this type of virus. The volume is organized according to viral families, with chapters first addressing ssRNA and dsRNA viruses, then ssDNA viruses, and finally dsDNA viruses.

Although the number of gene products and the chemical nature of the genome vary widely among these viruses, there are unifying themes. All these viruses have icosahedral symmetry, and all have at least one gene product in their capsids with the viral jellyroll fold. The fold is a beta-sandwich with unique topology (Chapman and Liljas 2003) found predominantly in viral capsid proteins. This is a clear indication that these viruses have a common ancestor, but the evolutionary relationship among the viruses is impossible to establish, since viruses undergo exceptional levels of horizontal gene exchange. All the viruses have a single jellyroll in their gene products, except adenovirus that has two jellyrolls fused together in a single gene encoding the major capsid protein. The jellyroll topology relates the viruses discussed here to non-enveloped, icosahedral viruses that infect bacteria and plants. Indeed, the fused jellyrolls of adenovirus have appeared in dsDNA virus structures infecting bacteria and archea, although the latter viruses have internal envelopes (Bamford et al. 2005).

All animal viruses must breach a membrane to deliver their genome or, for dsRNA viruses, the particle that synthesizes the genome, into the cell cytoplasm. DNA viruses must, in addition, get the genome into the nucleus. Enveloped viruses fuse their envelope membrane with a cellular membrane, creating a portal to transport the contents within the viral envelope into the cytoplasm. The mechanism for this event has been extensively investigated and is reasonably well understood (Harrison 2008). The process is initiated by the exposure of a viral "fusion peptide"

when the virus is in the proper location for genome delivery. Changes in the virion structure, often initiated by change in pH, lead to the exposure of the normally sequestered hydrophobic peptide. The fusion peptide anchors the viral membrane into the cellular membrane, initiating the fusion process.

As discussed in many of the chapters in this volume, non-enveloped viruses expose "lytic peptides" that interact with membranes and facilitate genome delivery through a mechanism that is yet to be determined (Banerjee and Johnson 2008). Generation of this peptide appears to be the result of convergent evolution, since most non-enveloped viruses have a novel mechanism for its origin. A common feature found in noda- (Odegard et al, this volume) and picornaviruses (Tuthill et al, this volume) is the autocatalytic cleavage of the viral subunit to create a covalently independent, but particle-associated, polypeptide. The C-terminal 44-residue polypeptide of Flock House Virus is transiently exposed in a pH-dependent manner. Maximum exposure and lysis of liposomes *in vitro* occurs at pH 6.5. Likewise, vp4 of picornaviruses results from post-assembly, autocatalytic cleavage, remains associated with the particle, and is transiently exposed. The N-termini of the picornavirus polypeptides are myristoylated, and this moiety targets them to membranes. Broad-spectrum antiviral activity in picornaviruses is associated with small molecules that inhibit the required dynamic character of the particle, preventing genome translocation across a cellular membrane.

A 675-residue capsid gene product in the dsRNA orthoreovirus, μ1, has a myristoylated N-terminus and undergoes an auto-catalytic cleavage between residue 42 and 43. A variety of data discussed in Danthi et al. in this volume implicate this polypeptide in membrane translocation of the entire inner core of reovirus. The dsRNA rotavirus uncovers its membrane active region through the trypsin cleavage of the VP4 spike proteins (Baker and Prasad, this volume). VP8 and VP5* are the resultant cleavage products, and when VP8 dissociates from VP5*, trimeric hydrophobic loops are exposed that interact directly with the target membrane. Vp5* then undergoes a dramatic conformational change that draws the target membrane into the proximity of the inner core.

The ssDNA parvoviruses (Parrish, this volume) do not have a lytic peptide to alter host cell membranes. This virus group has a phospholipase A2 activity associated with it, but like many other non-enveloped viruses, this activity is only activated after the particles have entered the endosome and the N-terminal residues of the Vp1 capsid protein are exposed through 5-fold axes. Regions of this portion of the capsid are also thought to have nuclear localization signals. Polyomavirus has a dsDNA genome, and the particle travels in the endosome all the way to the endoplasmic reticulum where enzymes resident there induce the exposure of the hydrophobic Vp2 protein that mediates particle translocation from the ER (Tsai and Qian, this volume). Finally, the dsDNA adenovirus devotes an entire gene product to the task of membrane translocation (Smith et al, this volume). Vp6 is composed of residues 34 239 in the virion following its processing during assembly by a virally encoded protease. It is released into the endosomic membrane during particle disassembly within the endosome, facilitating the release of a nucleoprotein complex that is then targeted to the nucleus.

The discussion above highlights two of the numerous features described in detail in these chapters and indicates the intriguing assortment of divergence and convergence associated with the evolution of non-enveloped virus particles. The reader of these chapters will be rewarded with a state-of-the-art description of the structure and function of this very important class of viruses and may well be stimulated to add to this developing story.

Summer 2010

John E. Johnson
Peter K. Vogt

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Flock House Virus: A Model System for Understanding Non-Enveloped Virus Entry and Membrane Penetration

Amy Odegard, Manidipa Banerjee, and John E. Johnson

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Abstract The means by which non-enveloped viruses penetrate cellular membranes during cell entry remain poorly defined. Recent findings indicate that several members of this group share a common mechanism of membrane penetration in which the virus particle undergoes programmed conformational changes, leading to capsid disassembly and release of small membrane-interacting peptides. Flock House Virus (FHV), a member of the *nodaviridae* family, offers some unique advantages for studying non-enveloped virus entry. The simplicity of the FHV capsid, coupled with a robust reverse genetics system for virus expression and an abundance of structural and biochemical data, make FHV an ideal model system for such studies. Here, we review the FHV atomic structure and examine how these molecular details provide insight into the mechanism of FHV entry. In addition, recent studies of FHV entry are discussed and a current model of FHV entry and membrane penetration is presented. A complete understanding of host cell entry by this minimal system will help elucidate the mechanisms of non-enveloped virus membrane penetration in general.

1 Introduction

Enveloped viruses utilize membrane fusion to bypass cellular membranes, while non-enveloped viruses employ alternative strategies to breach membrane barriers. Recent studies have advanced our understanding of this process and revealed some common themes (reviewed in Tsai 2007; Banerjee and Johnson 2008; Chandran and Nibert 2003). Non-enveloped viruses have specialized capsid associated polypeptides, which mediate membrane interactions. In many cases, these capsid proteins are assembled as inactive precursors and primed for membrane interactions by proteolytic cleavage, which is often autocatalytic. Once trafficked to the appropriate site of membrane penetration, non-enveloped virus capsids are triggered by cellular factors to adopt a membrane-active conformation in which the hydrophobic regions become exposed and/or membrane-lytic peptides are released from the virus particle; these newly exposed lipophilic moieties insert into and disrupt target membranes. The FHV mechanism of membrane disruption, in which the coat

protein is primed by proteolytic cleavage followed by programmed exposure/release of a small membrane-lytic peptide, fits an emerging common mode of non-enveloped virus membrane penetration. Considering its protein composition and genome organization, FHV is among the simplest of all non-enveloped viruses and serves as an excellent model system for understanding the molecular details of membrane penetration.

FHV is a prototype of the family *Nodaviridae*, whose members naturally infect insects and fish. FHV was originally isolated from the grass grub *Costelytra zealandica* (Coleoptera: Scarabaeidae) in New Zealand (Dearing et al. 1980; Scotti et al. 1983). FHV is a non-enveloped, icosahedral virus that contains two single-stranded, positive-sense RNA genome segments. FHV robustly infects cultured *Drosophila* cells, converting ~20% of the cell mass to virus by 48 h post-infection. A typical virus preparation produces ~20 mgs of virion particles. The ratio of particle to plaque-forming units is ~300:1.

The FHV virion initially assembles as a non-infectious provirion particle, made up of the two FHV RNA genome segments, RNA1 (3.1 kb) and RNA2 (1.4 kb), surrounded by 180 copies of a single precursor capsid protein α (44 kDa). Following capsid assembly, a maturation step ensues in which α undergoes autoproteolytic cleavage to generate the particle-associated cleavage products β (39 kDa) and γ (4.4 kDa) (Gallagher and Rueckert 1988). This autocatalytic cleavage event produces the mature virion particle and is required for infectivity (Schneemann et al. 1992). Several lines of evidence indicate that the small hydrophobic γ peptide facilitates membrane penetration during host cell entry (Bong et al. 1999, 2000; Janshoff et al. 1999; Maia et al. 2006; Banerjee et al. 2009; Odegard et al. 2009).

Here we detail the accumulated information pertaining to FHV cell entry and contemplate some critical, yet unanswered questions. We first review the FHV X-ray crystal structure and examine features predicted to be important during entry and membrane penetration. Next, we discuss a series of recent studies that have advanced our understanding of the FHV entry pathway, the mechanism of membrane penetration, and the function of the membrane lytic γ peptide. Based on this work, we present a model of FHV entry that is widely applicable to a diverse group of non-enveloped viruses.

2 Capsid Architecture and Autocatalytic Cleavage of Subunits

2.1 FHV Expression Systems

An enabling feature of FHV for entry studies is the ability to readily produce particles with a variety of mutations that package the corresponding mutant genome. Authentic and mutant FHV particles containing the cognate genome are prepared by transfecting positive sense RNA, derived from cDNA clones of the FHV genome,

into cultured *Drosophila melanogaster* cells. If the capsid protein mutations are assembly competent, mutant virion particles are produced, even if the mutant particles are non-infectious and cannot undergo a second round of infection. Thus, sufficient quantities of non-infectious particles can be generated for biological studies. An alternative expression system was established that generates virus-like particles (VLPs) (Schneemann et al. 1993). When expressed from a recombinant baculovirus, the coat protein α spontaneously assembles into VLP particles. VLPs are identical in protein composition to authentic FHV and undergo maturation cleavage normally (Schneemann et al. 1993). However, VLPs do not package viral RNA and contain random cellular RNAs, and are therefore non-infectious. A significant advantage of the insect cell based expression system is the production of milligram quantities of non-infectious particles for structural and biochemical studies. The transfection strategy generally produces only a fraction of a milligram of non-infectious particles.

2.2 Capsid Structure

The 30-nm authentic FHV virion consists of two single-stranded RNA genome segments enclosed by an icosahedral protein capsid (Fisher and Johnson 1993). The capsid shell has a triangulation number of 3 ($T = 3$), and each of the 60 icosahedral asymmetric units (iASU) consists of three subunits, denoted A, B, and C. The subunits are chemically identical polypeptides (protein α) placed in different structural environments in the capsid, thus giving rise to quasi-symmetry. Five A subunits form pentamers at the icosahedral fivefold axis of the capsid, whereas alternating B and C subunits at the icosahedral threefold axis of symmetry form quasi-hexamers (Fig. 1a). Subunits A, B, and C in each iASU are organized around the quasi-threefold axis of symmetry (Fig. 1b).

Each of the chemically identical subunits contains 407 amino acids that form an eight-stranded antiparallel β-barrel, or jellyroll motif (Fig. 1b) (Fisher and Johnson 1993), a fold common to several RNA viruses (Rossmann and Johnson 1989). Loops connecting the β strands of the subunits are exposed to the capsid surface, whereas the interior surface contains helices contributed by the N- and C-terminal portions of the subunits. The X-ray crystal structure of FHV refined to 2.7 Å (PDB # 2z2q) shows ordered density for amino acids 57 379 in subunit A, 57 377 in subunit B and residues 20 31 and 55 381 of subunit C (Fisher and Johnson 1993). A crystal structure of VLPs with a wildtype capsid protein sequence was refined at 3.5 Å (PDB # 2q26) and shows a capsid surface very similar to that of authentic FHV.

The bipartite positive sense RNA packaged inside the icosahedral capsid consists of RNA1 (3.1 kb), which codes for an RNA dependent RNA polymerase, and RNA2 (1.4 kb), which codes for the capsid protein (Scotti et al. 1983). A 387-nucleotide subgenomic RNA3, which corresponds to the 3′ end of RNA1, codes for a small protein B2 (Friesen and Rueckert 1981; Guarino et al. 1984). B2 has been shown to be an inhibitor of RNA silencing in virus-infected cells (Li et al. 2002; Chao et al. 2005).

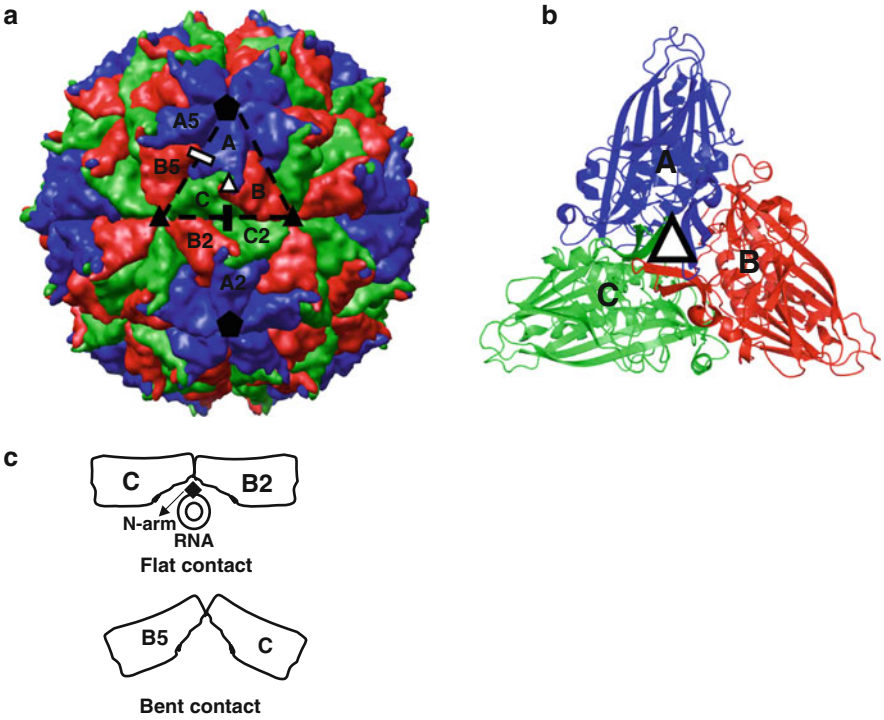

Fig. 1 FHV capsid architecture. (**a**) Surface representation of the FHV capsid based on the X ray structure at 2.7 Å. The FHV capsid is composed of 180 copies of the coat protein, organized as 60 icosahedral asymmetric units (iASUs). A single iASU is highlighted with a *dashed line*, and the three subunits in the iASU are designated A (*blue*), B (*red*), and C (*green*). The icosahedral twofold (*rectangle*), threefold (*triangle*), and fivefold (*pentagon*) symmetry axes are indicated as symbols in *black*. The quasi twofold (*rectangle*) and quasi threefold (*triangle*) fold symmetry axes are indicated as symbols with *black borders*. (**b**) Close up view of a single iASU containing subunits A, B, and C. The view is from the outside of the virus centered at the quasi threefold axis of symmetry. (**c**) Schematic of the bent and flat contacts between subunits. The N arm of the C subunit and the ordered duplex RNA, which stabilize the flat contact, are indicated

2.3 Capsid–RNA Interactions

The crystal structure of authentic virions shows 10 bp of well-ordered double-stranded RNA along each icosahedral twofold axis of symmetry, interacting with conserved lysines from the C and C2 subunits (Fig. 1a). The duplex RNA is attributed to secondary structure formed by the single-stranded RNA packaged inside the capsid. Analysis by cryo-electron microscopy (cryoEM) revealed that the encapsidated viral RNAs form a highly organized dodecahedral cage structure (Tihova et al. 2004), similar to what is seen in closely related Pariacoto virus (Tang

et al. 2001). Furthermore, it was shown that formation of the dodecahedral cage was independent of the sequence and length of the encapsidated RNA, and probably dictated by the FHV capsid protein, since the non-genomic, insect cell RNA packaged inside the VLPs also assumes an arrangement similar to the organization of the RNA genome inside authentic particles (Tihova et al. 2004). The N- and C-termini of the capsid protein are thought to mediate RNA interactions, as these regions have been shown to be required for RNA recognition and packaging (Schneemann and Marshall 1998; Marshall and Schneemann 2001).

The duplex RNA and the N-terminal peptide arm (N-arm; residues 20 31) of the capsid protein are ordered in the C subunits, but not the A and B subunits (Fisher and Johnson 1993), giving rise to different contacts across the icosahedral twofold and quasi-twofold axes (Fig. 1a). In the C subunit, the duplex RNA and N-arm are ordered and aid in the formation of a "flat intersubunit contact" between subunits C and B2 near the icosahedral twofolds (Fig. 1c). This interface is flat because the duplex RNA and the N-terminal peptide form a wedge between subunits that prevents bending. Near the quasi-twofold axis of symmetry, the N-arm and RNA are disordered, resulting in the formation of a "bent contact" between subunits (Fig. 1c). The duplex RNA and N-arm do not bind at this interface, allowing the subunits to form a bent contact.

2.4 Autocatalytic Cleavage and Production of the Gamma Peptide

FHV is initially assembled as an immature provirion particle, composed of 180 copies of the coat protein α. Following capsid assembly, α undergoes autoproteolytic cleavage to generate the mature, infectious virion (Gallagher and Rueckert 1988; Schneemann et al. 1992) (Fig. 2a). The mature virion contains two particle-associated cleavage products a large N-terminal fragment, β (363 amino acids), and a small C-terminal fragment, γ (44 amino acids) (Fig. 2b). While the central region of β forms the capsid shell (Fisher and Johnson 1993), the γ peptides form amphipathic helices that remain non-covalently associated with the capsid interior (Fisher and Johnson 1993). The γ peptides are membrane active and disrupt host cellular membranes during entry (Bong et al. 1999, 2000; Janshoff et al. 1999; Maia et al. 2006; Banerjee et al. 2009; Odegard et al. 2009).

Analysis of the residues positioned near the autocatalytic cleavage site led to a proposed mechanism of autoproteolytic cleavage (Fig. 2b). It is predicted that acid hydrolysis orchestrated by aspartate-75 (D75) results in the cleavage of the scissile bond between asparagine-363 (N363) and alanine-364 (A364) (Fig. 2b). Due to its local environment (Fig. 2), Asp-75 is highly protonated even at neutral pH, enabling it to act as a proton donor at near neutral pH conditions (Zlotnick et al. 1994). It is predicted that Asp-75 forms a hydrogen bond with the carbonyl oxygen of the Asn-363-Ala-364 peptide bond, making it susceptible to nucleophilic attack by a water molecule. This model is supported by the finding that mutating either the catalytic aspartate (D75) or the asparagine at position 363 (N363) (Fig. 2b)

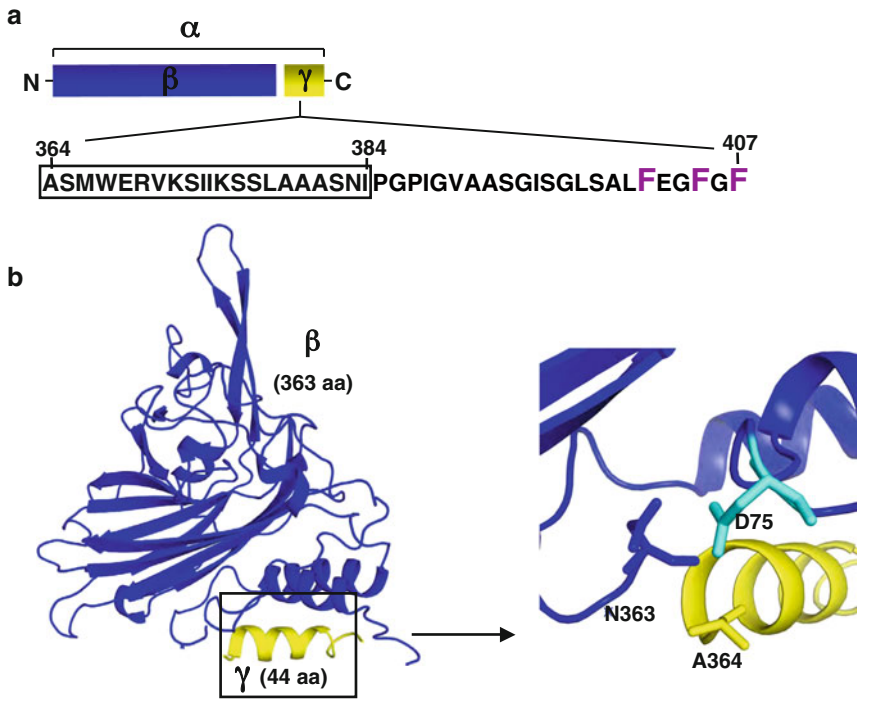

Fig. 2 FHV autocatalytic cleavage. (**a**) Diagram of the coat protein α and the autoproteolytic cleavage products, β and γ. The N terminal and C terminal positions are labeled. The sequence of the γ peptide is shown, and the N terminal amphipathic domain (*boxed*) and the C terminal RNA binding domain are indicated. The phenylalanine residues at the C terminus, which are essential during both virus entry and assembly, are highlighted in *magenta*. (**b**) Ribbon diagram of the capsid protein subunit (*blue*), with the γ peptide highlighted in *yellow*. A zoomed in view of the autocatalytic cleavage site, between asparagine 363 (N363) and alanine 364 (A364), and the catalytic aspartate residue (D75) (*in cyan*) is shown on the *right*. The amphipathic helix of the γ peptide is colored *yellow*

abrogates maturation cleavage and essentially eliminates infectivity (Schneemann et al. 1992; Zlotnick et al. 1994). The increased mobility of gamma after cleavage (Oliveira et al. 2000) is almost certainly essential for infection. D75N and N363T FHV, which could not be produced as authentic virions in quantities sufficient for crystallization, were generated in insect cells as VLPs (Fisher et al. 1993) and were structurally characterized (PDB # 2q23, 2q25). The immature VLPs were closely similar to authentic virions and the wildtype VLPs, but showed continuous density through the cleavage site and some changes in the conformation of the loop containing the catalytic D75 (Z. Chen and J.E. Johnson, unpublished observation). The cleavage generates a subtle change in which A364 separates by ~8 Å from N363. The position of N363 is virtually unchanged with cleavage.

2.5 *Structure and Organization of the γ Peptides*

The γ peptide has two separate domains an N-terminal amphipathic helix separated from a predominantly hydrophobic C-terminal region by a proline glycine proline turn (Fig. 2a). Residues 364 385 at the N-terminus, form a well ordered amphipathic helix in all three subunits, while the C-terminal region of γ is disordered and invisible in the crystal structure (Fisher and Johnson 1993). Several lines of evidence suggest that the amphipathic portion may directly interact with host cell membranes during virus entry (Bong et al. 1999, 2000; Janshoff et al. 1999; Maia et al. 2006). The C-terminal region of γ is essential for specific packaging of the FHV genomic RNA and phenylalanine residues at positions 402, 405, and 407 (Fig. 2a) were implicated in RNA packaging (Schneemann and Marshall 1998). The functionality of these regions will be discussed in detail later.

The amphipathic γ helices are located on the interior of the capsid in the X-ray structure of authentic FHV (Fig. 3a), with quasi-equivalence dictating marked differences in the structural organization of peptides from the A, B, and C subunits. A difference map between the electron density derived from a cryoEM reconstruction of FHV at 22 Å and the density of the FHV crystal structure computed at the same resolution revealed the dissimilarity in the interaction of γ with bulk RNA (Cheng et al. 1994). The γ peptides from the B and C subunits formed numerous contacts with the RNA about the icosahedral threefold axes; however, the peptides from the A subunits were positioned far from the bulk RNA and formed pentameric, bundle-like structures at the fivefold axes (Fig. 3b). It was hypothesized that the viral RNA could remain associated with the

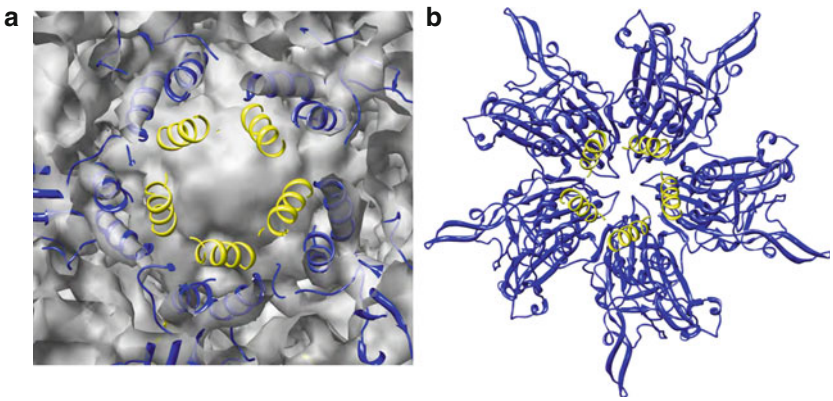

Fig. 3 Position of the γ peptides in the FHV capsid. (**a**) Cutaway view of an FHV capsid, with the X ray structure of the A subunits at the fivefold axis of symmetry (*in blue*) built into the EM density for the capsid (*in grey*). The γ peptides located in the capsid interior are shown in *yellow*. (**b**) Ribbon diagram of the FHV capsid centered at the fivefold axis of symmetry, viewed from the inside of the capsid looking out. The γ peptides (*yellow*) form pentameric helical bundles at the fivefold axis

γ peptides as they are released from the capsid interior, and that the RNA might travel along with the γ peptides to the site of membrane penetration or into the cytoplasm. Thus, in addition to its role as a membrane lytic factor, the γ peptides may function to deliver the RNA to the site of membrane penetration or into to the cytoplasm.

In spite of similar organization, the γ peptides in authentic virus particles and VLPs display slight differences in their biochemical behavior. The γ peptides in VLPs are slightly less restrained since they are more amenable to amino-acetylation (Bothner et al. 1999), and can more readily disrupt artificial membranes (M. Banerjee and J.E. Johnson, unpublished results) compared to their counterparts in authentic virus. The protein shell in VLPs is digested 7× faster than virion subunits when both are exposed to trypsin in solution (Bothner et al. 1999). It is probable that this difference is due to the stabilizing role played by authentic RNA suggesting that the genome has evolved for optimal biochemical interactions as well as for its genetic role. Regions of the capsid protein may make specific and stronger contacts with viral RNA than it can with random cellular RNA.

2.6 Metal Ion Binding Sites

The crystal structure of FHV showed calcium ions bound to specific regions in the virus capsid (Fisher and Johnson 1993). Five binding sites were initially detected per iASU by generating a difference electron density map between amplitudes from original FHV crystals and crystals soaked in 40 mM EGTA (Fisher and Johnson 1993). Upon refinement to 2.7 Å, weak density was established for a calcium ion at the quasi-threefold axis of relating subunits A, B, and C (Fig. 4a). Side chains of aspartate 249 and glutamate 251 and the corresponding quasi-threefold related amino acids in the iASU coordinate this calcium ion (Fig. 4b). Calcium ions were also detected at the three subunit interfaces, A B, B C, and A C, in the iASU (Fig. 4a). Each of the three quasi-equivalent Ca ions had virtually identical environments with the side chain of aspartate 161 from one subunit and the side chain of aspartate 221 and the main chain carbonyl of glycine 273 of a neighboring subunit coordinating the metal (Fig. 4c). FHV variants, such as FHV VLPs (PDB # 2q26), or the maturation defective mutants of FHV (PDB # 2q23 and 2q25) differed slightly from authentic FHV in terms of divalent cation binding. They contained strong density for one bound calcium ion and a sulfate ion at the quasi-threefold axis of symmetry, in addition to those at the subunit interfaces (Table 1).

It was suggested that calcium binding stabilizes the quaternary structure by strengthening the interaction between subunits in the iASU. Divalent cations have been detected bound to the capsids of several plant RNA viruses such as Cowpea Chlorotic Mottle Virus (CCMV). CCMV swells in the presence of a metal ion chelator such as EDTA, indicating a general "loosening" of the capsid in the absence of calcium, and possible disassembly and release of infectious genome

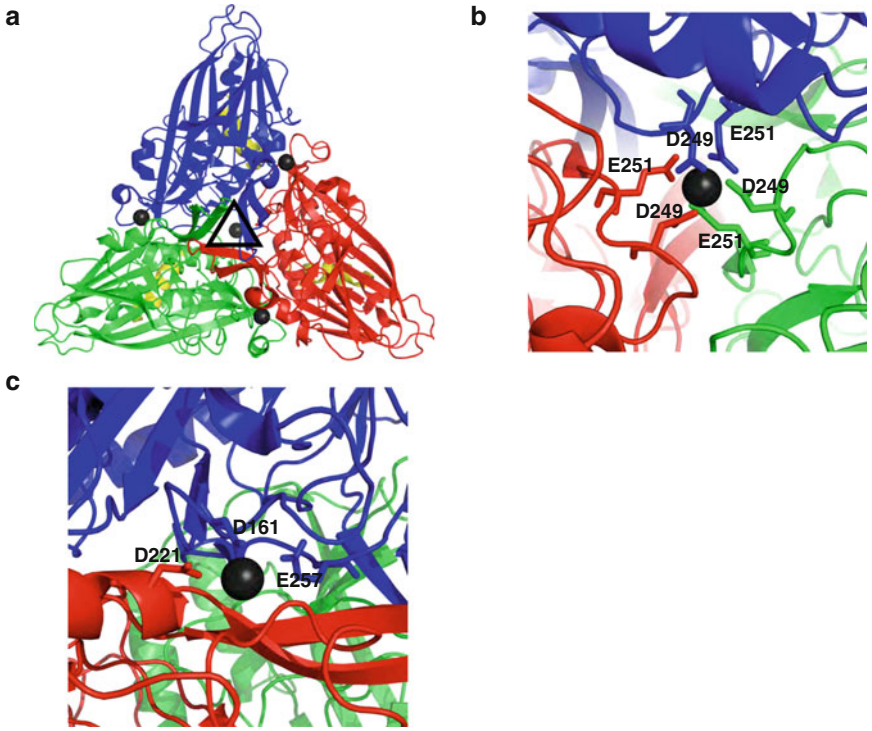

Fig. 4 Metal binding by FHV. (**a**) Calcium binding sites at the quasi threefold axis of symmetry and at the subunit interfaces in an icosahedral asymmetric unit (iASU) of FHV capsid. Bound calcium ions are shown as *black spheres*. The quasi threefold axis of symmetry is indicated as a *triangle with block borders*. (**b**) Calcium binding site at the quasi threefold axis of symmetry, coordinated by aspartate 249 and glutamine 251, and corresponding residues in the threefold related subunits. (**c**) Calcium binding site at the interface of subunits A and B, and the coordinating residues

Table 1 Metal binding sites in FHV

	PDB ID	Calcium at quasi threefold axis	Sulfate at quasi threefold axis	Calcium between subunits	Sulfate between subunits
Authentic FHV	2z2q	1 (weak)	0	3	3
FHV VLP	2q26	1	1	3	3
Maturation defective VLP (D75N/N363T)	2q25/2q23	1	1	3	3

(Speir et al. 1995; Speir et al. 2006). FHV particles prepared in the presence of metal chelators are purified as immature provirions (Schneemann et al. 1994), indicating a possible role for calcium in the assembly and maturation of the capsid (Table 2).

3 Early Events During FHV Cell Entry

Little information is available concerning the site of FHV entry in the natural host, as early events in infection have not been examined using insect models. To date, nearly all studies of FHV entry have been performed at the cellular level with cultured *Drosophila melanogaster* cells. Although recent studies have advanced our understanding of FHV cell entry, many details of the process remain unresolved. There is evidence that the virus initiates infection by binding to a host cell receptor(s) and enters into the cell by receptor-mediated endocytosis (Fig. 5). However, the FHV cell surface receptor(s) has yet to be identified and the route of entry remains poorly defined. Following attachment and uptake, FHV must penetrate the cell membrane and release its single-stranded RNA genome into the host cytoplasm, where viral replication takes place (Fig. 5). While recent studies have provided some insights into the mechanism of FHV membrane penetration, many questions remain. In particular, the role of host cell factors during FHV entry, the capsid structural changes that accompany membrane penetration, and the molecular details of virus membrane interactions remain to be fully elucidated.

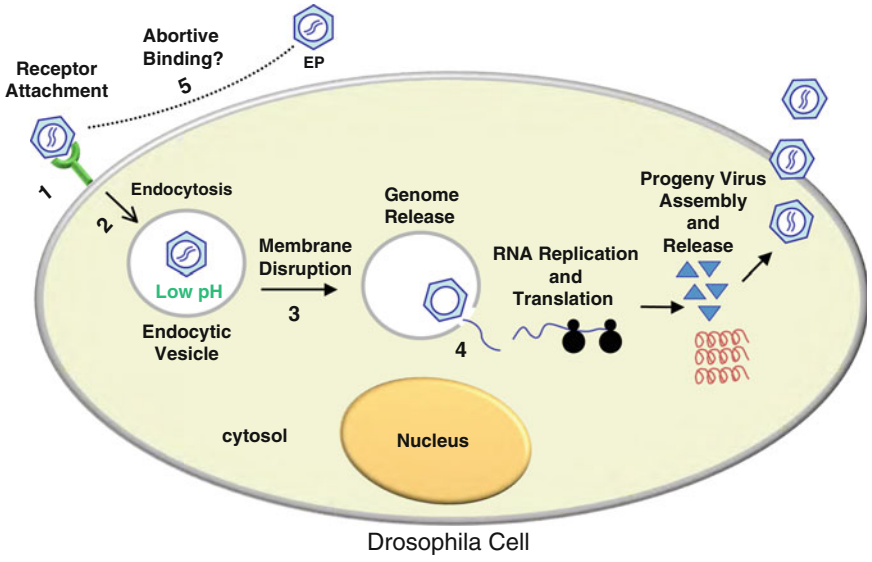

Fig. 5 A model of FHV cell entry: (1) attachment to host cell receptor, (2) uptake by receptor mediated endocytosis, (3) permeabilization of the endosomal membrane, and (4) release of the genomic RNA into the cytoplasm. The cell surface receptor is represented in *green* and viral RNA is shown in *blue*. Eluted particles (EP) are predicted to form upon dissociating from the receptor after initial binding (5)

3.1 FHV Cell Surface Receptor

Many steps of the FHV entry pathway, particularly the identity of the FHV cell surface receptor(s), have remained elusive. The existence of a specific cellular receptor is supported by the observation that FHV binding to *Drosophila* cells is saturable (Walukiewicz et al. 2006). Also, cell lines that are not normally susceptible to FHV infection can support replication when FHV mRNA is transfected into the cytoplasm of these cells (Ball et al. 1992), suggesting that these cells lack a functional FHV receptor.

3.2 Route of FHV Entry

A recent study demonstrated that FHV enters cells via an acidic, endocytic pathway as NH_4Cl and bafilomycin A1, inhibitors that raise endocytic pH, blocked FHV infection (Odegard et al. 2009). Furthermore, FHV particles that were preincubated at pH 6.0 prior to infection remained infectious in the presence of NH_4Cl and bafilomycin A1, no longer requiring acidic endosomal pH during infection. This indicates that low endocytic pH is not required during FHV entry if virus particles have already been exposed to low pH *in vitro*, and provides further support that NH_4Cl and bafilomycin A1 specifically block infection by inhibiting endosomal acidification. These findings provide the first evidence that FHV enters cells via an acidic endocytic pathway and that low pH is required for FHV infection (Fig. 6).

4 FHV Membrane Penetration

4.1 Structure-Based Model of FHV Membrane Penetration

Following receptor binding and uptake, the FHV single-stranded RNA genome must cross the cellular membrane to enter into the host cytoplasm. A model of FHV membrane penetration was proposed based on the FHV X-ray crystal structure and biochemical studies. In the X-ray crystal structure, the γ peptides are located inside the capsid shell with residues 364 385 forming amphipathic helices (Fisher and Johnson 1993) (Figs. 2a and 3a, b). Subsequent studies showed that the FHV capsid is dynamic with γ transiently exposed to the exterior of the capsid (Bothner et al. 1998). These findings led to a structure-based model of FHV membrane disruption in which the dynamic γ peptides are reversibly exposed to the surface of the capsid (Bothner et al. 1998), "sampling" the environment until they encounter the appropriate cellular trigger. The virus is then activated to undergo an irreversible conformational change in which the

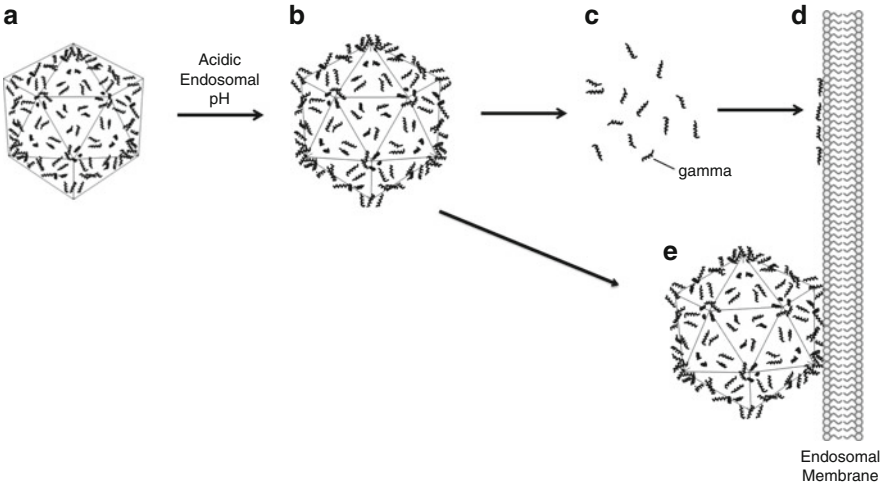

Fig. 6 Current model of FHV membrane penetration. Prior to infection, the γ peptides (*shown as helices*) are non covalently associated with the interior of the FHV capsid (**a**). During uptake into the host cell, FHV is exposed to acidic pH within the endocytic compartment. Acidic endosomal pH triggers the virus to undergo partial disassembly and the γ peptides are externalized (**b**) and/or released from the virus particle (**c**). The liberated γ peptides (**d**) or particle associated γ peptides (**e**) insert into and create a local disruption of the endosomal membrane to facilitate translocation of the RNA or nucleocapsid into the cytoplasm

γ helical bundles located at each fivefold axis are externalized and the amphipathic helices insert into the target membrane (Fisher and Johnson 1993; Cheng et al. 1994) (Fig. 6), forming a conduit for the viral RNA to enter the cytoplasm. While this is an attractive structure-based model, more evidence is required to support it.

4.2 In Vitro *Studies of FHV Membrane Penetration*

The hypothesis that the γ peptides mediate FHV membrane penetration is supported by the finding that a synthetic version of the γ peptide, termed γ_1, spontaneously partitions into lipid bilayers and increases the membrane permeability of liposomes (Bong et al. 1999). The γ_1 peptide consists of the 21 N-terminal residues of the 44-residue γ peptide, corresponding to the amphipathic alpha helical domain (Bong et al. 1999). Biophysical experiments indicate that the γ_1 peptide is organized as a random coil in solution and adopts a kinked helical conformation upon inserting into membrane bilayers (Bong et al. 2000; Janshoff et al. 1999; Maia et al. 2006). Studies of the γ_1 peptide bound to model membranes demonstrate that it is positioned parallel to the lipid bilayer, with the hydrophobic face of the helix packed against the membrane surface (Janshoff

et al. 1999; Bong et al. 2000). These findings suggest that the γ_1 peptide inserts only into the outer leaflet of the lipid bilayer, favoring a model in which the peptide causes localized disruption of the membrane rather than forming a membrane-spanning pore. However, it has been shown that in the context of the virus particle, the full-length γ peptide is positioned differently than the γ_1 peptide (Banerjee et al. 2009).

4.3 *FHV Membrane Lytic Activity Is Triggered by Low Endocytic pH*

Many enveloped and non-enveloped viruses that enter cells by acid-dependent endocytosis utilize low pH as a trigger for capsid disassembly or protein conformational changes related to membrane fusion or penetration. It was recently shown, using artificial liposomes as a model membrane system, that acidic pH promotes FHV membrane penetration *in vitro* (Odegard et al. 2009). FHV was found to mediate the highest level of liposome disruption at pH 6.0, corresponding to the pH found within early endosomes (pH $\sim$ 5.9 6.0) or late endosomes (pH $\sim$ 5.6 6.0) (Mellman et al. 1986; Yamashiro and Maxfield 1984). Furthermore, FHV particles incubated at pH 6.0 bound ANS with greater affinity than at pH 7 indicating increased hydrophobicity, which correlated with the optimal pH of FHV membrane lytic activity. These findings support a model in which FHV enters cells by endocytosis and is activated by acidic pH to adopt a membrane active conformation (Figs. 5 and 6).

4.4 *FHV Autoproteolytic Cleavage Is Required for Membrane Disruption*

Mutant FHV particles that do not undergo autocatalytic cleavage are not infectious (Schneemann et al. 1992). It was hypothesized that cleavage-defective particles are non-infectious because these particles do not contain a dissociable form of the lipophilic γ peptide, and as a result, cannot facilitate membrane penetration during entry. In support of this model, a recent study demonstrated that cleavage-defective mutants have a decreased capacity to disrupt model membranes *in vitro* (Odegard et al. 2009). Furthermore, perturbation of cleavage-defective particles by low pH or heating did not enhance membrane lytic activity, suggesting partial capsid disassembly is not sufficient to restore membrane activity and the γ peptides must dissociate from the particle.

While cleavage-defective particles fail to disrupt membranes, these particles were shown to undergo a pH-dependent increase in hydrophobicity, which peaked at pH 6.0, the same pattern observed for wild-type FHV particles (Odegard et al. 2009).

This result suggests that cleavage-defective particles may undergo some of the structural transitions associated with low pH triggered membrane penetration. As part of the structural changes that accompany FHV membrane interactions, it is likely that the γ peptides are externalized from their previously buried position inside the capsid. The finding that the γ peptide region of cleavage-defective particles is transiently exposed to the capsid surface, similar to what is observed in wild-type virus particles (Bothner et al. 1999), further supports the idea that these particles can undergo some of the conformational changes associated with membrane interactions.

4.5 Working Model of FHV Entry and Membrane Penetration

The findings described herein support a model of FHV entry and membrane penetration that is widely applicable to a diverse group of non-enveloped viruses. The FHV capsid is primed for entry-related disassembly and membrane interactions by post-assembly autoproteolytic cleavage, which generates the small membrane-interacting γ peptide. Prior to infection, the γ peptides are largely sequestered inside the capsid shell, with occasional "breathing" or exposure to the capsid surface. We propose that FHV initiates infection by attaching to a host cell surface receptor(s) and proceeds to enter into the cell via receptor-mediated endocytosis. This receptor-binding event may evoke a conformational change that initiates uncoating or renders the virus capsid susceptible to disassembly once exposed to low pH within the endocytic pathway. We predict exposure to low endocytic pH during cell entry induces the capsid to adopt a membrane-active conformation in which the γ peptides are externalized or released from the virus particle, and ultimately, these liberated or particle-associated γ peptides facilitate disruption of the endosomal membrane (Figs. 5 and 6). This model fits a common paradigm of non-enveloped virus entry in which virus penetration proteins undergo primed and triggered conformational changes, leading to capsid disassembly and release of small membrane-interacting peptides (Chandran and Nibert 2003; Tsai 2007; Banerjee and Johnson 2008).

4.6 Do Particle-Associated or Released γ Peptides Mediate Membrane Interactions?

It is not clear whether particle-associated γ peptides interact with membranes or if γ peptides must be released from particles to facilitate membrane disruption. Cleavage-defective particles, which do not contain a dissociable form of the γ peptide, are virtually non-infectious (Schneemann et al. 1992) and have compromised membrane lytic activity (Odegard et al. 2009), suggesting release of the γ peptides may be required for FHV membrane disruption. Furthermore, in contrast to wild-type FHV, cleavage-defective particles do not display enhanced membrane lytic activity when

the capsid is perturbed by exposure to low pH or heating (Odegard et al. 2009). This result suggests partial capsid disassembly of cleavage-defective particles is not sufficient to establish robust membrane lytic activity and the γ peptides must dissociate from the particle.

However, there is also evidence to support the alternative model in which particle-associate γ peptides facilitate membrane disruption. It has been established that the γ peptide region of both wild-type and cleavage-defective particles is transiently exposed to the capsid surface (Bothner et al. 1999). The finding that wild-type and cleavage-defective particles are competent to mediate low levels of membrane disruption at neutral pH (Odegard et al. 2009) suggests that the γ peptides may interact with membranes when surface exposed.

5 Entry-Intermediate Particle Types

During entry the FHV capsid undergoes systematic disassembly and conformational changes and, in the process, distinct cell entry intermediates are generated. Based upon the model for FHV entry, several different intermediates are possible (Fig. 5). Detailed characterization of these intermediates and their respective structural rearrangements will provide insights into the mechanism of FHV entry and membrane penetration, which can then be applied to related non-enveloped viruses.

5.1 The Eluted Particle: A Putative Entry Intermediate

During a normal FHV infection, a fraction of virus particles are eluted from cells. These particles, termed eluted particles, are thought to represent a distinct entry intermediate that has undergone some or all of the structural rearrangements that accompany virus entry into the host cell (Figs. 5 and 7). Following attachment to *Drosophila* cells, approximately 30% of input FHV particles are routinely recovered as eluted particles in the non-cell associated supernatant (Walukiewicz et al. 2006).

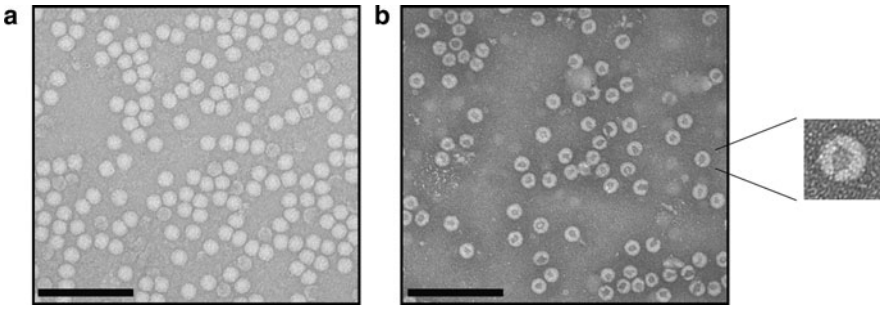

Fig. 7 Negative stain electron micrographs of (**a**) native FHV and (**b**) eluted particles. *Bar* 200 nm

Further analysis revealed that eluted particles had a dramatically different phenotype than native virions (Fig. 7). Eluted particles were unable to rebind to fresh *Drosophila* cells and, as a result, were no longer infectious. Also, eluted particles were found to aggregate when exposed to a pH below 6.2, while native virions were stable under the same conditions. In addition, eluted particles were shown to have lost approximately 25% of the γ protein from the capsid (Walukiewicz et al. 2006). Perhaps the most striking difference was observed when eluted particles were visualized by negative stain EM; unlike native virions, eluted particles were permeable to stain (Fig. 7). Furthermore, the staining pattern of eluted particles was not uniform suggesting that these particles have adopted an asymmetric conformation. These observations indicate that eluted particles are biochemically and structurally distinct entities and probably are an authentic FHV cell entry intermediate.

5.2 Additional Entry Intermediate Particle Types?

Since the FHV receptor has yet to be identified, entry intermediates formed upon receptor binding have not been studied directly. However, a FHV receptor binding intermediate may resemble eluted particles, which are thought to form upon dissociating from the receptor after initial binding (Walukiewicz et al. 2006). Another entry intermediate may form when FHV particles are exposed to low endocytic pH during cell entry. It was recently shown that exposure to low pH within endosomes triggers the virus to adopt a membrane-active conformation (Odegard et al. 2009). While the nature of this conformational change remains to be fully characterized, FHV particles were shown to expose hydrophobic regions when incubated at endocytic pH *in vitro*. When low-pH treated FHV particles were examined by negative stain electron microscopy, no gross conformational changes were apparent and these particles remained intact. Further biochemical and structural analysis will be necessary to fully characterize this putative entry intermediate. It remains possible that a combination of cellular factors contributes to FHV uncoating, and accordingly, a wide variety of intermediate particles may exist. Further elucidation of the pathway of FHV entry and the cellular components involved will allow us to continue to examine the nature of FHV entry intermediates.

6 Functional Domains of γ Involved in Entry

The N-terminal amphipathic helix of γ, which is structurally similar to the fusion peptides of enveloped viruses (Maia et al. 2006), is thought to mediate membrane interactions during cell entry (Bong et al. 1999; Maia et al. 2006). The C-terminal region, however, was primarily implicated in RNA packaging (Schneemann and Marshall 1998). A recent study has also shown an essential role for the γ C-terminus during virus entry (Banerjee et al. 2009). The multifunctional nature of this

44-residue peptide (Fig. 2a) is probably a reflection of the small capsid size of FHV, causing γ to carry out vital roles during both virus entry and assembly.

6.1 γ Trans-Complementation

The γ peptide can be supplied in trans from VLPs to rescue the infectivity of non-infectious, cleavage-defective versions of FHV (Walukiewicz et al. 2008). FHV particles containing point mutations D75N or N363T do not undergo autocatalytic cleavage (Schneemann et al. 1992), and therefore do not contain functional γ peptides. These immature, mutant particles are non-infectious (Schneemann et al. 1992), presumably because γ is not available to mediate membrane penetration during cell entry. When cultured *Drosophila* cells are co-infected with immature FHV and VLPs containing γ, the infectivity of the mutant FHV particles is restored to wild-type levels (Walukiewicz et al. 2008). Although the molecular mechanism of this process is not clearly understood, the necessity for mature VLPs, containing cleaved γ, for trans-complementation signifies that γ peptides from VLPs facilitate the release of cleavage-defective genome in the cytosol (Fig. 5). In addition, this assay demonstrates that if membrane penetration activity is supplied in trans, cleavage-defective FHV particles are competent for downstream replication events. Similar studies of related non-enveloped viruses have also demonstrated the infectivity of entry-defective particles can be rescued by supplying membrane penetration activity in trans. For example, infectivity of a reovirus entry mutant was enhanced by co-infecting with genome-deficient particles (Odegard et al. 2004), and the infectivity of a mutant, entry-defective parvovirus was rescued by co-infection with adenovirus (Farr et al. 2005).

In the FHV system, γ trans-complementation was utilized as a quantitative assay to judge the effectiveness of truncated or mutated γ peptides compared to the wildtype version, in promoting virus entry (Banerjee et al. 2009). This assay, which measures the amount of ^{35}S-labeled progeny virus produced upon co-infection of *Drosophila* cells with otherwise non-infectious VLPs and immature FHV, quantifies the effect of γ specifically during entry *in vivo*, separate from its other functions in the virus life cycle.

6.2 Entry Related Function of γ C-Terminus

The γ trans-complementation assay was used concurrently with an *in vitro* liposome disruption assay to confirm an essential role for the C-terminus of γ during virus entry. Dye-loaded liposomes are a well-established model system to study virus membrane penetration and have been used to study the entry mechanisms of several non-enveloped viruses, including adenovirus, rotavirus, and several

members of the picornavirus family. γ, as a synthetic peptide (Maia et al. 2006), or supplied from authentic virus (Odegard et al. 2009) or VLPs, punctures liposomes and releases fluorescent dye. VLPs lacking all or part of the γ C-terminus, but containing the N-terminal amphipathic helix in its entirety (Fig. 2a), can neither rescue the infectivity of immature FHV in a trans-complementation assay, nor release dye from liposomes *in vitro*. Single, separate mutation of the C-terminal phenylalanine residues (Fig. 2a) to alanines also affects both *in vivo* entry and *in vitro* membrane disruption by particles. The linear correlation in the results of these assays indicates that the C-terminus of γ is required for endosomal membrane disruption during FHV entry. Interestingly, γ isolated from particles, but containing the same mutations, is as effective in liposome disruption as the wildtype peptide. This suggests that the mutations in the C-terminus of γ might affect the correct organization of the peptide in the capsid and that this organization is essential for entry (Banerjee et al. 2009).

6.3 Calcium Site Mutations Affect Membrane Penetration

FHV capsid elements, apart from the γ peptide, have been shown to affect membrane disruption by FHV. The calcium coordinating glutamate and aspartate residues were mutated to glutamine and asparagine residues, resulting in a drastic decrease in the amount of calcium bound to the FHV capsid. The mutations did not affect the assembly and maturation cleavage of either the authentic virions or VLPs. Although the calcium binding sites (Fig. 4a) are distant from the location of the γ amphipathic helix in FHV crystal structure, calcium site mutations affected the function of γ and host cellular entry by FHV particles in a unique manner (Banerjee et al. 2010). The influence of the integrity of calcium binding sites on the biological function of γ during virus entry is now being investigated.

6.4 Structure: Function Correlation of FHV Mutants

A hybrid structural and biological approach was utilized recently to characterize the mutations in FHV capsid protein, and to provide a structural explanation of entry-related biological phenomenon. The effect of mutations on the organization of γ on the inner capsid surface of FHV was examined with X-ray crystallography and electron cryo-microscopy (CryoEM), and the extent of cellular membrane disruption during entry was concurrently determined through biological and biochemical means (Banerjee et al. 2009). An 8.8-Å cryoEM reconstruction of particles lacking the C-terminus of γ revealed that the N-terminal amphipathic helices of gamma are disordered at the fivefold axis of symmetry (Banerjee et al. 2009), and that the pentameric helical bundle (Fig. 3b) is not visible. That these particles are unable to

disrupt artificial membranes *in vitro* and promote entry *in vivo*, suggests an essential role for the correct organization of γ for its biological function during entry. A similar correlation between the structural organization of γ and its ability to disrupt cellular membranes during entry was also found for FHV particles containing mutations at the calcium binding sites (Banerjee et al. 2010).

6.5 Does Quasi-Symmetry Dictate Separate Roles for γ from Different Subunits?

The striking difference in the structural organization of γ at quasi-equivalent locations indicates that quasi-symmetry may dictate separate functions for γ from different subunits in the iASU. The γ peptides from the A subunits form pentameric helical bundles at the fivefold axis (Fig. 3a, b), and it has been suggested that this class of γ is primarily responsible for interacting with cellular membranes (Cheng et al. 1994). This hypothesis is partially supported by a recent study, where disorder in the pentameric helical bundles corresponded to decreased membrane disruption during virus entry (Banerjee et al. 2009). An ordered pentameric bundle might be involved in forming a channel through cellular membranes for deposition of the viral genome. The γ peptides from the B and C subunits contact bulk RNA inside the capsid, and are organized in a manner unlike those in the A subunits (Cheng et al. 1994). It is possible that the B and C subunit peptides have a separate function during virus entry. The C-terminus of γ specifically packages viral RNA in capsids during assembly, which indicates a direct interaction of this region of γ with FHV RNA. The N-terminal amphipathic helix of γ also constitutes a mitochondrial targeting signal and guides an attached GFP molecule to mitochondria in transfected HeLa cells (H.E. Walukiewicz and J.E. Johnson, unpublished observation). It is possible that this targeting signal in B and C subunit **γ** peptides guides the FHV genome associated with the C-terminus of the peptides to the mitochondria, the replication site for FHV, in infected *Drosophila* cells.

Table 2 Remaining questions for future research

- What is the identity of the FHV cell surface receptor?
- What are the cellular triggers that initiate membrane penetration?
- Do particle associated or released γ peptides mediate membrane interactions, which regions of γ insert into the membrane bilayer, and does γ insert into membranes as a monomer or oligomer?
- Does FHV form a small membrane spanning pore or causes a large scale disruption of the membranes?
- Are the RNA genome segments or the entire nucleocapsid translocated across the membrane into the cytoplasm?
- Does γ serve additional functions in addition to membrane disruption, such as facilitating the delivery of genomic RNA to the cytoplasm?
- What is the mechanism of γ trans complementation?

7 Concluding Remarks

The mechanism of host cell entry by non-enveloped viruses is an unresolved area in molecular virology. The overall simplicity of the FHV capsid and genome, along with the wealth of available high resolution structural information, make FHV an ideal candidate for understanding how non-enveloped viruses enter and infect cells. The proposed mechanism of FHV membrane disruption, in which the coat protein is primed by proteolytic cleavage followed by programmed capsid conformational changes and release of a small membrane-interacting peptide, fits an emerging common mode of non-enveloped virus membrane penetration. As described here, recent studies have lead to significant advances in the understanding of FHV entry and support a model of virus entry that is widely applicable to a diverse group of non-enveloped viruses.

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The Caliciviruses

Umesh Katpally and Thomas J. Smith

Contents

Abstract The caliciviruses are by far the major cause of non-bacterial gastroenteritis, highly infectious, and have a rapid and severe onset of symptoms. Studies on this family of viruses have been hampered by the lack of animal model and tissue culture system. However, recent advances in protein expression systems and the development of a mouse norovirus animal model has led to rapid advances in our understanding of these viruses with regard to structure and the host immune response. Our current understanding of this important family of viruses is reviewed here.

1 Immunity Against Human Calicivirus Infection and Prospects for a Vaccine

Efforts to vaccinate against human calicivirus-induced illness have been initiated due to the widespread incidence and epidemic nature of infection. The need for such a vaccine is especially apparent in the military setting, where outbreaks of NV

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infection have hampered military operations in numerous instances (Sharp et al. 1995). In addition, elderly and immunocompromised individuals, if not the population as a whole, may benefit from such a vaccine. Based on the incomplete understanding of a protective immune response to calicivirus-induced illness, the requirements for an efficacious vaccine are unclear. In addition, the multiple antigenic classes of human Caliciviruses may prove to be a hindrance in vaccination to the family of viruses collectively.

Studies of calicivirus infection in humans have yielded somewhat confusing results. In volunteer studies, the incubation time is 10 51 h with a meantime of 24 h (Blacklow et al. 1972; Dolin et al. 1971, 1972; Steinhoff et al. 1980; Wyatt et al. 1974). Surprisingly, symptoms only last 24 48 h and viral shedding occurs concomitantly with illness, ceasing 72 h after the onset of symptoms (Thornhill et al. 1975). Also contrary to most viral infections, adults are more susceptible (with infection rates at least 80% in some studies) than children suggesting that previous exposure does not ensure protection against subsequent infection (Kaplan et al. 1982). Indeed, one volunteer study suggested that individuals can exhibit either short term or long term resistance after exposure to caliciviruses (Parrino et al. 1977). The short-term resistance, 6 14 weeks post infection, is serotype specific and represents traditional viral resistance. These individuals, however, were susceptible 27 42 months after the initial infection. In contrast, some individuals did not exhibit symptoms after either the first or second exposures, nor did they present a serological response to the infection. The speed of viral clearance and the lack of humoral response suggest that adaptive immunity may not play a crucial role in protection. Indeed, some studies have even suggested that higher titers of serum antibodies may lead to higher susceptibility to disease (Graham et al. 1994). However, recent studies in natural settings have suggested that repeated exposures to NV can induce natural immunity (Black et al. 1982; Ryder et al. 1985) and that protection from disease is correlated to antibody titers (Nakata et al. 1985). The lack of immunity from a single infection is not uncommon among mucosal diseases. Some of these apparent contradictions may be due to the role carbohydrates play in cellular recognition and viral pathogenesis as reviewed below.

A good animal model for calicivirus infection has been rabbit hemorrhagic disease virus (RHDV, type species of *Lagovirus* genus). In one interesting case, RHDV accidentally escaped from Wardang Island off the coast of Yorke Peninsula, South Australia during trials in 1995 and reached Queensland in the same year. Ever since rabbits were released in Australia in 1859, they have wreaked tremendous ecological havoc in Australia. Therefore, the losses in the rabbit population due to RHDV were not at all unwelcome. Since there were only two rabbits, both tracked constantly with radio transmitters, the spread of the virus to the mainland had to have occurred via insect transmission. RHDV does not kill rabbits less than 3 weeks of age, and affects only 60% of rabbits 3 6 weeks of age. If the rabbits are infected by RHDV and survive, the appear to develop antibodies to the virus and are resistant to challenge later in life. Therefore, RHDV has a larger impact on the

local population if introduced after rabbits have bred and the young are old enough to be affected by the virus. The resistance observed in the natural population certainly suggests that adaptive immunity can control the infection. Indeed, vaccination of rabbits against RHDV infection has been accomplished through intramuscular injection of RHDV VLPs plus adjuvant (Laurent et al. 1994). All animals in the experimental groups survived the challenge with a lethal dose of virus if vaccinated 15 days prior to the challenge while 9 out of the 10 rabbits survived the challenge 5 days post vaccination. Interestingly, the one susceptible animal was also the only one with a negligible antibody titer post-vaccination, supporting the idea of a protective humoral response in calicivirus infection.

In the case of mouse norovirus (MNV-1), a cell culture system, an infectious clone, and an animal model have all been developed so that the immune response to a calicivirus infection can be detailed (Wobus et al. 2006). It was found that mice that are unable to develop mature T and B cells (RAG $^{-/-}$ mice) did not die but rather these mice developed a persistent MNV-1 infection (Karst et al. 2003). These studies therefore inferred that the innate immune system might be sufficient for resistance to MNV-1 infection and that adaptive immunity is required for viral clearance. This is an attractive hypothesis since usual 24-h incubation periods and 24 48-h time courses of human norovirus diseases seem too short of a time period for an adaptive immune response. In contrast to the RAG $^{-/-}$ results, STAT1 $^{-/-}$ mice [STAT1 is involved in signaling through both the IFNαβ and IFNγ receptors; (Katze et al. 2002)], and mice lacking both IFNαβ and IFNγ receptors succumbed to a lethal infection by MNV-1. In a subsequent publication, the Virgin group demonstrated that, while the innate immune response was necessary to survive an MNV-1 challenge, antibodies are crucial for the clearance of the infection (Chachu et al. 2008). Further, they demonstrated that viral clearance could be facilitated by an adoptive transfer of anti MNV-1 antibodies. Therefore, as with human calicivirus infection, the immune response to MNV-1 is complex and most certainly represents a synergism between the adaptive and innate components of the immune system.

Based on promising results in animals, phase I trials were done to test oral immunization of humans with NV VLPs (Ball et al. 1999). Edible vaccines in which NV VLPs are expressed in either transgenic tobacco leaves or potato tubers have also been examined (Mason et al. 1996). Oral dosing of mice with either NV VLPs produced from tobacco leaves and partially purified or potato tubers expressing the NV capsid protein, developed serum IgG, while the VLPs in tobacco extracts also stimulated secretory IgA production. Potato tubers were also immunogenic when fed to human volunteers (Tacket et al. 2000). Current vaccination studies are aimed at determining if higher doses of VLPs or use of a mucosal adjuvant would enhance the immune response to the non-replicating particles, and if cross-protective immunity is induced to antigenically distinct human caliciviruses. It is not known whether induction of antibody responses is the sole or even most important aspect of immunity that should be targeted for vaccines.

2 Calicivirus Structure

The calicivirus virion is made up of a single major capsid protein. While this is common among plant viruses it is very unusual among animal viruses. Calicivirus capsid proteins range in molecular weight from 56 to 76 kDa (Clarke et al. 1997; Jiang et al. 1993). A major advance in the study of calicivirus structure was the finding that VLPs are found in the supernatant of insect cells infected with a recombinant baculovirus expressing the NV capsid protein (Jiang et al. 1990). NV VLPs have been suggested to be antigenically similar to native virions (Green et al. 1993; Jiang et al. 2002). VLP self-assembly is independent of ORF3, and both the full-length capsid protein of 58 kDa and a smaller 34-kDa cleavage product are detected in infected insect cells (Jiang et al. 2002). The three-dimensional structures of two calicivirus capsids, NV (Prasad et al. 2000) and the primate calicivirus Pan-1 (Prasad et al. 1994), have been determined. Initially, cryoelectron microscopy (cryo-EM) was used to analyze rNV VLPs (Prasad et al. 1994) and Pan-1 virions containing RNA (Prasad et al. 1994). Subsequently, the crystallographic structure of VLPs of NV was determined (Prasad et al. 1999). These studies found that the viruses exhibit $T = 3$ icosahedral symmetry with the particles being comprised of 180 copies of a single capsid protein. With architectures very much like the plant Tombusviridae, the capsid protein is comprised of the N-terminus (N), shell (S), and protruding domain (P). The N-terminus of the NV capsid protein is buried in the particles while the C-terminal amino acids are exposed on the VLP surface. The S domain is composed of an eight-stranded ß-sandwich fold and forms the contiguous shell around the RNA genome. This shell domain is connected by a flexible hinge to a "protruding" (P) domain at the C-terminal half of the capsid protein. Much like the Tombusviridae, dimers of these subunit protruding domains are found at the icosahedral twofold axes (C C subunit dimers) and encircling the fivefold axes (A B subunit dimers). Unlike the Tombusviridae, however, the protruding domain can be further divided into two subdomains, P1 and P2. P1 forms a stem region connecting the shell domain to a globular head region (P2). Much like viruses within the Tombusviridae family, the shell domain shows high amino acid sequence homology among NLVs and forms a continuous ~300-Å diameter protein shell. P1 shows moderate sequence diversity among NLVs, while P2, located at the outermost extreme of the capsid, has a highly variable amino acid sequence. Since a monoclonal antibody (mAb) that binds to P2 was found to block attachment of rNV VLPs, it is thought that P2 is responsible for cellular recognition (White et al. 1996).

More recently, the crystal structure of the intact calicivirus, San Miguel sea lion virus (SMSV), has been determined (Chen et al. 2006). SMSV is the prototype of the *Vesivirus* genus of the Caliciviridae family of which feline calicivirus (FCV) is also a member. The X-ray structure of SMSV is very similar to the previously determined structure of a recombinant calicivirus with respect to capsid architecture and domain organization of the major capsid protein, VP1. Compared to the atomic structure of the NV VLPs, there appear to be additional points of flexibility

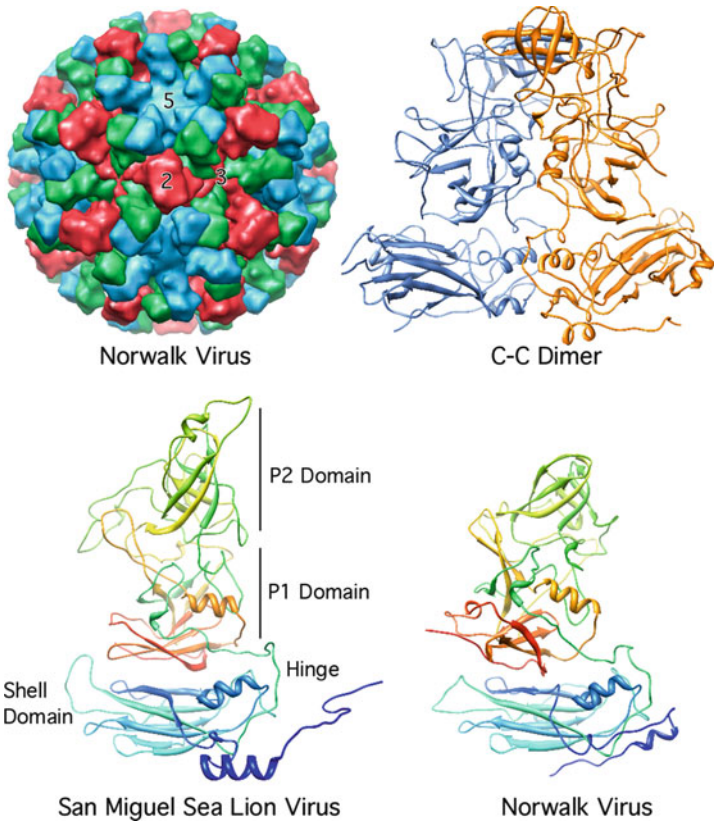

Fig. 1 Structure of the Caliciviridae family. The figure at the *top left* is a surface rendered image of the Norwalk virus VLP (Prasad et al. 1999) where subunit A is colored *blue*, B is *green*, and C is *red*. The *upper right* figure shows how the protruding domains from adjacent subunits form dimeric spikes. The *lower* two figures are ribbon diagrams of single subunits of the Vesivivirus San Miguel sea lion virus (Chen et al. 2006) and the Norovirus, Norwalk virus (Prasad et al. 1999) where the ribbons are colored *blue* to *red* as the chain extends from the amino to carboxyl termini

between the domains of VP1 that likely impacts the relative orientation of the domains. As shown in Fig. 1, the P1/P2 domains in SMSV are more perpendicular to the shell surface and results in most of the P domain dimer interactions to be mediated by the outermost P2 domain. The authors suggested that this inherent flexibility might increase antigenic diversity while maintaining the same domain organization. In addition, the outermost loops of the P2 domain are significantly larger than those observed in NV and, together with differences in orientation, gives the protruding domains a more rounded appearance compared to the "flat-topped" spikes of NV.

A number of studies have suggested that the hypervariable P2 domain might have a role in antigenicity and host cell receptor interactions. Indeed, the antibody/RHDV (Thouvenin et al. 1997) and, more recently, the MNV-1/Fab (Katpally et al. 2007)

complexes have been analyzed using cryo-electron microscopy and have shown that antibodies do indeed bind to the outer most tips of the P2 domains. Using the structure of SMSV, they were able to map the neutralizing epitopes of other vesiviruses to several highly exposed loops within the conserved polypeptide fold of P2 suggesting that antigenic diversity can be achieved by incorporating amino-acid changes into these loops while not affecting the conserved regions involved in the dimer interface.

In addition to VLPs with $T = 3$ symmetry, a population of smaller particles is also found in some NV VLP preparations (e.g., Green et al. 1997; Jiang et al. 1995; Leite et al. 1996; White et al. 1997) and stool samples from calicivirus-infected individuals (Taniguchi et al. 1981). The smaller particles of NV VLPs with a diameter of 23 nm contain the full-length 58-kDa NV capsid protein and are similar antigenically and biochemically to the 38-nm VLPs (White et al. 1997). However, it is interesting to note that the smaller particles must differ in some fundamental way since they are far more sensitive to proteolytic cleavage than the larger, $T = 3$ particles. It was suggested that the smaller particles are formed from 60 copies of the capsid protein and exhibit a $T = 1$ symmetry. The same $T = 1$ symmetry was hypothesized for the small 27-nm RHDV VLPs observed after a small N-terminal deletion and epitope insertion in the capsid protein since these particles are antigenically indistinguishable from their larger counterparts (Nagesha et al. 1999). Viral particles of two sizes are also observed in liver homogenates of rabbits infected with RHDV and worms infected with an insect calicivirus (Hillman et al. 1982). The insect calicivirus particles isolated from insect waste consist mainly of a 28-nm type while particles isolated from infected larvae consist of both 28-nm and 38-nm species (Hillman et al. 1982). The biological role, if any, of these particles is not clear.

A recent study has examined the structure and function of particles formed by expressed VA387 norovirus P domain protein (Tan et al. 2008). When the P domain is expressed with and without the shell-protruding domain linker region, small ~200-Å diameter particles are observed. These particles cross react with antibodies to NV and exhibit very similar blood group antigen recognition patterns as the virus-like particles formed by full length capsid protein. From the ~8.4-Å cryo-TEM image reconstruction, the particles apparently have octahedral symmetry and are composed of 12 P domain dimers. This is clearly another example of how the formation of the P domain dimer is independent of either the shell domain or icosahedral architecture, and forms the functional unit with regard to glycan recognition and epitope display.

3 Feline Calicivirus Interactions with Its Receptor

Feline calicivirus (FCV) causes respiratory illness and stomatitis in cats (Radford et al. 2007). While the human noroviruses bind to histo-blood group antigens (Harrington et al. 2002; Marionneau et al. 2002), FCV interacts with α-2,6-sialic acid (Stuart and Brown 2007). In both cases, these glycan interactions are thought

to be important but unlikely to represent the sole cellular receptor for the virus. Feline junctional adhesion molecule 1 (fJAM-1) has been identified as the functional receptor for FCV (Makino et al. 2006). It is important to note that, to date, this is the only protein receptor identified for the caliciviruses. This was clearly demonstrated by studies showing that transfection of the fJAM-1 gene into non-permissive cells transferred sensitivity to infection that was blocked by antibodies to fJAM-1. JAM-1 is a member of the immunoglobulin-like superfamily of proteins found on the surface of leukocytes and blood platelets and is thought to regulate the formation of tight junctions in epithelial and endothelial cells. Domain deletion and mutagenesis experiments have mapped the virus interactions to outermost D1 domain (Ossiboff and Parker 2007). It is interesting to note that bacterially expressed fJAM-1, which is devoid of any glycan modifications, is able to inhibit virus binding to cells (Ossiboff and Parker 2007). This would suggest that the sialic acid on fJAM-1 is not necessary for binding of the receptor to FCV.

The cryo-TEM structure of FCV complexed with fJAM-1 has been determined to a resolution of $\sim$18 Å (Bhella et al. 2008). As reviewed in Fig. 1 and akin to that seen with the plant virus family, the Tombusviridae, there are two types of protruding domain dimers: the A/B subunit dimers that surround each of the fivefold axes and the C/C dimer found at the icosahedral twofold axes. While the general structure agreed well with the atomic structure of SMSV, it is interesting that either alone or complexed with fJAM-1, the C/C dimers were less ordered than the A/B dimers. In particular, there was apparent disorder around the flexible junction between the S and P domains of the capsid protein.

The structure of human JAM-1 (hJAM-1) has been determined to atomic resolution (Prota et al. 2003). In the crystal cell, the two D1 Ig-like domains interact at $\sim$90° angles akin to what is observed in antibody structures. The D2 domains interact with different copies of the hJAM-1 proteins in an anti-parallel fashion. For a number of reasons, including possible interactions with reoviruses, it was suggested that these D1 interactions might be dynamic in nature. The structure of human JAM-1 was altered to reflect the sequence of fJAM-1 and fitted into the electron density envelope of the FCV/fJAM-1 complex (Bhella et al. 2008). Unlike the interactions observed in the crystal structure of hJAM-1, the model suggests that two copies of fJAM-1 bind to the outermost portion of the P2 dimers in a crossed manner such that the D1 domain from one bound molecule interacts with the D2 domain of the other. The current model, however, places these two domains fairly close together in some regions. Again, this model places a single D1 domain at the viral surface rather than the D1 dimers observed in the crystal structure. fJAM-1 interacts with the known hypervariable antigenic loops of P2 and those residues in fJAM-1 involved in these contacts have been shown to be important for virus binding. These authors also suggest that the P domains rotated by $\sim$13° in a counter clockwise fashion and the shell domains rotated by $\sim$18° in a clockwise manner upon receptor binding. While these are interesting observations, they are rather subtle and, as with the receptor fitting, will require higher resolution reconstructions for further interpretation.

4 Interaction Between Noroviruses and Polysaccharides

It has been shown that susceptibility of an individual to NV infection is related to the expression of particular carbohydrates (Lindesmith et al. 2003). NV VLPs bind to the H, Lewis, and A histo-blood group antigens; the variation in which is determined by the individual's expression of various glycotransferase enzymes [for a review, see Hutson et al. (2004)]. The expression of these carbohydrate moieties is also tissue and developmentally specific. For example, the highest levels of expression on the small intestinal villi lie at the outermost tip. Other than evidence correlating the sensitivity of individuals with their blood antigen grouping, the biological importance of this interaction has come from studies demonstrating that sera from convalescent patients blocks the hemagglutination reaction. Using the structural model for NV, this phenomenon has been further articulated through mutagenesis studies (Tan et al. 2003). These researchers have proposed that there is a pocket in the P2 domain that contains an RGD/K motif that binds the histo-blood group antigens. This was subsequently supported by findings that the isolated, expressed P domain by itself forms a dimer that is capable of binding to these carbohydrate moieties (Tan et al. 2004). Other noroviruses display different ABH and Lewis carbohydrate specificities (Harrington et al. 2002; Huang et al. 2003; Hutson et al. 2004). As reviewed above, the previous volunteer study demonstrated that a number of individuals were susceptible to repeated challenges of NV while others were resistant to infection. These results suggest that natural resistance and the occasional lack of correlation between an antibody response and protection is likely due to genetically determined expression of particular carbohydrate moieties (Lindesmith et al. 2003). Therefore, the importance of blood group carbohydrates in infection with human noroviruses is clear, but whether these carbohydrates represent a viral receptor or are sufficient for infection is not clear since there is no cell culture system for human noroviruses. It should be noted that a very small proportion (4 6%) of specifically bound VLPs is internalized in various cells (White et al. 1996). While this interaction may be sufficient for infection, it is lower than the level of internalization of positive control viruses such as rotaviruses.

Interestingly, a growing number of viruses have been shown to interact with the target cell via carbohydrate moieties as apparent co-receptors or, in some cases, independent of cell surface proteins. There are several non-enveloped viruses that, while they require protein receptors for internalization, use heparin sulfate as attachment factors: Theiler's virus (Reddi and Lipton 2002) [peripheral nerve protein, PO (Libbey et al. 2001)], adenovirus 2 [coxsackievirus-adenovirus receptor (Bergelson et al. 1997)], adeno-associated virus 2 [α5 integrin (Summerford et al. 1999)], FMDV [αvβ3 (Perinstein et al. 1995), αvβ6 (Jackson et al. 2001), and αvβ1 integrins (Miller et al. 2001)], and human papillomavirus 16 [α6 integrin (Evander et al. 1997)]. Interestingly, it has been shown that some FMDV serotypes (Sa-Carvalho et al. 1997) and the alphavirus, Sindbis virus (Klimstra et al. 1998), become attenuated when grown in tissue culture and this is concomitant with acquiring the ability to bind heparin sulfate. Indeed, heparin-binding FMDV can

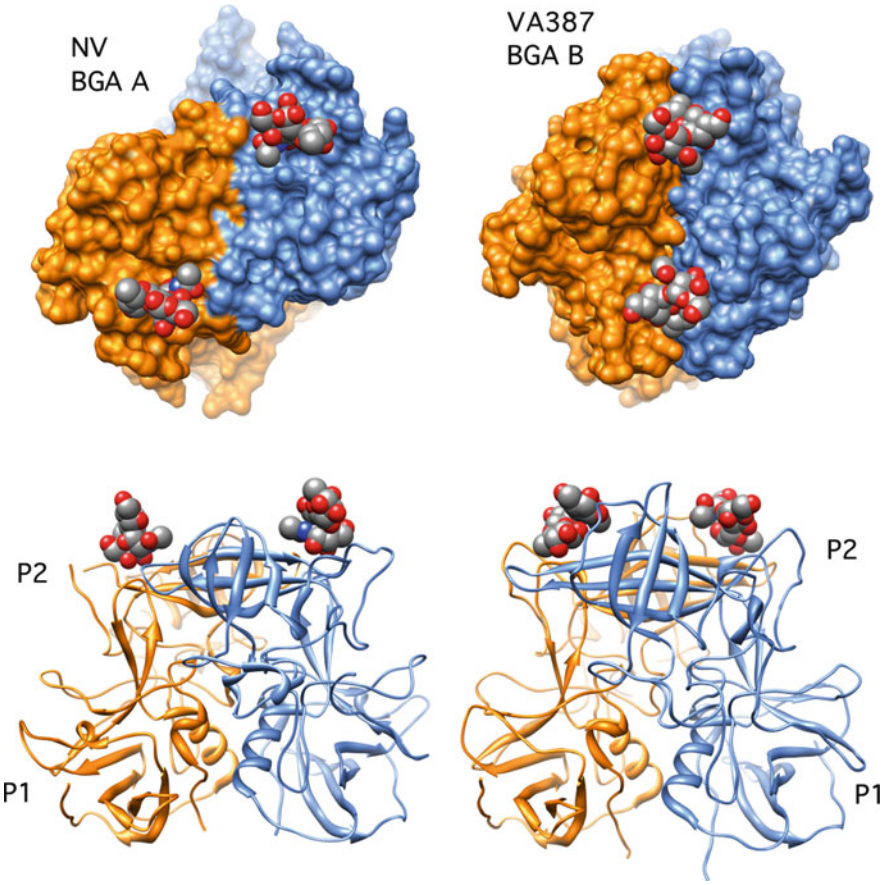

Fig. 2 The locations of carbohydrate binding sites on the P domains of the Noroviruses. The *upper* two figures show the top view of the P domain dimers (*orange* and *blue*) with the atoms of the bound trisaccharides shown as *spheres*. *Below* are the side views of the same structures with the proteins represented as ribbon diagrams. The *left side* of the diagram is o f NV bound with a trisaccharide representing blood group antigen A (Bu et al. 2008) while the figures on the *right* are of VA387 bound with a trisaccharide representing blood group antigen B (Cao et al. 2007)

actually enter the cell independent of the protein receptor via caveola-mediated endocytosis (O'Donnell et al. 2008). It, therefore, seems likely that the Caliciviruses similarly use cell surface polysaccarides to enhance the efficiency of the attachment process by increasing the duration of the initial interactions.

Recently, there have been a number of structures of norovirus P domains complexed with simple saccharides (Fig. 2) (Bu et al. 2008; Cao et al. 2007; Choi et al. 2008). By cloning the P domains separate from the full-length capsid protein, these groups were able to obtain crystals that diffract to high resolution and therefore more amenable for detailed studies of oligosaccaride P domain interactions. NV, the archetype genogroup I norovirus, recognizes A-type and H-type

human blood group antigens while VA387, a genogroup II norovirus, recognizes a larger array of blood group antigens. In the first structure (Cao et al. 2007), the P domains from VA387 were crystallized in the presence of trisaccharides representing blood group antigens A (α-L-Fuc-(1→2)-[α-D-GalNAc-(1→3)]-D-Gal) and B (α-L-Fuc-(1→2)-[α-D-Gal-(1→3)]-D-Gal). These glycans bind to essentially the same location: at the interface between the two subunits and at the outer most tip of the P2 domain. The ability to recognize both types of glycans is likely due to the fact that most of the protein/ligand interactions are via the fucose moiety that is common to both. While the binding pocket lies at the P2 dimeric interface, the predominant interactions lie on one side of the dimer. Subsequently, similar studies were performed on Norwalk P domains (Bu et al. 2008). One of the major differences between NV and VA387 is that VA387 can bind to both A and B blood group antigens while NV can only bind to A antigens. This is well explained by the structure that shows that, while blood group antigen A binds to NV in approximately the same location as was observed in VA387, the majority of the interactions are formed between the α-GalNAc moiety and the P2 domain the defining difference between the A and B blood group antigens. The structural basis for carbohydrate recognition by NV was further explored in a subsequent publication (Choi et al. 2008). In this study, they determined the structure of both A and H blood group antigens complexed with NV P domains. The H blood group polysaccaride is quite different than the A type with the terminal sugar being an α-fucose followed by a β-galactose. Researchers found that these two sugars together replaced the interactions made by the single α-GalNAc moiety in the A group antigen. They proposed that it is the hydrophobic interaction that both glycans make with a conserved tryptophan (375) and conserved hydrogen binding patterns that are the common to both glycans. It is, therefore, a very interesting case of where there is ligand specificity yet two quite different ligands bind to the same site. This is perhaps akin to different antibodies binding to the same epitope with quite different paratopes.

5 Attenuation of NMV-1 Pathogenesis

The Virgin laboratory has found that MNV-1 can readily attenuate (Wobus et al. 2004) when passaging and amplifying different plaque purified strains of MNV-1 in RAW cells. This attenuation process was likely due to adaptation in RAW cells since the titer increased by 10-fold after the three passages. What is particularly interesting is the location of the mutations that occur during this attenuation/adaptation process. The first mutation occurs in the ORF1 region and lies in the gene that is analogous to the picornaviral 3A. This is not entirely surprising since in the picornaviruses, 3A is thought to help assemble replication complexes in the cell and thereby help activate the RNA-dependent polymerase. Mutations here decrease the lethality of MNV-1 by ∼50%. After the second passage, a second mutation was found in that region, but now there was an additional mutation (a LYS to GLU

mutation) in the capsid protein. After the third passage, this capsid mutation had completely overtaken the wild-type form of the virus.

It is fairly common that virulent viruses become attenuated during passaging in cell culture. The attenuation mutation in MNV-1 is of particular interest because of where it lies in the capsid protein structure. As reviewed in Fig. 1, the capsid of NV is similar to the Tombusviridae in that there are protruding domains that form dimers at the icosahedral C C and A B interfaces. This attenuation mutation lies fairly deep within the interface region between the P2 domains. In the case of Sindbis virus, the location of the attenuation mutant was in the glycoprotein E2 that had been suggested to be directly involved in receptor interaction (Klimstra et al. 1998). This mutation may be more indirectly involved in receptor binding. It is also possible that such attenuation mutations in the capsid could affect stability, assembly, or uncoating such that the production rate of virus particles *in vivo* is affected. One argument against this mutation purely affecting assembly is that VLPs of NV do indeed assemble even when both P1 and P2 have been eliminated (Bertolotti-Ciarlet et al. 2002).

6 The Cryo-TEM Structure of the *T* 3 MNV-1 Capsid

The MNV-1 system has a great deal of potential since it can be propagated in a cell culture system, aspects of its pathogenesis and the host immune response can be examined in an animal model, large amounts of virus can be readily produced, neutralizing monoclonal antibodies have been isolated, and an infectious clone has been developed (Wobus et al. 2006). As reviewed above, the only other structure of an intact virus determined to date is the San Miguel sea lion virus (SMSV) that is a member of the *Vesivirus* genus. Therefore, it was first necessary to determine the structure of MNV-1 for comparison to the other members of the *Caliciviridae* family. Surprisingly, even from the initial examination of the MNV-1 electron density (Fig. 3), it was quite apparent that the structure of MNV-1 was significantly different than either the NV VLP or the SMSV intact particle. While the P domains of NV VLPs rest upon the shell domain, there was a large gap in the electron density between the shell and protruding domains of MNV-1. When the NV VLP structure was overlaid onto the MNV-1 density, it was clear that the entire P1 domain lies in that gap between the P and S domains and that the electron density for the MNV-1 protruding domains extended far beyond the outer extents of the P2 domains. At first, it seemed possible that this difference might be due to artifacts such as scale factor errors. However, this was immediately dismissed since the inner and outer extents of the shell domains in MNV-1 match extremely well to that of the VLP NV model. Therefore, it appears that the MNV-1 P domains lift up off the shell (S domains) to form a second proteinaceous layer.

To examine this further, a model for MNV-1 was constructed by modifying the structure of NV. Because of extensive protein protein interactions, it was assumed

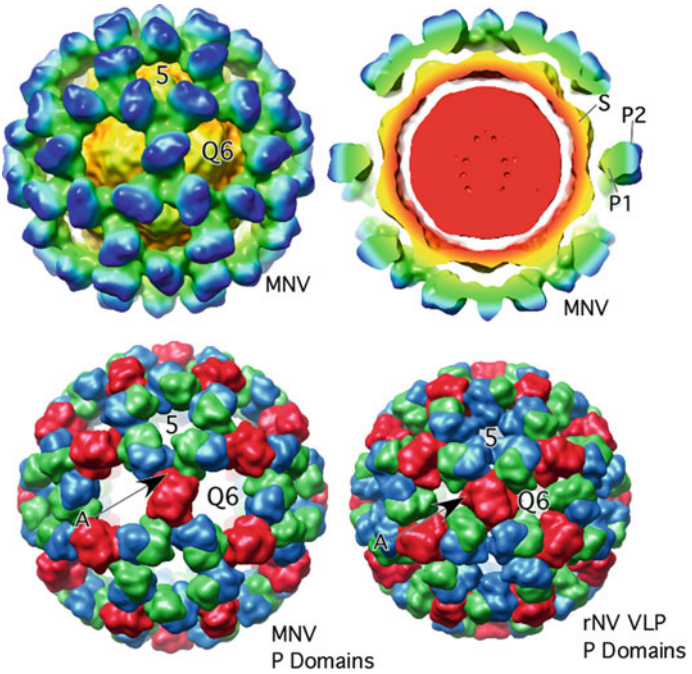

Fig. 3 Structure of the mouse norovirus, MNV 1. The *top* two panels are of the cryo EM image reconstructions of the authentic virus, MNV 1. The coloring is according to radius and the figure on the *right* shows a slice through the approximate middle of the virus. Note that the P1 domain is quite removed from the surface of the shell formed by the S domain. The *bottom* two figures show the P domains of the MNV 1 and those of the VLPs formed by the recombinant protein of NV. Note that the domains of MNV 1 are rotated (*arrow A*) and lifted off the surface to form an outer shell with a larger diameter compared to NV

that the A B and C C protruding domain interactions were not significantly different than NV. When the A B and C C P dimers moved as rigid bodies, they fit extremely well into the "floating" P domain density map. The fact that these structures fit well into the density strongly supports the contention that the gap between the P and S domains is due to either a large error in scale factor or contrast transfer function correction. There is, in fact, a handedness to the protruding domains themselves in that the P1 domain sticks out at an angle compared to the top P2 domain (Fig. 3). The hand that best accommodated this "twist" was used for modeling. The protruding domains had to be rotated by $\sim 40^{\circ}$ in a clockwise fashion and lifted up by ~ 16 Å in order to fit into the "floating" shell of protruding domains. This increased the outer radius by $\sim 10\%$. Secondly, the holes at the quasi sixfold axes opened up significantly. Finally, the rotation of the P domains established new contacts among the P1 domains that likely stabilize this new outer shell. In contrast, the P domain dimers in NV VLPs essentially interact at the tip via the P2 domains with the P1 domains flaring out at the base. Therefore, there was relatively little contact between the P1 domain dimers of the capsid subunits in NV VLP.

One possible origin for the unique conformation of MNV compared to prior structures is that all of the other norovirus structures to date have been of recombinant VLPs. It may be that these VLPs do not undergo the same assembly and maturation processes as authentic virions. Arguing against this idea is that the crystal structure of authentic SMSV (Chen et al. 2002) did not have this "floating P domain" shell. However, the P domains of this *Vesivirus* are in a confirmation that is quite distinct from that observed in the crystal structure of rNV VLP and therefore the structure observed here with MNV may be norovirus genus-specific. It is also possible that the crystallization conditions used for SMSV and rNV VLP favored the more compressed state of the viral shell.

Since the unusual, flexible connecting loop between the P1 and S domains is common to all *Caliciviruses*, it seems possible that this conformational transition is also shared amongst these viruses. This proposed conformation change may be similar to the structural changes that reoviruses undergo during the various stages of the infection process (Sturzenbecker et al. 1987). Perhaps the more compressed state of the viral shell observed in rNV VLP represents a more stable conformation that insures spread among organisms while the MNV structure might represent an activated and perhaps more infectious state.

An alternative explanation for the difference between the MNV-1 structure and prior calicivirus structures is that this conformation is only observed in MNV-1. This may be akin to the differences amongst the rhinovirus serotypes where capsid transitions are easier, for example, in HRV14 than in HRV16 (Katpally and Smith 2007; Olson et al. 1993). While HRV14 rapidly uncoats in the presence of its receptor (Olson et al. 1993) and extrudes the normally buried VP1 and VP4 N-termini at room temperature (Katpally and Smith 2007), HRV16 only exhibits such dynamics at significantly higher temperatures (Katpally and Smith 2007; Olson et al. 1993). Similarly, perhaps this structure in MNV-1 is unique because it is more facile than the other caliciviruses or VLPs. The biological role of this "enlarged" state of MNV is still unclear but the availability of an animal model, a cell culture system, and an infectious clone make its elucidation a tractable problem for further study.

7 The Cryo-TEM Structure of MNV-1 Complexed with Fab's

The only other image reconstruction of an antibody bound to a Calicivirus was that of RHDV VLP complexed with whole antibody as reviewed above (Thouvenin et al. 1997). As a way to further detail possible changes in the virion upon antibody-mediated neutralization, the structure of the Fab A6.2/MNV-1 complex (Fig. 4) was determined using cryo-TEM methods (Katpally et al. 2007). It was immediately evident that the P domains in this complex were in the same conformation as that observed in the MNV-1 virus structure alone. Indeed, when this electron density was overlaid with that of the unbound virion, the only significant difference was that belonging to the bound Fab. This suggests that neutralization caused by this Fab does not induce gross conformational changes in the virion.

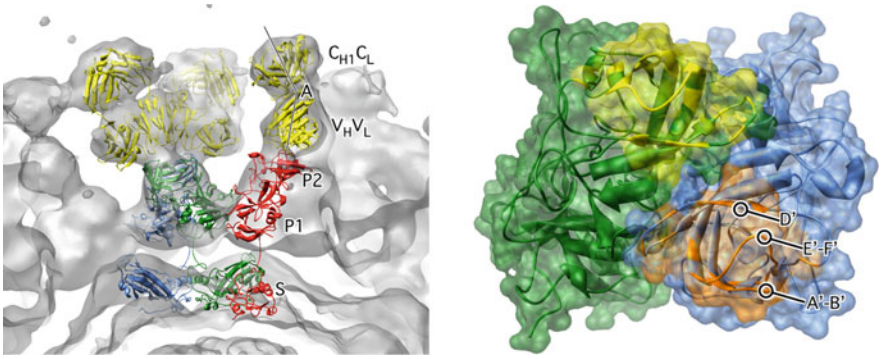

Fig. 4 The structure of MNV complexed with a neutralizing Fab. The figure on the *left* shows the fitting of the separate domains of NV into the cryo TEM envelope (*grey transparent surface*) of the MNV 1/Fab 6.2 complex. The A, B, and C subunits of NV are colored *blue*, *green*, and *red*, respectively. The Fab is represented by a *yellow* ribbon diagram and the approximate dyad axes in the variable and constant regions are noted by *black lines*. Note that the P domains are clearly lifted off the surface of the shell by ~16 Å. The *right* figure shows a top view of the P domains with the approximate Fab contact points highlighted in *orange* and *yellow*. In this figure, the structure of NV (Prasad et al. 1999) was used to model MNV 1 and Fab17 (Liu et al. 1994) was used to model the Fab structure

Further, this was important validation of the apo structure since this data was collected using different virus preparations made at different times. This strongly argues that the gap between the P domains and the S domains is real. Finally, since the density of the bound Fab fragment was as strong as the protruding domains, it was also apparent that a Fab was bound to each of the 180 P2 domains and that this "floating P domain" conformation is immunologically relevant.

To facilitate interpretation of this cryo-TEM structure, the modified NV VLP structure from above and that of a known Fab [Fab1 to HRV14; (Che et al. 1998)] were placed into the electron density. As shown in Fig. 4, the models fit extremely well. Indeed, the elbow angle (the angle between the variable and constant domains) was clearly visible (labeled "A" in the bottom left panel). The two Fabs bound to each P2 domain dimer at a glancing angle and are so close to each other that the density from the two sets of variable domains tend to merge. The Fab arms of an IgG are remarkably flexible and can bind bivalently to virions (e.g., Smith et al. 1993). The angles of the bound Fabs seen here suggest that the IgGs could bind with both arms within the A B or C C dimers but with some difficulty. It is still possible that some IgGs could bridge between the two types of dimers, but this would preclude having all of the antibodies bound in a bivalent manner as was observed in HRV14 (Smith et al. 1993) where the binding of the Fab fragments by themselves bound in such an orientation where little to no conformational changes were required to model the intact IgG onto the structure. Indeed, binding of the mAb1 antibody to the same epitope was with a slight twist in orientation that prevented bivalent binding of mAb1(Che et al. 1998).

The Fab A6.2/MNV-1 structure lends further evidence that the "floating" P domains represent the true structure of MNV-1. Firstly, the structure of the Fab fits well in the Fab density, demonstrating that the P domains are indeed at that higher radius and the magnification is accurate. Secondly, the Fabs served to define the outer boundary of the protruding domains and proved that there are no problems with the contrast transfer function corrections that could yield weak density immediately above the shell domains.

Using the model of the modified NV VLP structure and the bound Fab, it is possible to map the epitope regions on the virus (Fig. 4). This is only an approximation of the epitope region since neither the virus nor antibody atomic structures have been determined. The bound antibody mainly contacts the protrusion formed by the A′ B′ and the E′ F′ loops. These loops are on the most extreme tips of the outer P2 domain. If the antibodies bound anywhere else on the top surface of the P2 domains, it seems unlikely that each copy of capsid protein would be bound with a Fab. Interestingly, it seems possible that an IgG could bind bivalently to the A B or C C dimers. However, as was the case with one of the antibodies to HRV14 (Che et al. 1998), this would take significant rearrangement of the Fab arms and therefore may make inter-particle cross-linking more likely. Nevertheless, there were no detectable differences between the MNV-1 and the MNV-1/Fab cryo-TEM structures.

Clearly, the Caliciviridae represent an important family of viruses that exhibit very interesting pathology. Over the past 15 years, there has been impressive progress made to understand all aspects of these viruses on a number of fronts. With all of the appropriate tools in place, it seems likely that this will accelerate in the years to come.

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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 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.

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	?

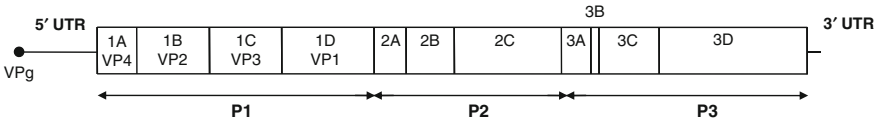

Fig. 1 Picornavirus genome and polyprotein organization. The *boxed area* represents the single open reading frame (ORF) with untranslated regions (UTR) at each end. Translation and proteo lytic processing produce primary products P1, P2, and P3. The P1 polyprotein is the capsid precursor and contains 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 environ ment 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)

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

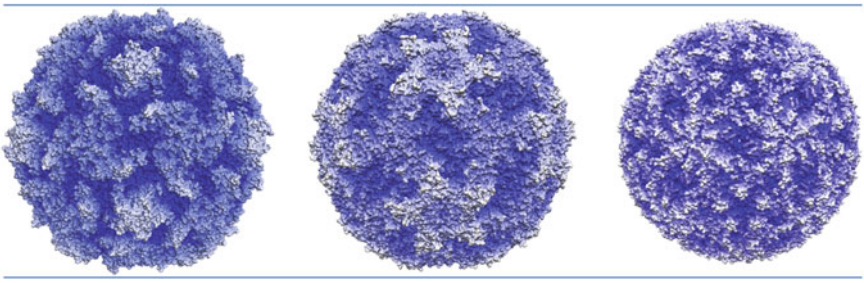

Fig. 2 Picornavirus capsid structures. Radial depth cued images of picornavirus particles with a color gradient from innermost (*dark blue*) to outermost (*white*) surfaces. From *left* to *right*: poliovirus (enterovirus), 32 nm in diameter with five pointed *star shape* at the fivefold axes, deep "canyon" 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

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 outbreaks 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 ($\sim$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 pathway-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 microglobulin 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 exposed to acidic pH as assessed 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 ($t_{1/2} \sim$ 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 ($\sim$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 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 icosahedrally 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.

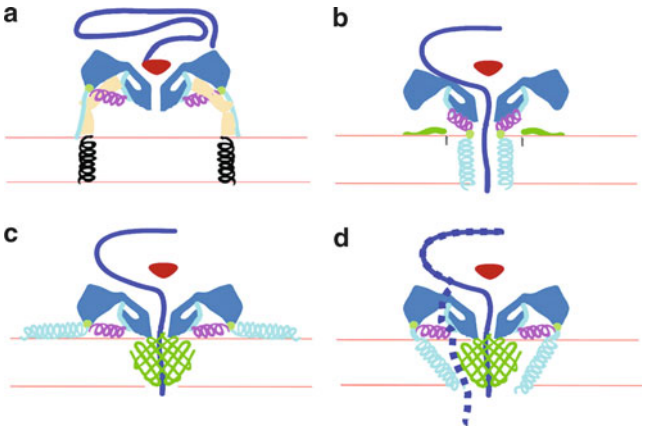

Fig. 3 Working models for poliovirus entry. A cross section of a portion of the capsid is shown in *dark blue*, VP4 is *green*, and the N terminus of VP1 is *cyan* and *magenta*. (**a**) Native poliovirus binds its receptor, Pvr (ectodomains 1 3, tan; transmembrane domain, *black helix*), and at physiological 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

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

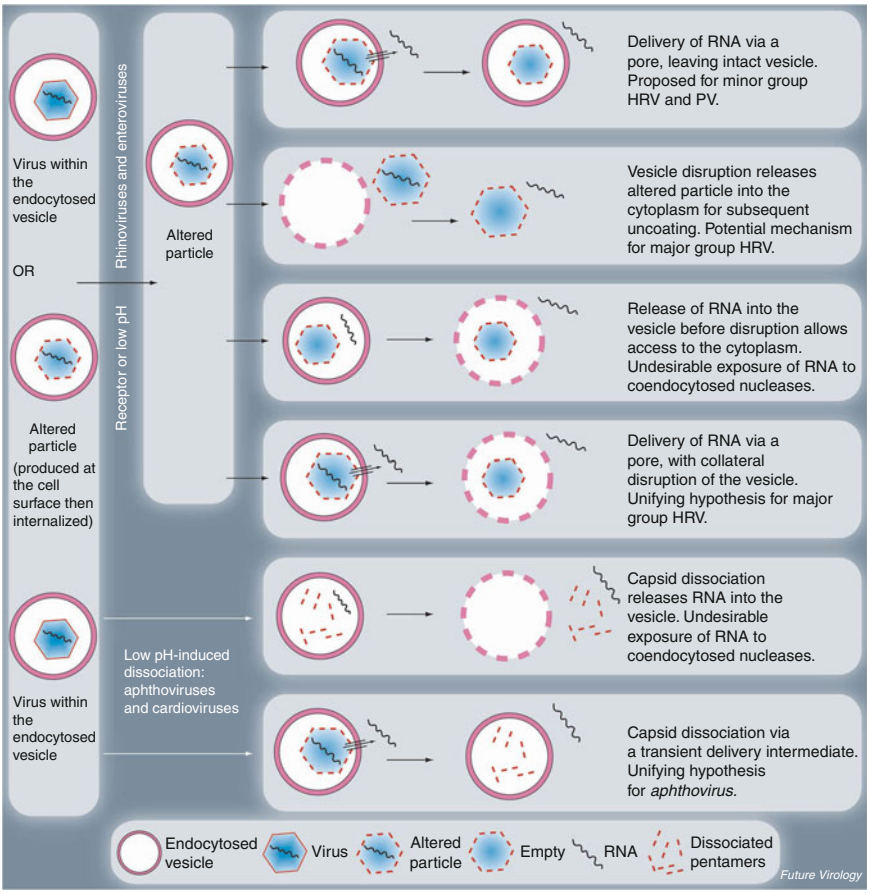

Fig. 4 Possible scenarios for picornavirus structural changes and genome delivery to the cytosol during endocytosis. Adapted from Tuthill et al. (2007), with permission from Future Medicine

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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From Touchdown to Transcription: The Reovirus Cell Entry Pathway

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Abstract Mammalian orthoreoviruses (reoviruses) are prototype members of the *Reoviridae* family of nonenveloped viruses. Reoviruses contain ten double-stranded RNA gene segments enclosed in two concentric protein shells, outer capsid and core. These viruses serve as a versatile experimental system for studies of virus cell entry, innate immunity, and organ-specific disease. Reoviruses engage cells by binding to cell-surface carbohydrates and the immunoglobulin superfamily member, junctional adhesion molecule-A (JAM-A). JAM-A is a homodimer formed by extensive contacts between its N-terminal immunoglobulin-like domains. Reovirus attachment protein σ1 disrupts the JAM-A dimer, engaging a single JAM-A molecule by virtually the same interface used for JAM-A homodimerization. Following attachment to JAM-A and carbohydrate, reovirus internalization is promoted by β1 integrins, most likely via clathrin-dependent endocytosis. In the endocytic compartment, reovirus outer-capsid protein σ3 is removed by cathepsin proteases, which exposes the viral membrane-penetration protein, μ1. Proteolytic processing and conformational rearrangements of μ1 mediate endosomal membrane rupture and delivery of transcriptionally active reovirus core particles into the host cell cytoplasm. These events also allow the ϕ cleavage fragment of μ1 to escape into the cytoplasm where it activates NF-κB and elicits apoptosis. This review will focus on mechanisms of reovirus cell entry and activation of innate immune response signaling pathways.

1 Introduction

The mammalian reoviruses are members of the *Reoviridae* family, which includes the important human pathogens rotavirus and Colorado-tick fever virus (Schiff et al. 2007). Like other *Reoviridae* members, reoviruses are nonenveloped, icosahedral particles that contain a segmented, double-stranded (ds) RNA genome surrounded by concentric protein shells (Schiff et al. 2007). These viruses are ubiquitous and display a broad host range, resulting in infection of wide variety of mammals including humans (Virgin et al. 1997). However, reovirus causes disease primarily in the very young (Mann et al. 2002; Tardieu et al. 1983; Tyler et al. 2004). These viruses have served in some respects as prototypes for the study of the *Reoviridae* due to the availability of isolates that display dissimilar phenotypes, the ability to perform genetic analysis using reassortant viruses and reverse genetics, and the existence of a murine model of virus-induced disease.

Initiation of reovirus infection requires deposition of the genome-containing inner capsid (known as the core) into the cytoplasm. Delivery of this rather large cargo (~70 nm in diameter) requires an exquisitely timed and regulated series of events both at the host cell surface and within host endosomes. The virus must attach to host cells, internalize and traffic to cellular endosomes, undergo proteolytic disassembly to expose the viral membrane-penetration apparatus, and penetrate host cell membranes for delivery of the viral core into the cytoplasm. These early events during reovirus infection activate innate immune signaling pathways. Here, we describe our current understanding of each of these steps in the cell entry pathway used by reovirus.

2 Structural Analysis of Reovirus Virions and Attachment Protein σ1

Reovirus particles are approximately 850 Å in diameter and consist of two concentric protein shells, the inner core and outer capsid (Dryden et al. 1993; Schiff et al. 2007) (Fig. 1). The reovirus genome consists of ten segments of dsRNA, which range in length from ~1.2 to ~3.9 kilobases. The genome segments are named based on size, with three large (L), three medium (M), and four small (S) segments. Reovirus proteins are designated according to the encoding gene segments, *lambda* (λ) for L, *mu* (μ) for M, and *sigma* (σ) for S. The reovirus inner core has $T = 1$ symmetry and is primarily formed by a shell of 60 asymmetric dimers of λ1 and 150 monomers of σ2 (Reinisch et al. 2000). Pentameric turrets of λ2, a capping enzyme and conduit for viral transcripts exiting the core, are located at the icosahedral vertices of the reovirus particle and span both the inner core and the outer capsid

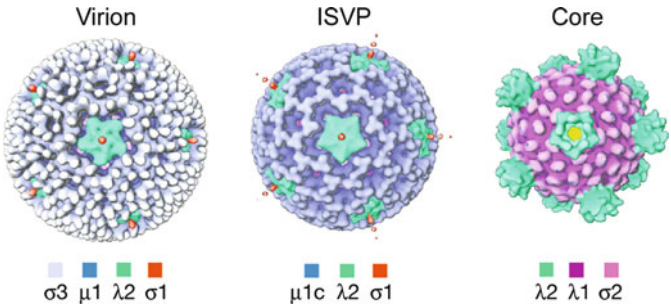

Fig. 1 Reovirus disassembly intermediates. Surface shaded representations of cryo EM image reconstructions of reovirus are shown, as viewed along a twofold axis of symmetry. Density corresponding to σ1 can be seen extending from turrets of λ2 at the icosahedral axes of virions and ISVPs. Cores lack σ1. Image adapted from Dryden et al. (1993)

(Bartlett et al. 1974; Cleveland et al. 1986; Dryden et al. 1993; Fausnaugh and Shatkin 1990; Furuichi et al. 1976; Gillies et al. 1971; Luongo et al. 1998, 2000; Mao and Joklik 1991; Reinisch et al. 2000). Minor core components include μ2 (~24 copies) and λ3 (12 copies) (Coombs 1998; Dryden et al. 1998). Each copy of viral RNA-dependent RNA polymerase λ3 is associated with three monomers of λ1 and occupies a single icosahedral vertex in the inner core (Drayna and Fields 1982; Starnes and Joklik 1993; Tao et al. 2002; Zhang et al. 2003). Surrounding the core is the outer capsid, which has quasi $T = 13$ (*laevo*) icosahedral symmetry (Metcalf 1982) and is composed of 200 heterohexamers of the membrane-penetration protein, μ1, and its protective cap, σ3 ($\mu1_3\sigma3_3$) (Dryden et al. 1993; Liemann et al. 2002; Metcalf 1982). Extending from a λ2 turret at each vertex of a reovirus particle is a trimer of σ1, the viral attachment protein, which is released during entry (Chappell et al. 2002; Dryden et al. 1993; Fraser et al. 1990; Furlong et al. 1988; Strong et al. 1991).

The reovirus σ1 protein mediates binding to cellular receptors (Barton et al. 2001b; Chappell et al. 2000) and influences target-cell selection in the infected host (Weiner et al. 1977, 1980). The 455 amino acids of strain T3D σ1 fold into a trimer approximately 480 Å long and 90 Å wide at its broadest point, with a globular C-terminal head, a central body, and a slender N-terminal tail (Chappell et al. 2002; Fraser et al. 1990; Guglielmi et al. 2006) (Fig. 2). Residues 310 455 comprise the head, which is constructed from two Greek-key motifs that assemble into an eight-stranded β-barrel (Chappell et al. 2002; Schelling et al. 2007). With the exception of the loop connecting β-strands D and E (D E loop), which contains a 3_{10} helix, loops connecting individual strands of the β-barrel are very short. N-terminal to the σ1 head, residues 246 309 form repeating units of two antiparallel β-strands

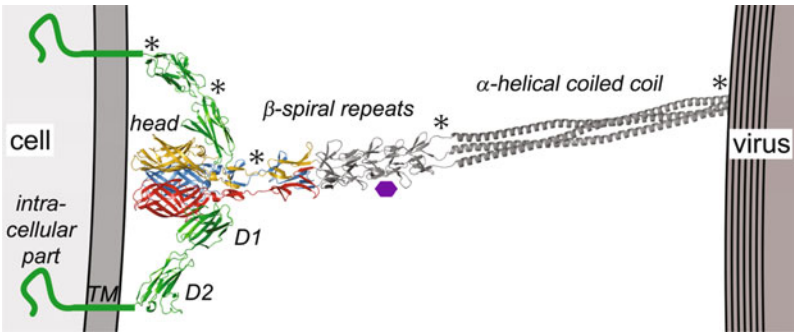

Fig. 2 Model of reovirus attachment to JAM A on the cell surface. A ribbon trace model of full length T3D σ1, extending from a schematic virion, with the known structure of the C terminus (Chappell et al. 2002) in *tricolor* and the prediction for the N terminus in *gray*. The predicted SA binding site (Chappell et al. 2000; Dermody et al. 1990) is marked with a *hexagon*. The extracellular domains D1 and D2 of JAM A (Prota et al. 2003) and schematic representations of the transmembrane (TM) and intracellular domains are shown in *green*. *Asterisks* indicate regions of flexibility. For clarity, only two JAM A monomers are shown bound to σ1. Figure and legend modified from Kirchner et al. (2008)

connected by short loops. Three such units assemble into a triple β-spiral, which is a motif observed to date only in viral fibers, including the adenovirus fiber (van Raaij et al. 1999), bacteriophage PRD1 P5 (Merckel et al. 2005), avian reovirus attachment protein σC (Guardado et al. 2005), and mammalian reovirus T3D σ1 (Chappell et al. 2002). In addition to the three β-spiral repeats observed in the crystallized σ1 fragment, sequence analysis suggests that the remainder of the T3D σ1 body (residues 167 249) contains an additional five N-terminal β-spiral repeats (Chappell et al. 2002; Guglielmi et al. 2006). Alternatively, these residues may form a combination of β-spiral repeats and α-helical coiled-coil, as suggested by sequence analysis (Chappell et al. 2002; Guglielmi et al. 2006; Nibert et al. 1990) and an observed narrowing in this region in a composite negative-stain electron micrograph (EM) (Fraser et al. 1990). The structure of the N-terminal tail, residues ∼1 160, of σ1 is unknown. However, a repeating heptad sequence motif is predictive of an amphipathic α-helix, which likely assembles into an α-helical coiled-coil in the trimer (Chappell et al. 2002; Guglielmi et al. 2006; Nibert et al. 1990). The extreme N-terminus of σ1 does not contain any obvious sequence motifs. It is hydrophobic and anchors the protein into the pentameric turret formed by λ2 in the reovirus virion (Dryden et al. 1993; Furlong et al. 1988). This symmetry mismatch suggests an interaction of limited strength, which may aid in σ1 release during viral disassembly (Stehle and Dermody 2003).

The σ1 molecule possesses discrete regions of flexibility along its length (Chappell et al. 2002; Fraser et al. 1990) (Fig. 2). One site of substantial flexibility in T3D σ1 is contributed by a four-residue insertion between the two most C-terminal β-spiral repeats (Cavalli et al. 2004; Chappell et al. 2002). Sequence alignments suggest that σ1 of reovirus prototype strains T1L and T2J each contain a six-residue insertion at the same position (Chappell et al. 2002). This insertion appears to correspond to a region of flexibility observed just below the σ1 head in EM images (Fraser et al. 1990). A second region of flexibility observed at the midpoint of σ1 may correspond to the transition between the predicted α-helical coiled-coil region of the tail and the β-spiral-containing body, and a final region of flexibility close to the N-terminus likely represents the virion insertion domain (Chappell et al. 2002; Fraser et al. 1990; Guglielmi et al. 2006; Nibert et al. 1990).

Reovirus σ1 undergoes significant conformational alterations during viral disassembly (Dryden et al. 1993; Furlong et al. 1988; Nibert et al. 1995). Some serotype 3 reoviruses, including T3D, can be cleaved by intestinal proteases (Nibert et al. 1995). Cleavage occurs in the σ1 body (Chappell et al. 1998), just N-terminal to the first β-spiral repeat in the crystal structure (Chappell et al. 2002). This proteolytic cleavage enhances viral hemagglutination capacity, suggesting an unmasking or conformational change in the sialic acid (SA)-binding region of the molecule (Nibert et al. 1995). This idea is supported by the observation of σ1 molecules with either single- or multilobed head regions (Fraser et al. 1990), which suggests that σ1 may exist in both "open" and "closed" conformations.

Although neither the precise mechanism nor the nature of σ1 conformational changes is understood, structural studies of σ1 provide clues about how these changes might occur. A cluster of six conserved aspartic acid residues on a rigid

β-hairpin at the base of the σ1 head, sandwiched between hydrophobic residues that block access to solvent, forms the main contact area between monomers in the trimer (Chappell et al. 2002; Schelling et al. 2007). Of the two aspartic acid residues contributed by each monomer, one (Asp346) is neutralized by a salt-bridge interaction with a nearby residue, while the other (Asp345) is not (Chappell et al. 2002; Schelling et al. 2007). The three Asp345 side chains closely appose each other at the center of the trimer in an otherwise hydrophobic environment. Since accumulation of negative charge in this region is predicted to destabilize the trimer (Cavalli et al. 2004), and a D345N mutation results in σ1 trimers with a structure indistinguishable from wild-type (Schelling et al. 2007), it is likely that Asp345 is protonated in the σ1 crystal structure (Chappell et al. 2002), representing the "closed" conformation of σ1. This conformation might form during crystallization at near-neutral pH and physiologically in conditions of low pH, similar to those encountered in the endocytic compartment during reovirus entry (Schelling et al. 2007). Thus, the aspartic acid sandwich motif may contribute to σ1 conformational rearrangements by acting as a molecular switch that mediates the oligomeric state of the σ1 head, depending on environmental pH (Schelling et al. 2007).

3 Reovirus Attachment Is Mediated by Cell-Surface Sialic Acid and Junctional Adhesion Molecule-A

Similar to viruses from a broad array of families that use carbohydrates as receptors (Olofsson and Bergstrom 2005), cell-surface SA serves as a receptor for several serotype 3 reovirus strains, including prototype strain T3D (Barton et al. 2001a; Chappell et al. 2000; Gentsch and Pacitti 1985, 1987; Paul et al. 1989). T3D exhibits a reduced capacity to agglutinate erythrocytes following treatment with neuraminidase, which removes terminal SA moieties (Gentsch and Pacitti 1987). Preincubation of either L cells with neuraminidase or virus with sialosides also significantly diminishes T3D binding (Gentsch and Pacitti 1985; Paul et al. 1989). SA residues linked in either α2,3 or α2,6 configurations effectively block serotype 3 reovirus binding to L cells (Paul et al. 1989). Reovirus T3D binds to sialoglycophorin, but not to asialoglycophorin, with an avidity of $\sim 5 \times 10^{9}$ M (Barton et al. 2001a), which is a property mediated by the σ1 protein (Chappell et al. 2000). Thus, SA functions as a serotype 3 reovirus receptor in cultured cells. In addition, SA binding also serves an important role in reovirus tropism and pathogenesis *in vivo* (Barton et al. 2003). An SA-binding strain of reovirus, but not a non-SA-binding strain, causes bile duct injury in newborn mice and exhibits 1,000-fold greater binding capacity for human cholangiocarcinoma cells, which are derived from bile duct epithelium.

Although the structure of σ1 in complex with SA is not yet available, studies using expressed proteins indicate that the region of T3D σ1 required for SA binding resides near the midpoint of the body, while a region just N-terminal to the head domain of T1L σ1 binds carbohydrate (Chappell et al. 2000). For both T1L and T3D, interactions with carbohydrate are mediated by a region of predicted β-spiral

(Chappell et al. 2002). The capacity to bind SA is essential for reovirus infection of murine erythroleukemia (MEL) cells (Chappell et al. 1997; Rubin et al. 1992). Adaptation of non-SA-binding reoviruses to growth in MEL cells results in amino acid substitutions at residues 198, 202, and 204 of σ1 that confer SA-binding capacity on the resultant viruses (Chappell et al. 1997). Molecular modeling of the σ1 body, based on available structure and sequence data, suggests that these residues are surface-exposed and proximal to one another in the predicted β-spiral region (Chappell et al. 2002). Thus, residues 198, 202, and 204 are likely to contribute to an SA-binding site in T3D σ1.

In addition to SA, reovirus also binds junctional adhesion molecule-A (JAM-A, also known as F11R/JAM/JAM1), a member of the immunoglobulin superfamily (Barton et al. 2001b; Martin-Padura et al. 1998; Williams et al. 1999). JAM-A was identified as a reovirus receptor using a genetic screen and subsequently shown to bind directly to the σ1 head domain with nanomolar affinity (Barton et al. 2001b; Schelling et al. 2007). Human and murine homologs of JAM-A, but not JAM family members JAM-B or JAM-C, serve as receptors for all reovirus serotypes and strains tested to date (Barton et al. 2001b; Campbell et al. 2005; Prota et al. 2003). The role of JAM-A as a reovirus receptor *in vivo* has been examined using JAM-A-null mice (Antar et al. 2009). Following peroral inoculation, JAM-A is dispensable for reovirus growth in the intestine. However, it is required for infection of vascular endothelial cells and promotes efficient hematogenous dissemination of reovirus to sites of secondary infection. Thus, JAM-A serves as a high-affinity reovirus receptor in cultured cells and *in vivo*.

Structural and biochemical studies highlight the regions and specific interactions that mediate reovirus engagement of JAM-A (Campbell et al. 2005; Chappell et al. 2002; Forrest et al. 2003; Guglielmi et al. 2007; Kirchner et al. 2008; Prota et al. 2003) (Fig. 3). The largest area of conserved residues in σ1 forms the D E and F G loops in

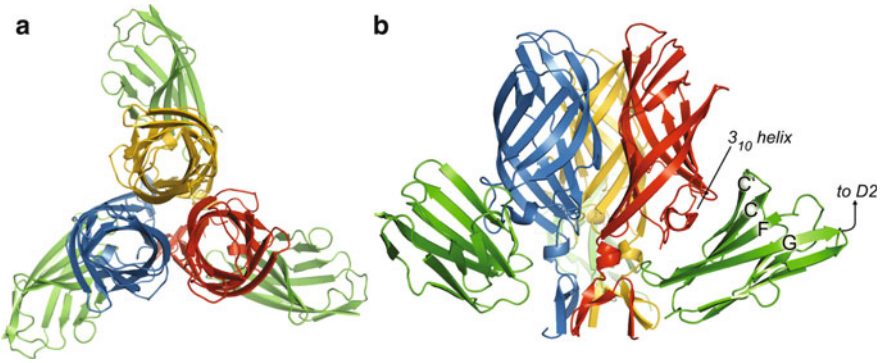

Fig. 3 Crystal structure of the σ1 JAM A complex. (**a** and **b**) Ribbon drawings of a complex formed between the trimeric σ1 head domain and monomeric JAM A D1, viewed along the threefold symmetry axis (**a**) and from the side (**b**). Monomers of the σ1 head are shown in *blue*, *red*, and *yellow*; JAM A D1 is shown in *green*. Secondary structure elements are labeled. Figure and legend modified from Kirchner et al. (2008)

the head domain (Campbell et al. 2005; Chappell et al. 2002). The crystal structure of the T3D σ1 head domain in complex with the JAM-A D1 domain reveals that residues in this region, centered at the D E loop and its 3_{10} helix, form the largest area of JAM-A contact (Kirchner et al. 2008). Interactions in this area are highly polar and involve residues Thr380, Gly381, and Asp382. A second, unpredicted area of JAM-A contact resides within the σ1 body, just N-terminal to the head domain. Interactions in this region are largely hydrophobic and involve β-spiral residues Tyr298 and Arg316, the α-helical turn that connects the β-spiral with the β-barrel, and Pro377.

The D1 domain of JAM-A is required for high-affinity binding to σ1 (Forrest et al. 2003; Guglielmi et al. 2007; Prota et al. 2003). Mutation of individual JAM-A D1 domain residues Arg59, Glu61, Lys63, Leu72, Tyr75, and Asn76, which lie in or adjacent to the dimer interface, diminishes or abolishes σ1 binding and reovirus infectivity (Guglielmi et al. 2007). Concordantly, the structure of the σ1-JAM-A complex shows that each σ1 trimer binds three independent JAM-A monomers (Kirchner et al. 2008). Contacts primarily involve the JAM-A dimer interface and a conserved region at the base of the σ1 head (Chappell et al. 2002; Kirchner et al. 2008) (Fig. 3). In addition, the structure of the σ1-JAM-A complex also identifies residues bound by σ1 that are found just outside the dimer interface of JAM-A (Kirchner et al. 2008). These residues may serve as initial contact points for σ1 and facilitate disruption of the JAM-A homodimer to allow interaction of σ1 with the JAM-A dimer interface. It is also possible that a cavity in the JAM-A dimer interface renders the homodimer intrinsically unstable, thereby promoting its disruption by σ1. Regardless of the mechanism, the σ1-JAM-A interaction is thermodynamically favored, as the K_D is approximately 1,000-fold lower than the K_D of the JAM-A homodimer interaction (Guglielmi et al. 2007; Kirchner et al. 2008).

Reovirus employs a multistep mechanism of viral attachment in which a low-affinity interaction with SA serves to tether the virion to target cells and precedes a high-affinity interaction with JAM-A (Barton et al. 2001a). This strategy for adhesion to host cells is used by members of unrelated virus families (Berger et al. 1999; Dragic et al. 1996; Montgomery et al. 1996; Ugolini et al. 1999). In some cases, such as with HIV, initial receptor engagement leads to conformational changes in the viral attachment protein that permit coreceptor engagement (Sattentau and Moore 1991). It is not known whether binding to SA induces structural changes in σ1, which affect its capacity to interact with JAM-A. However, it is clear that SA binding is not a necessary prerequisite for JAM-A binding, as non-SA-binding reoviruses are capable of binding JAM-A (Barton et al. 2001b).

4 Internalization of Reovirus Virions into the Endocytic Pathway Is Mediated by β1 Integrins

Following attachment to cell-surface carbohydrate and JAM-A, reovirus is internalized by receptor-mediated endocytosis (Borsa et al. 1979, 1981; Ehrlich et al. 2004; Maginnis et al. 2006, 2008; Sturzenbecker et al. 1987) (Fig. 4). Expression of

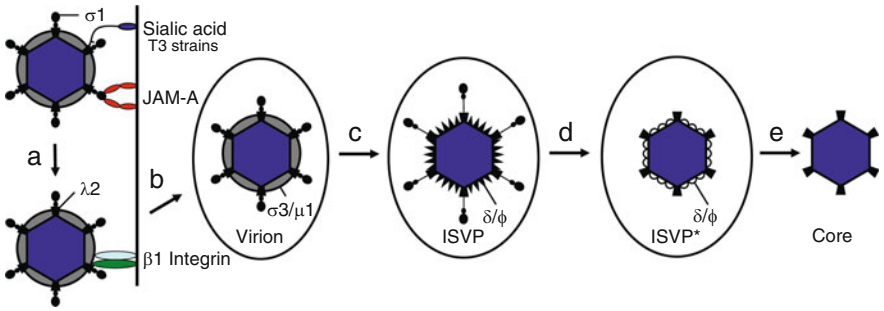

Fig. 4 The reovirus cell entry pathway. (**a**) Following attachment to cell surface carbohydrate (α linked sialic acid for serotype 3 reoviruses) and JAM A, reovirus virions enter cells by receptor mediated endocytosis. (**b**) Within the endocytic compartment, the viral outer capsid undergoes acid dependent proteolysis. (**c**) The first disassembly intermediate is the ISVP, which is character ized by loss of σ3 and cleavage of μ1C into particle associated fragments δ and ϕ. (**d**) The ISVP then undergoes further conformational changes to form the ISVP*. The ISVP* is characterized by conformational rearrangements of the μ1 fragments to expose hydrophobic residues, release of μ1N, and loss of attachment protein σ1. (**e**) The μ1 cleavage fragments mediate penetration through the endosomal membrane, releasing the transcriptionally active core into the cytoplasm

a JAM-A truncation mutant lacking a cytoplasmic tail allows reovirus to infect nonpermissive cells (Maginnis et al. 2006), suggesting that molecules other than JAM-A mobilize the internalization apparatus that promotes reovirus cell entry. Based on similarities in the structures of the reovirus and adenovirus attachment proteins and receptors (Stehle and Dermody 2004), it was hypothesized that reovirus and adenovirus employ similar integrin-dependent internalization mechanisms to enter cells. In keeping with this hypothesis, reovirus λ2 protein contains conserved integrin-binding motifs, RGD and KGE (Breun et al. 2001; Seliger et al. 1987). These sequences are displayed on surface-exposed loops of λ2 (Reinisch et al. 2000), where they could interact with integrins. Interestingly, the λ2-encoding L2 gene segment is genetically linked to viral shedding in infected mice and spread to littermates (Keroack and Fields 1986), suggesting a role for λ2 in reovirus-induced disease.

Treatment of cells with antibodies specific for β1 integrin reduces reovirus infection, while antibodies specific for the other integrin subunits expressed on permissive cells, including those specific for α integrin subunits, have no effect (Maginnis et al. 2006). However, antibodies specific for β1 integrin do not alter infection by *in-vitro* generated infectious subvirion particles (ISVPs) (Maginnis et al. 2006), which directly penetrate the plasma membrane and do not require endocytosis (Hooper and Fields 1996; Lucia-Jandris et al. 1993). These findings suggest that β1 integrin blockade inhibits endocytic uptake of virions. In comparison to β1 integrin-expressing cells, β1-null cells are substantially less susceptible to infection by reovirus virions, while infection by ISVPs is equivalent in both cell types (Maginnis et al. 2006). Diminished reovirus replication in β1-null cells

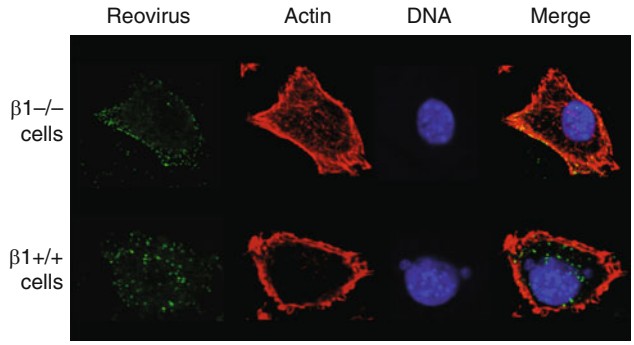

Fig. 5 β1 Integrin enhances reovirus entry into cells. GD25 ($\beta1^{-/-}$) and GD25β1A ($\beta1^{+/+}$) cells were chilled, adsorbed with strain T1L virions, and incubated at 4°C for 1 h. Nonadherent virus was removed, warm medium was added, and cells were incubated at 37°C for the times shown. Cells were fixed, stained for reovirus (*green*), actin (*red*), and DNA (*blue*), and imaged using confocal immunofluorescence microscopy. Representative digital fluorescence images of the same field are shown in each row. Figure and legend modified from Maginnis et al. (2006)

correlates with diminished viral uptake (Fig. 5), indicating that β1 integrin is required for efficient reovirus cell entry.

Most available evidence suggests that reovirus is internalized by a clathrin-dependent pathway (Borsa et al. 1979, 1981; Ehrlich et al. 2004; Maginnis et al. 2008; Sturzenbecker et al. 1987). Reovirus virions are observed to colocalize with clathrin in living cells (Ehrlich et al. 2004), and treatment of cells with chlorpromazine, a clathrin-specific chemical inhibitor, inhibits reovirus internalization and infection (Maginnis et al. 2008). However, both clathrin- and caveolin-dependent mechanisms can be employed by some viruses to enter host cells (Laniosz et al. 2008; Querbes et al. 2006). Although there are no published reports of clathrin-independent uptake strategies for reovirus, a role for caveolae in reovirus cell entry has not been conclusively excluded.

NPXY motifs in the β1 integrin cytoplasmic tail play a key role in sorting reovirus within the endocytic compartment. NPXY motifs are found in the cytoplasmic domains of many receptors (Chen et al. 1990; Davis et al. 1986; Oleinikov et al. 2000) and recruit adaptor protein 2 or disabled protein 2 (Morris and Cooper 2001; Oleinikov et al. 2000) to initiate clathrin assembly at the plasma membrane. Substitution of a tyrosine with a phenylalanine residue in either or both β1 integrin NPXY motifs (NPXF) results in inefficient internalization of reovirus virions and diminished infectivity (Maginnis et al. 2008). Infection of cells expressing NPXF β1 integrin results in distribution of virions to lysosomes where they are degraded, suggesting that the β1 integrin NPXY motifs target reovirus to the precise endocytic organelle that permits functional disassembly. Cellular signaling networks that respond to reovirus and facilitate its uptake and endocytic transport are unknown.

5 Removal of Outer-Capsid Protein σ3 by Cathepsin Proteases Initiates the Reovirus Disassembly Cascade

In cellular endosomes, reovirus virions undergo stepwise disassembly to form discrete intermediates, the first of which is the ISVP (Borsa et al. 1981; Chang and Zweerink 1971; Silverstein et al. 1972; Sturzenbecker et al. 1987) (Figs. 1 and 4). ISVPs are characterized by the loss of σ3, a conformational change in σ1, and cleavage of μ1 to form δ and ϕ. The rate-limiting step in reovirus disassembly is the proteolytic removal of σ3 (Baer and Dermody 1997; Sturzenbecker et al. 1987). Proteolysis of σ3 is dependent on acidic pH in some cell types (Dermody et al. 1993; Sturzenbecker et al. 1987) and endocytic cysteine proteases (Baer and Dermody 1997). Cathepsins B and L catalyze reovirus disassembly in fibroblasts (Ebert et al. 2002). Both enzymes are optimally active at acidic pH and serve functions in extracellular matrix formation, antigen presentation, and apoptosis (Chapman et al. 1997). These enzymes also mediate cell entry of several other viruses, including Ebola virus (Chandran et al. 2005), Hendra virus (Pager and Dutch 2005), and SARS coronavirus (Huang et al. 2006). Cathepsin S, a neutral pH cysteine protease required for processing internalized antigens (Riese et al. 1996), mediates uncoating of some reovirus strains in a macrophage cell line (Golden et al. 2004). It is possible that the broad tissue tropism displayed by reovirus is determined in part by the multiple host proteases capable of mediating its disassembly, analogous to highly pathogenic influenza viruses that disseminate systemically by utilization of alternative proteases for hemagglutinin processing (Goto and Kawaoka 1998; Stieneke-Grober et al. 1992).

Proteolytic enzymes also are required for reovirus infection following peroral inoculation of mice (Bass et al. 1990; Bodkin et al. 1989). Reovirus virions are converted to ISVPs in the intestinal lumen by the resident serine proteases chymotrypsin and trypsin. ISVPs produced in this fashion infect intestinal M cells to allow systemic dissemination of reovirus in the host (Amerongen et al. 1994). ISVPs generated by chymotrypsin or trypsin *in vitro* or in the gut lumen (Bass et al. 1990; Bodkin et al. 1989) are indistinguishable from ISVPs generated by cathepsin B or cathepsin L *in vitro* or in the endocytic compartment of cells (Baer et al. 1999; Ebert et al. 2002).

Sequences in σ3 that influence its susceptibility to proteolysis have been identified through studies of viruses selected during persistent infection (PI viruses) or mutant viruses selected for resistance to either cysteine protease inhibitor E64 (D-EA viruses) (Ebert et al. 2001) or ammonium chloride (ACA-D viruses) (Clark et al. 2006). These viruses exhibit accelerated kinetics of disassembly and harbor a Tyr→His mutation at amino acid 354 near the C-terminus of the protein (Clark et al. 2006; Ebert et al. 2001; Wetzel et al. 1997) (Fig. 6). Cryo-EM image analysis of a PI virus with an isolated Y354H mutation reveals a structural alteration in σ3 at a hinge region located between its two major domains (Wilson et al. 2002). These findings suggest that the C-terminus of σ3 regulates susceptibility of the protein to cleavage.

The σ3 C-terminus also dictates strain-specific differences in the susceptibility of σ3 to proteolytic attack (Jané-Valbuena et al. 1999, 2002). The σ3 protein of

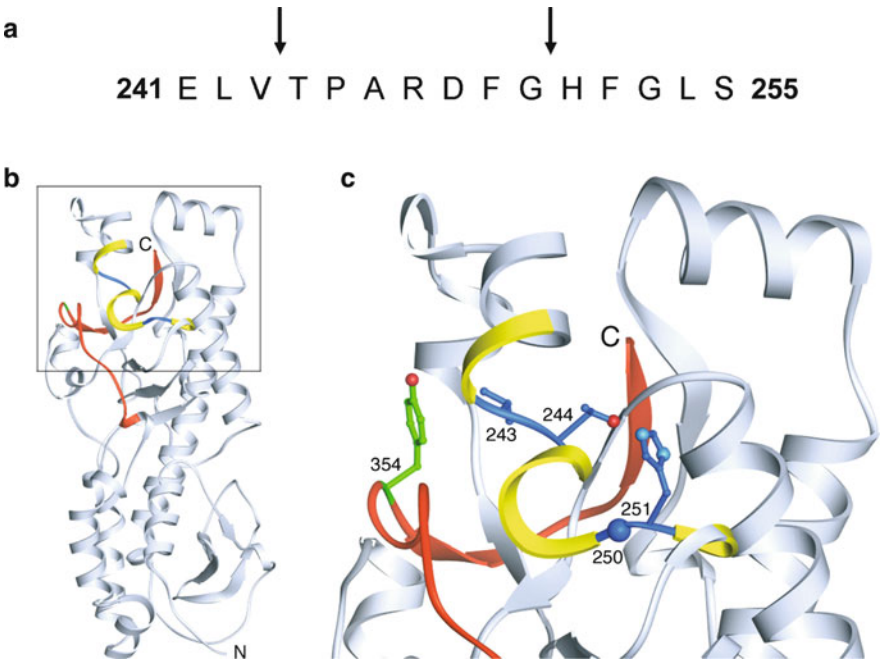

Fig. 6 The σ3 protein is a target for cathepsin proteolysis. (**a**) The primary amino acid sequence of σ3 from amino acids 241 to 255 is shown. *Arrows* highlight cathepsin L cleavage sites identified by N terminal sequencing of σ3 cleavage products following treatment of reovirus strain T1L with cathepsin L *in vitro*. (**b**) Cathepsin L cleavage sites are highlighted in the crystal structure of σ3. A ribbon diagram of the crystal structure of T3D σ3 (Olland et al. 2001) is displayed on the *left*. The cathepsin L cleavage sites in T1L are depicted in *blue* between amino acids 243 and 244 and between 250 and 251. Surrounding residues, from amino acids 241 to 253, are shown in *yellow*. The C terminal residues of σ3, from amino acids 340 to 365, are colored *red*. Amino acid 354, which is altered in PI, D EA, and ACA D viruses, is colored *green*. The virion distal end of σ3 is at the *top* of the figure, and the virion proximal end and N terminus are at the *bottom*. (**c**) An enlarged view of the boxed region of σ3 indicated in panel B is shown using the same color scheme. Amino acids 243, 244, 250, 251, and 354 are depicted in ball and stick representation. Figure and legend modified from Ebert et al. (2002)

strain T1L is cleaved more rapidly than that of T3D. Analysis of ISVPs recoated with chimeric σ3 proteins generated from T1L and T3D revealed that the C-terminus is primarily responsible for the rate of σ3 proteolysis. Moreover, sequence polymorphisms at residues 344, 347, and 353 in σ3 contribute to this effect (Jané-Valbuena et al. 2002).

Treatment of reovirus virions *in vitro* with either cathepsin B or cathepsin L leads to an initial cleavage of σ3 at a terminus (Ebert et al. 2002). Since sequence polymorphisms in the σ3 C-terminus determine susceptibility to proteolysis, the initial cleavage of σ3 probably occurs in this region. During proteolysis by cathepsin L, subsequent cleavages occur between residues 243 244 and 250 251 (Ebert et al.

2002) (Fig. 6a). These cleavage sites are physically located near the C-terminus in the σ3 crystal structure (Olland et al. 2001) (Fig. 6b, c). Because of this proximity, the small end fragment released following initial cathepsin L cleavage likely exposes the cleavage sites between residues 243 244 and 250 251, rendering them sensitive to proteolysis. The C-terminus therefore appears to control access to internal, proteolytically sensitive sites in σ3. Because reovirus disassembly in some cell types is acid-dependent (Dermody et al. 1993; Sturzenbecker et al. 1987), the C-terminus might be primed for movement at acidic pH. Mutations near the C-terminus, like Y354H, may alter the conformation of the protein to allow improved access to these cleavage sites and thus accelerate outer capsid disassembly (Wilson et al. 2002). High-resolution structural analysis of Y354H-σ3, which is currently ongoing, will enhance an understanding of σ3 proteolysis.

6 Penetration of Endosomal Membranes by Reovirus Is Mediated by Outer-Capsid Protein μ1

Studies to assess the capacity of reovirus entry intermediates to penetrate artificial lipid bilayers, model membranes of erythrocytes, or membranes of cells that support reovirus infection indicate that ISVPs but not virions or cores mediate membrane penetration (Borsa et al. 1979; Chandran and Nibert 1998; Chandran et al. 1999, 2001; Hooper and Fields 1996; Lucia-Jandris et al. 1993; Tosteson et al. 1993). Such studies led to the idea that ISVPs or a related subviral particle is the membrane-active intermediate in the reovirus entry pathway. Since ISVPs differ from cores by the presence of outer-capsid proteins σ1 and μ1 (Coombs 1998; Dryden et al. 1993), and because cores recoated *in vitro* with μ1 alone are capable of membrane penetration (Chandran et al. 1999), these findings point to a role for the μ1 protein in membrane penetration. This biochemical evidence is also supported by several genetic studies. Differences in membrane-penetration efficiency displayed by reovirus strains T1L and T3D segregate with the μ1-encoding M2 gene segment (Chandran et al. 2002; Lucia-Jandris et al. 1993). Additionally, viruses selected for resistance to denaturants such as ethanol contain mutations within the M2 gene segment and display alterations in membrane penetration capacity (Chandran et al. 2002; Danthi et al. 2008b; Hooper and Fields 1996; Wessner and Fields 1993). Together, these data demonstrate a function for the μ1 protein in membrane penetration.

The μ1 protein folds into four distinct domains (Fig. 7a). Domains I, II, and III are primarily α-helical and show no homology with other proteins. Domain IV forms a jelly-roll β-barrel commonly found in the capsid proteins of many nonenveloped viruses (Harrison 2001). This domain interacts extensively with similar domains of the neighboring μ1 molecules and with σ3. The μ1 protein also contains three proteolytic cleavage sites (Fig. 7b). These include an autocatalytic cleavage site at amino acid 42, which separates μ1N and μ1C, a cleavage site at

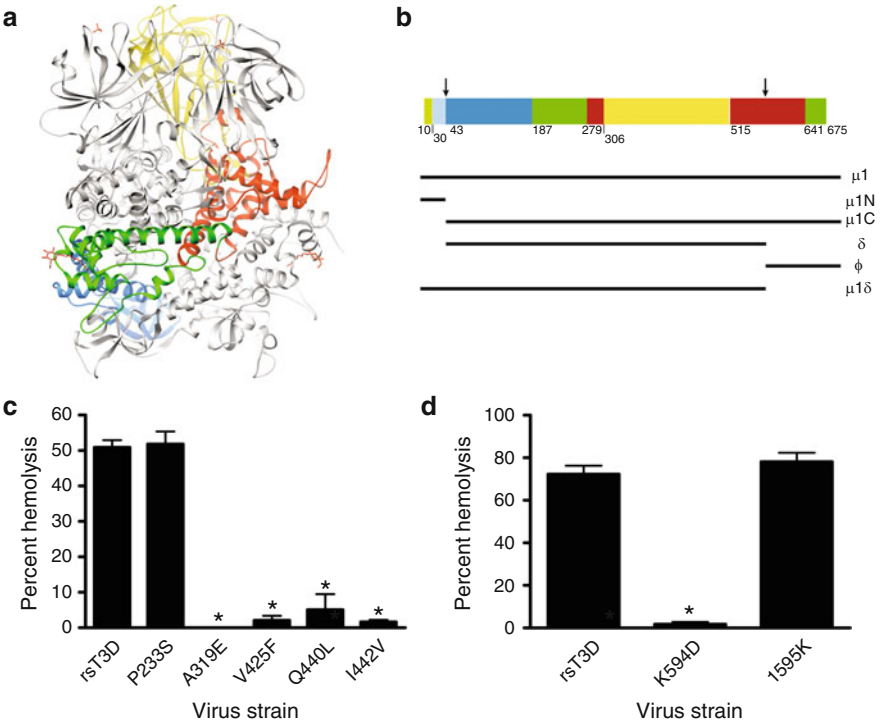

Fig. 7 The μ1 protein mediates membrane penetration. (**a**) Ribbon diagram of the crystal structure of the T1L μ1 trimer without bound σ3. One μ1 subunit is colored by domain (domain I, *light* and *dark blue* [μ1N, μ1C]; domain II, *light* and *dark green* [μ1N, μ1C]; domain III, *red*; domain IV, *yellow*); the other two μ1 subunits are shown in *gray*. The β octyl glucosides and sulfate ions present in the structure are shown in *red* and *yellow*. (**b**) Domain segmentation of the amino acid sequence as determined from the three dimensional structure. The domain color code is as depicted in (a). Cleavage sites are indicated by *arrows*. Figure modified from Liemann et al. (2002). (**c** and **d**) A 3% v/v solution of bovine erythrocytes was incubated with 5.4×10^{10} ISVPs of wild type rsT3D or the indicated μ1 δ (c) or μ1 ϕ (d) mutant at 37°C for 1 h. Hemolysis was quantified by determining absorbance of the supernatant at 415 nm. Hemolysis following treat ment of an equal number of cells with virion storage buffer or virion storage buffer containing 1% TX 100 was considered to be 0 or 100%, respectively. Results are expressed as mean percent hemolysis for triplicate samples. *Error bars* indicate SD. *, $P < 0.05$ as determined by Student's *t* test in comparison to rsT3D. Figure modified from Danthi et al. (2008a, b)

approximately amino acid 580, which releases the δ and ϕ fragments, and a cleavage site at the C-terminus that releases an ~10 amino acid peptide (Chandran et al. 2003; Mendez et al. 2003; Nibert and Fields 1992; Odegard et al. 2004). While the physiologic roles of both the δ ϕ and the C-terminal cleavages are unclear, studies using reovirus cores recoated with a μ1N μ1C cleavage-resistant μ1 mutant indicate that cleavage of μ1 to generate μ1N and μ1C is required for membrane penetration and virion infectivity (Odegard et al. 2004). Since μ1N is released from

viral particles, it is postulated that cleavage of μ1 is required for release of μ1N, which then interacts with membranes as a function of its myristate moiety to effect membrane penetration (Ivanovic et al. 2008). The requirement for the release of a small hydrophobic peptide for membrane penetration is strikingly similar to the entry mechanisms employed by other nonenveloped viruses such as adenoviruses (Wiethoff et al. 2005), nodaviruses (Schneemann et al. 1992; Walukiewicz et al. 2008), and picornaviruses (Danthi et al. 2003).

In the native μ1 structure present in virions and ISVPs, the myristoylated μ1N fragment is buried inside a hydrophobic cavity in the α-helical pedestal formed by portions of domains I, II, and III (Liemann et al. 2002; Zhang et al. 2005). Based on these studies, massive conformational rearrangements resulting in unwinding of the μ1 trimer must be required to release μ1N during cell entry (Liemann et al. 2002; Zhang et al. 2006). Evidence for conformational changes in particle-associated μ1 following interaction of ISVPs with membranes or when exposed to high salt concentrations has led to the identification of an ISVP-like entry intermediate in the reovirus cell entry pathway (Chandran et al. 2002). This intermediate, referred to as ISVP*, is characterized by changes in the conformation of the μ1 δ fragment, loss of the σ1 protein, and an increase in the overall hydrophobicity of the particle (Chandran et al. 2002). Thus, the μ1 protein associated with ISVPs is in a metastable state primed to undergo conformational changes to assume a more hydrophobic structure capable of interaction with membranes. While it is not understood how these conformational changes in μ1 are triggered, it is thought that interaction of an anion-binding site in domain IV with phospholipid head groups in endosomal membranes might trigger the requisite rearrangements in μ1 that reveal the myristoylated μ1N and the internal hydrophobic residues (Liemann et al. 2002). At high particle concentrations, ISVP* conversion is regulated by a positive feedback mechanism in which μ1N, which is released during ISVP* formation, promotes ISVP-to-ISVP* conversion of the remaining particles (Agosto et al. 2008). Acceleration of ISVP* formation by μ1N is dependent on temperature and target flexibility, suggesting that particle dynamics are required to expose a μ1N interaction domain (Agosto et al. 2008). Since such particle concentrations are unlikely to be achieved following a low multiplicity viral infection, it remains unclear how these findings translate to ISVP* formation in cellular endosomes during viral entry.

Genetic studies using ethanol-resistant or thermostable mutants indicate that μ1 residues affecting the overall stability of the virus also regulate membrane-penetration efficiency (Chandran et al. 2002; Danthi et al. 2008b; Hooper and Fields 1996; Wessner and Fields 1993). These and other stability-altering residues identified in thermostable reovirus mutants (Middleton et al. 2007) are located between residues 383 and 612 of μ1 and map to either domain IV that forms the jelly-roll β-barrel or the α-helical portions of domain III that lie just below the β-barrel structure. Since these μ1 domains participate in interactions between neighboring μ1 monomers, these residues are thought to modulate viral stability by preventing unwinding of the μ1 trimer (Liemann et al. 2002). Consistent with an increase in μ1 protein rigidity in ethanol-resistant and thermostable mutants, viral cores recoated with mutant μ1 proteins, or recombinant reoviruses containing single amino acid

substitutions in μ1 in an otherwise wild-type background, display diminished ISVP-to-ISVP* conversion and have defects in membrane penetration (Wessner and Fields 1993; Hooper and Fields 1996; Chandran et al. 2002; Middleton et al. 2007; Danthi et al. 2008a, b) (Fig. 7c, d). These studies suggest that a central region of μ1 involved in intermolecular interactions is an important regulator of the ISVP-to-ISVP* transition. In addition to these residues, changes in the C-terminal ϕ fragment also control viral stability (Middleton et al. 2007) and affect membrane penetration by reducing the efficiency of ISVP-to-ISVP* conversion (Danthi et al. 2008a). While it is not clear how ϕ residues modulate these properties, since both μ1N and ϕ are released from the virus particle during ISVP* formation (Ivanovic et al. 2008), it is likely that conformational rearrangements in μ1 during ISVP* formation are not restricted to the δ domain but also involve the μ1N and ϕ domains. Therefore, amino acid substitutions within ϕ that negatively affect its conformational flexibility would likely prevent the μ1 reorganization required for ISVP* formation. Biochemical and structural characterization of additional mutant viruses that may be affected to varying degrees in the capacity to undergo μ1 conformational changes may identify as yet unknown intermediates during ISVP-to-ISVP* conversion and offer insights into mechanisms that promote the elaborate remodeling of μ1 required for membrane penetration.

Analogous to the picornaviruses (Danthi et al. 2003), reovirus forms small, size-selective pores in erythrocyte model membranes (Agosto et al. 2006). Both μ1N and ISVP*s associate with erythrocyte membranes (Agosto et al. 2006; Ivanovic et al. 2008), but μ1N is capable of pore formation in the absence of other viral components (Ivanovic et al. 2008). While ϕ also associates with membranes (Ivanovic et al. 2008), its recruitment does not result in membrane penetration. These findings are consistent with the observation that viruses incapable of δ ϕ cleavage can penetrate membranes and are fully infectious (Chandran and Nibert 1998; Chandran et al. 1999). Since pore formation by μ1N is enhanced by the presence of ϕ, it is possible that ϕ functions as a μ1N chaperone and facilitates membrane penetration by reovirus (Ivanovic et al. 2008). Pores formed by released μ1N fragments are considerably smaller than those required to allow the viral intermediate to traverse the membrane (Agosto et al. 2006). Therefore, it is not clear how pore formation in model membranes relates to membrane penetration during cell entry. Analogous to erythrocyte membrane rupture, pore formation may result in osmotic lysis of endosomes in which viral particles are present. Alternatively, the initial small pore formed by the virus might recruit cellular factors that produce larger pores or channels through which the viral intermediate can translocate.

Both the viral core and the δ fragment of μ1 are found in the cytoplasm following reovirus entry into host cells (Chandran et al. 2003). While δ is found distributed diffusely throughout the cytosol, viral cores display a more punctuate cytoplasmic localization (Chandran et al. 2003). These observations suggest that the δ fragment disassociates from the ISVP* either during or immediately after membrane penetration. This idea is supported by the evidence that reovirus cores are transcriptionally active in the cytoplasm and that activation of transcription requires complete removal of the μ1 fragments. Removal of δ from cores is thought to be

accomplished by direct interaction of δ with the host chaperone Hsc70 via an ATP-dependent process (Ivanovic et al. 2007). Based on the finding that chaperones can translocate proteins across membranes (Young et al. 2004), it is possible that concomitant with removal of particle-associated δ, Hsc70 also aids in transport of the viral core across membranes (Ivanovic et al. 2007). Additional experiments are required to reveal the precise mechanism by which host membranes are breached by reovirus.

7 Reovirus Entry Evokes Innate Immune Responses that Trigger Cell Death

Reovirus infection elicits apoptosis of cultured cells and *in vivo*. Apoptosis induction by reovirus requires activation of innate immune transcription factors NF-κB and IRF-3 (Connolly et al. 2000; Hansberger et al. 2007; Holm et al. 2007) (Fig. 8). In cultured cells, reovirus-induced apoptosis does not require de novo synthesis of viral RNA and protein (Connolly and Dermody 2002; Danthi et al. 2006), indicating that the proapoptotic stimulus is contained within infecting viral capsids. Consistent with these findings, strain-specific differences in the capacity of reovirus to induce apoptosis segregate genetically with the viral S1 and M2 gene segments (Connolly et al. 2001; Tyler et al. 1995, 1996), which encode σ1 and μ1, respectively (McCrae and Joklik 1978; Mustoe et al. 1978). Antibody-dependent uptake of reovirus virions in an entry process that does not require JAM-A and SA leads to apoptotic cell death, indicating that signaling pathways triggered by σ1-receptor interactions are dispensable for reovirus-induced apoptosis (Danthi et al. 2006). Regardless of the receptors used to mediate attachment, initiation of prodeath signaling following reovirus infection requires viral disassembly in cellular endosomes (Danthi et al. 2006), suggesting an essential function for the μ1 protein in apoptosis induction.

Introduction of single amino acid substitutions into the δ region of μ1 decreases the capacity of the resultant mutant viruses to effect membrane penetration, mobilize NF-κB, and evoke apoptosis (Danthi et al. 2008b) (Fig. 8c). These findings suggest that the membrane-penetration and apoptosis induction-functions of μ1 are linked and that the δ region of μ1 is an essential modulator of both processes (Danthi et al. 2008b). It is possible that membrane penetration directly initiates proapoptotic signals. Alternatively, membrane penetration might allow delivery of the μ1 cleavage fragments into the cytoplasm where prodeath signaling is elicited. Two lines of evidence support the latter possibility. First, plasmid-driven expression of the μ1 ϕ domain in the cytoplasm is sufficient to induce apoptosis (Coffey et al. 2006). Second, recombinant viruses with engineered substitutions within ϕ are diminished in NF-κB activation and apoptosis (Danthi et al. 2008a) (Fig. 8d). Importantly, a membrane-penetration-proficient ϕ mutant is impaired in the capacity to activate prodeath signaling, indicating that ϕ modulates apoptosis independent of an effect on membrane penetration (Danthi et al. 2008a). Based on these findings, it appears likely

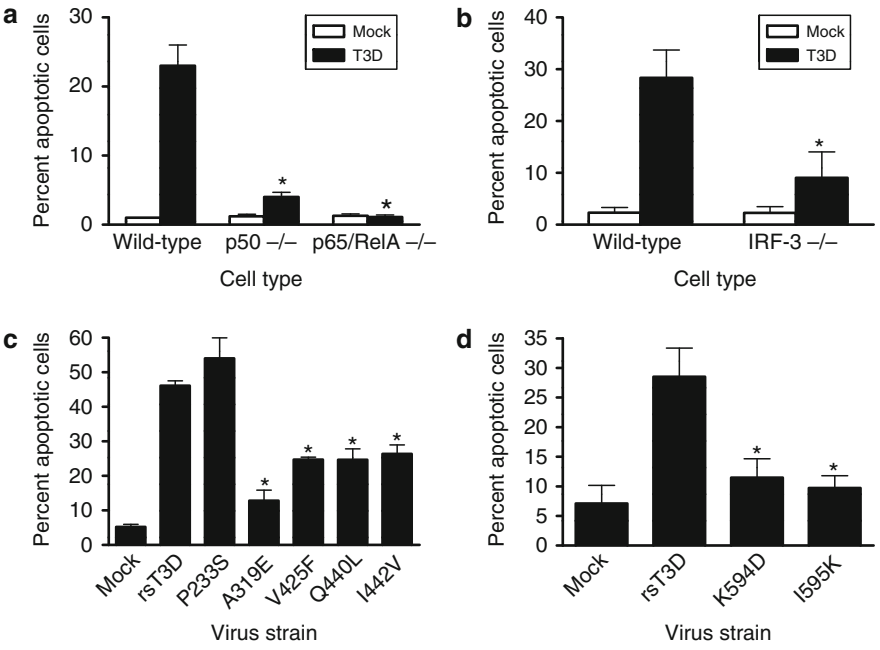

Fig. 8 Reovirus entry triggers apoptosis dependent on NF κB and IRF 3. (**a** and **b**) Wild type cells or cells lacking NF κB p50, NF κB p65/RelA, or IRF 3 were either mock infected or infected with T3D at an MOI of 100 PFU/cell. After incubation at 37°C for 48 h, cells were stained with acridine orange. The results are expressed as the mean percentage of cells undergoing apoptosis for three independent experiments. *Error bars* indicate SD. *, $P < 0.05$ as determined by Student's *t* test in comparison to T3D infected wild type cells. Figure modified from Connolly et al. (2000) and Holm et al. (2007). (**c** and **d**) HeLa cells were infected with rsT3D or each μ1 δ (c) or μ1 ϕ (d) mutant at an MOI of 100 PFU/cell. Following 48 h incubation, the percentage of apoptotic cells was determined by staining with acridine orange. Results are expressed as the mean percentage of apoptotic cells for triplicate samples. *Error bars* indicate SD. *, $P < 0.05$ as determined by Student's *t* test in comparison to rsT3D. Figure modified from Danthi et al. (2008a, b)

that cytoplasmic delivery of ϕ subsequent to membrane penetration initiates prodeath signaling following reovirus infection (Danthi et al. 2008a).

IRF-3 activation following reovirus infection requires the RIG-I pathogen sensor and the IPS-1 adaptor protein (Holm et al. 2007). Interestingly, unlike other viral systems, these host proteins are dispensable for reovirus-induced NF-κB activation (Holm et al. 2007). Since activation of IRF-3 also does not require viral RNA synthesis and occurs during viral entry, it is thought that viral genomic dsRNA triggers these signaling pathways (Holm et al. 2007). Empty reovirus particles devoid of genome can stimulate NF-κB but not IRF-3, providing additional support for the idea that NF-κB and IRF-3 are activated following reovirus infection via distinct mechanisms (Connolly et al. 2000; Holm et al. 2007). Since reovirus empty particles are capable of eliciting apoptosis (Connolly and Dermody 2002) but do not lead to IRF-3 activation (Holm et al. 2007), IRF-3 appears to play a contributory but

nonessential role in reovirus-induced apoptosis. Precise mechanisms by which the products of reovirus disassembly activate innate immune response signaling networks are unknown.

8 Conclusions and Future Directions

The process of cell entry is poorly understood for many pathogenic viruses. This gap in knowledge has been a significant impediment to the rational design of antiviral agents and vaccines that target distinct steps in the entry process. Studies of mammalian reovirus have uncovered discrete attachment and internalization receptors, a function for cathepsin proteases in disassembly, an intricate mechanism for protein-membrane interactions, and a framework for activation of innate immune response signaling. Many of these functions are shared by other viral pathogens, suggesting conserved mechanisms of cell entry that should be amenable to common therapeutic approaches. However, there is much more to learn.

The current model of σ1-JAM-A interactions at the cell surface suggests that structural characteristics of σ1 may facilitate concurrent engagement of JAM-A and carbohydrates by appropriately positioning the receptor-binding domains. Indeed, studies of adenovirus fiber, which is structurally homologous to σ1 (Stehle and Dermody 2003), have highlighted the importance of length and flexibility in viral tropism (Wu et al. 2003). However, the contributions of σ1 length and flexibility to reovirus receptor engagement have not been explored. In the structure of a σ1-JAM-A complex, the σ1 head forms a trimer. Yet, there is evidence to suggest that σ1 may at times exist in a partially detrimerized conformation (Fraser et al. 1990; Schelling et al. 2007). An improved understanding of the role of the unusual aspartic acid sandwich trimerization motif may lend insight into the contributions of trimer instability to reovirus attachment and entry.

Reovirus serotypes display striking differences in pathogenesis that are directly linked to σ1 (Tyler et al. 1986; Weiner et al. 1977, 1980). However, each of the reovirus serotypes uses JAM-A as a receptor (Campbell et al. 2005). Considering previous studies of reovirus interactions with JAM-A and carbohydrates, we think there are three possible explanations of σ1-mediated serotype-specific differences in reovirus tropism and disease. First, the carbohydrate specificity of a particular strain of reovirus might direct infection to specific cells or tissues. In support of this idea, serotype 3 reovirus strains that vary in SA utilization also vary in disease pathogenesis in the hepatobiliary system (Barton et al. 2003). While there is some evidence that T1L binds SA in intestinal loops (Helander et al. 2003), the exact nature of the carbohydrate coreceptors used by reovirus serotypes 1 and 2 remains undefined. Second, variations in the interaction kinetics or affinity of a particular σ1 serotype for JAM-A might contribute to differences in tropism. While there is overlap among JAM-A residues required for reovirus T1L, T2J, and T3D binding, there is evidence that the binding sites in JAM-A for these viruses differ (Guglielmi et al. 2007). Furthermore, sequence

alignments reveal that the residues in T3D σ1 that contact JAM-A in the complex (Kirchner et al. 2008) are not entirely conserved among reoviruses of all serotype (Campbell et al. 2005; Chappell et al. 2002). On a cell that expresses only low levels of JAM-A, differences in receptor interaction kinetics or affinity might determine whether or not reovirus can initiate infection. Third, JAM-A might serve as a serotype-independent reovirus receptor at some sites within the host, and other, as yet unidentified, receptors confer serotype-dependent tropism in the central nervous system. Indeed, studies using non-SA-binding reovirus to infect JAM-A-null mice provide support for this hypothesis and point specifically to the existence of unidentified receptors in both the intestine and the central nervous system (Antar et al. 2009). Future exploration of each of these possibilities will help clarify the role of σ1 in reovirus pathogenesis.

Internalization of reovirus requires β1 integrin (Maginnis et al. 2006), but it is not known whether reovirus directly engages β1 integrin to initiate internalization or induces interactions between JAM-A (or other receptors) and β1 integrin to activate the uptake machinery. Furthermore, it is unclear whether activation of signaling pathways is required to trigger reovirus internalization by β1 integrin. Studies using mutant β1 integrin constructs suggest that NPXY motifs within β1 integrin direct transport of reovirus to the subcellular compartment for disassembly and membrane penetration (Maginnis et al. 2008). However, the composition of the endocytic machinery recruited by the NPXY motifs that directs reovirus to the appropriate endocytic organelle for disassembly is yet to be identified. Since early steps in reovirus replication influence several stages of reovirus host interactions (Virgin et al. 1997), it is possible that engagement of β1 integrin influences reovirus pathogenesis. However, a function for β1 integrin in reovirus disease is unproven.

Disassembly of reovirus, which results in proteolytic removal of the σ3 outercapsid protein, is essential for exposure of the viral membrane-penetration apparatus. While this process must be precisely controlled to ensure efficient infection, mechanisms that underlie this regulation are not understood. It is not known whether the low pH environment of the endocytic compartment is merely required for optimal activity of endocytic proteases that catalyze reovirus disassembly or also functions to trigger the conformational changes in σ3 that lead to its degradation. The σ3 protein contains multiple cathepsin protease cleavage sites that may be sequentially employed to facilitate its timely removal (Ebert et al. 2002). However, it is not known whether a temporal pattern of cleavage site utilization exists for σ3. Cathepsins B and L are expressed in the intestine, liver, heart, and brain (Turk et al. 2001), which serve as sites for reovirus infection in newborn mice (Barton et al. 2003; O'Donnell et al. 2005). Cathepsin S is largely restricted to cells and tissues of the immune system (Chapman et al. 1997), which may influence reovirus replication in Peyer's patches during enteric infection (Fleeton et al. 2004; Morrison et al. 1991). Definition of cathepsin function in reovirus pathogenesis awaits the results of ongoing studies of reovirus infection using cathepsin-deficient mice.

Recent studies have provided important insights into mechanisms by which reovirus mediates membrane penetration. While a few residues within μ1 δ and ϕ that regulate conformational changes required for membrane penetration have been identified by analysis of mutant viruses (Chandran et al. 2002; Danthi et al. 2008a, b), it is not known how the conformational alterations of the membrane-penetration apparatus liberate μ1N, which mediates membrane penetration (Ivanovic et al. 2008). Additionally, the domains of μ1 reorganized during disassembly are only partially identified. The mechanism by which interaction of μ1N with the membrane results in pore formation and how its pore-forming capacity is enhanced by its interaction with ϕ also remain to be elucidated (Agosto et al. 2006; Ivanovic et al. 2008). Finally, it is not apparent how formation of small pores in membranes results in translocation of the reovirus core across the membrane.

Early steps in reovirus infection activate innate immune response transcription factors NF-κB and IRF-3 (Connolly et al. 2000; Holm et al. 2007), which drive the apoptotic response following reovirus infection (Connolly et al. 2000; Hansberger et al. 2007; Holm et al. 2007; O'Donnell et al. 2006). The activation of NF-κB is modulated by the μ1 ϕ domain subsequent to membrane penetration (Danthi et al. 2008a). However, the precise mechanism by which μ1 ϕ evokes NF-κB activation is unclear. Neither the fate of the ϕ fragment following entry of reovirus into host cells nor the cellular sensors that detect ϕ to trigger the prodeath function of NF-κB is known. Activation of IRF-3 following reovirus infection is dependent on the recognition of viral genomic dsRNA by RIG-I and IPS-1 (Holm et al. 2007), but how the genomic dsRNA escapes from the viral core for detection by RIG-I is not evident.

In addition to enhancing an understanding of fundamental aspects of entry mechanisms employed by nonenveloped viruses, studies of reovirus cell entry are also pertinent to the development of optimal reovirus-based oncolytic and vaccine vectors. Reovirus infects transformed cells much more efficiently than it does nontransformed cells (Duncan et al. 1978). Based on initial success in using reovirus for tumor killing in animal models (Coffey et al. 1998; Hirasawa et al. 2002), reovirus is currently undergoing clinical trials as a virotherapeutic for aggressive and refractory human tumors (Stoeckel and Hay 2006). Since reovirus undergoes primary replication in intestinal tissue with few or no symptoms in humans (Tai et al. 2005) and is now amenable to genetic modification (Kobayashi et al. 2007), it also is an excellent candidate for development of a multifunctional vaccine modality to elicit mucosal immunity. Therefore, understanding the precise mechanisms by which reovirus attaches to host cells and initiates an infectious cycle will allow reovirus to be strategically engineered to facilitate retargeting to distinct host cells or enhance the efficiency of cell entry for a variety of therapeutic applications.

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Rotavirus Cell Entry

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Abstract Infecting nearly every child by age five, rotaviruses are the major causative agents of severe gastroenteritis in young children. While much is known about the structure of these nonenveloped viruses and their components, the exact mechanism of viral cell entry is still poorly understood. A consensus opinion that appears to be emerging from recent studies is that rotavirus cell entry involves a series of complex and coordinated events following proteolytic priming of the virus. Rotaviruses attach to the cell through sialic acid containing receptors, with integrins and Hsc70 acting as postattachment receptors, all localized on lipid rafts. Unlike other endocytotic mechanisms, this internalization pathway appears to be independent of clathrin or caveola. Equally complex and coordinated is the fascinating structural gymnastics of the VP4 spikes that are implicated in facilitating optimal interface between viral and host components. While these studies only begin to capture the basic cellular, molecular, and structural mechanisms of cell entry, the unusual features they have uncovered and many intriguing questions they have raised undoubtedly will prompt further investigations.

1 Introduction

Rotaviruses are complex, relatively large (~1,000 Å), nonenveloped icosahedral viruses, which constitute a separate genus in the family Reoviridae (Estes and Kapikian 2006). This family, comprised of twelve genera, exhibits a diverse host range from plants to animals, including humans (Mertens et al. 2005). One of the distinguishing characteristics of these viruses is that their genomes consist of multiple segments of double-strand RNA (dsRNA) often enclosed by multiple capsid layers. The number of dsRNA segments, typically 10 12, varies among viruses in different genera. In these viruses, generally, each dsRNA segment encodes a single protein. Because host cells do not have the capability to transcribe dsRNA, these viruses have evolved to carry the enzymatic machinery to transcribe the dsRNA segments into capped mRNA molecules within the intact capsid (Hill et al. 1999; Lawton et al. 2000; Pesavento et al. 2006). Thus, in addition to facilitating the cell entry processes, which may vary in details from one genus to the other depending upon the host, the capsid architecture in these viruses is designed to carryout endogenous transcription of dsRNA segments and extrusion of the nascent transcripts.

Rotaviruses are the major causative agent of severe gastroenteritis in young children and animals (Estes and Kapikian 2006). These viruses exhibit enormous genetic and strain diversity. In addition to point mutations and gene rearrangements, genetic reassortment, similar to influenza viruses, between cocirculating strains contribute to the expanding diversity of rotaviruses (Cunliffe et al. 2002; Matthijnssens et al. 2008; Ramig 1997; Taniguchi and Urasawa 1995). Based on antigenic specificity and seroepidemiological studies, rotaviruses are broadly classified into seven groups (A G) (Estes and Kapikian 2006); only groups A C are

known to infect humans. Annually, an estimated 600,000 children die from rotavirus-induced gastroenteritis worldwide (Parashar et al. 2006). In the US, the estimated annual health care cost for rotavirus infections is about one billion dollars (Tucker et al. 1998). Group A rotavirus is the most common type and causes more than 90% of infections in humans. Nearly every child in the world is infected with group A rotavirus by the age of five. Infection can occur throughout life, although subsequent infections are usually asymptomatic. However, symptomatic reinfections may be due to a different rotavirus strain, serotype, or groups, such as group B rotavirus (also called adult diarrhea rotavirus), which has caused outbreaks in people of all ages in China, India, and Bangladesh (Krishnan et al. 1999; Mackow 2002; Penaranda et al. 1989b; Saif and Jiang 1994; Sanekata et al. 2003; Sen et al. 2001). Group C rotaviruses have been associated with rare and sporadic cases of diarrhea in children in Japan, England, and other countries (Penaranda et al. 1989a). Because of their prevalence and significantly larger disease burden, group A rotaviruses have been the major focus of molecular and structural biology studies; as a result, much of our understanding of rotavirus biology pertains to this group of rotaviruses. Although nongroup A rotaviruses are likely to share many replication strategies with group A rotaviruses, it remains to be seen whether the mechanisms underlying their cell entry processes differ from group A rotaviruses because of their ability to infect both children and adults.

Because of the enormous global health impact and disease burden, there has been a significant push toward development of effective vaccines against rotavirus (Angel et al. 2007; Parashar and Glass 2009). A live-attenuated rhesus-rotavirus-tetravalent vaccine was licensed in 1998 for use in the United States and was found to be quite effective at preventing diarrhea caused by group A rotavirus. However, it was subsequently withdrawn from the market due to temporal association with a bowel disease called intussusception. Recently, a pentavalent human bovine reassortant rotavirus vaccine and a monovalent attenuated human rotavirus vaccine have been licensed (reviewed in Greenberg and Estes (2009)). These have been shown to be relatively safe and effective in children in the United States. Their efficacy in developing countries and their effectiveness against expanding strain diversity, however, remains to be evaluated (Gentsch et al. 2005; Hyser and Estes 2009; Santos and Hoshino 2005). The mechanism by which these vaccines induce protection is not understood and needs further studies. Detailed understanding of the mechanisms underlying rotavirus pathogenesis including the factors that govern cell entry may provide useful insight into improving these vaccines or generating alternative strategies to counter rotavirus infection.

Rotavirus transmission is usually through the fecal oral route. It exhibits a restricted tropism by infecting only the mature enterocytes on the villi of small intestine (Estes and Kapikian 2006). Infection causes alterations in the normal digestive and absorptive functions of these cells, resulting in severe diarrhea. One of the rotavirus nonstructural proteins, NSP4, which exhibits novel toxin-like properties, is strongly implicated in the pathophysiology of rotavirus-induced diarrhea (Estes and Morris 1999; Lorrot and Vasseur 2006; Ramig 2004). Studies in animal models have indicated that rotavirus spreads extraintestinally and virus

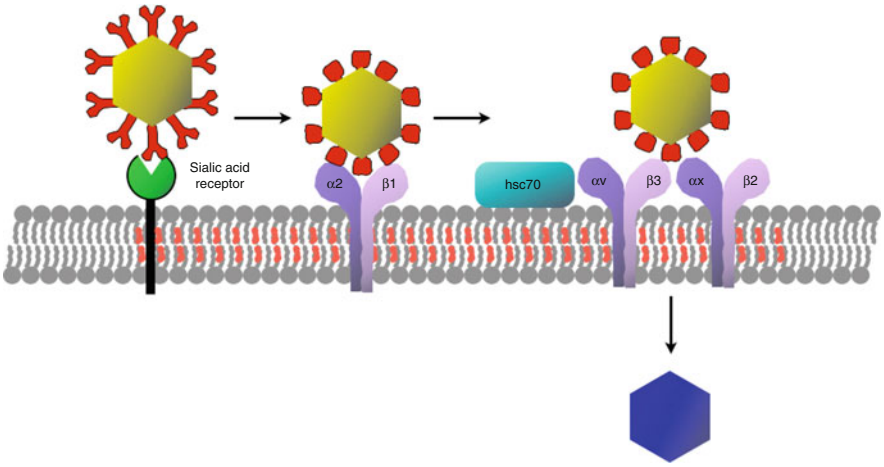

Fig. 1 Cell entry. A summary of the suggested major events during rotavirus cell entry is shown (adapted from Lopez and Arias 2006). Following protease priming, initial cell contact is mediated through SA containing cellular receptors localized on a lipid raft. This interaction involves the VP8* domain of the spike. Subsequently, interactions with integrin α2β1 followed by hsc70 and other integrins take place during postattachment events. During internalization, the VP4 and VP7 layers are removed and release the DLP into the cell

has been detected in extraintestinal sites, including mesenteric lymph nodes, bile ducts, lung, liver, and kidney (Blutt and Conner 2007; Ramig 2007). These sites can support rotavirus replication, particularly, in immunocompromised individuals, but whether such extraintestinal infections contribute to disease remains unclear. In cell culture systems, rotaviruses bind to variety of cell lines; however, infectivity has generally been studied in epithelial cells of renal and intestinal origin (Ciarlet and Estes 2001; Lopez and Arias 2006). Although the molecular basis of how rotavirus enters host cells and determinants of tissue and cell-type specific tropism still remain poorly characterized, a consensus opinion that appears to be emerging from various studies using cell culture systems is that rotavirus entry is a complex process involving coordinated interactions with multiple receptors (Fig. 1) (Lopez and Arias 2006; Mendez et al. 1999). This chapter reviews the current status of our understanding of the molecular and structural basis of rotavirus cell entry.

2 Rotavirus Structure

Rotavirus consists of 11 segments of dsRNA (Estes and Kapikian 2006). Each of the 11 segments in rotavirus codes for one protein with the exception of segment 11, which codes for two proteins. Of these 12 proteins encoded by the viral genome, six are structural (VPs) and six are nonstructural (NSPs). The protein(s) encoded by each of the rotavirus genes are well established (reviewed in Estes and Kapikian

(2006)). Although earlier electron cryo-microscopy (cryo-EM) studies at moderate resolution provided a detailed picture of the complex architecture of rotavirus (reviewed in Pesavento et al. (2006)), with recent advances in cryo-EM imaging and data processing procedure, a significantly higher resolution description of the capsid has been possible. These studies together with X-ray crystallographic analyses of various rotavirus structural proteins and more recently the crystal structure of the double layered particle (McClain et al. 2010) have begun to reveal intricate structural details of the capsid components, enabling a better characterization of the structure function relationships (Dormitzer et al. 2004; Li et al. 2009; Mathieu et al. 2001; Monnier et al. 2006; Zhang et al. 2008).

Like several other structurally characterized members in the Reoviridae (Grimes et al. 1998; Nakagawa et al. 2003; Reinisch et al. 2000; Shaw et al. 1996), rotavirus structure is predominantly based on $T = 13$ icosahedral symmetry. The mature infectious rotavirus particle has three concentric capsid layers that enclose the genomic RNA and is often referred to as TLP (triple-layered particle) (Li et al. 2009; Prasad et al. 1996) (Fig. 2a). One hundred and thirty-two aqueous channels, ~140 Å deep, span the outer two capsid layers at all the five- and six-coordinated positions of a $T = 13$ left-handed icosahedral lattice. Additionally, 60 spikes, each 120 Å long, located at the six-coordinated positions that are closer to fivefold axes emanate from the virion surface (Li et al. 2009; Prasad et al. 1988, 1990; Yeager et al. 1990).

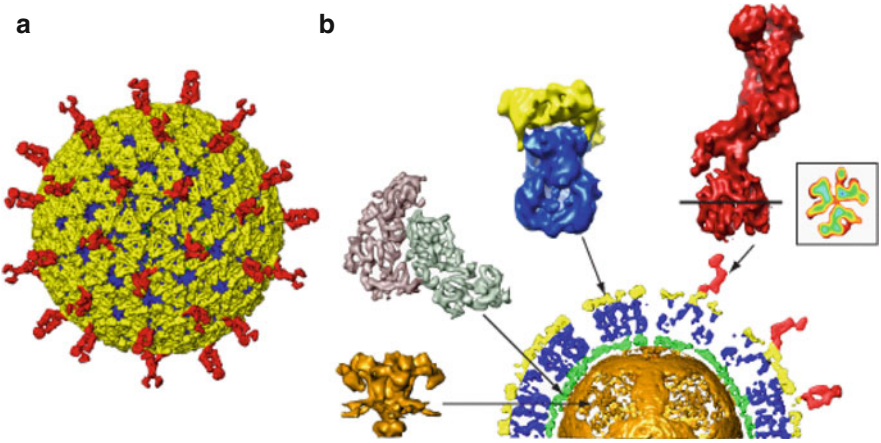

Fig. 2 Rotavirus structure. (**a**) The 9.5 Å resolution cryo EM of rotavirus is shown. The protein layers are radially colored. (**b**) A composite of the rotavirus structural protein components are shown along with a radial hemisphere section of the virus. From *left* to *right*, as well as from inner to outermost location, the RNA dependent RNA polymerase (VP1 and VP3) are shown in *orange*, the VP2A/B dimer is shown in *purple* and *green*, a VP6 trimer is shown in *blue*, a VP7 trimer is shown in *yellow* and a VP4 spike is shown in *red*. The *inset* image shows a layer of the VP4, indicated by a *line* in the adjacent VP4 image, of the threefold base domain situated in between the VP7 and VP6 layers

2.1 VP4 Spikes

The outer layer of rotavirus is made of two proteins VP7, a glycoprotein (38 kDa), and VP4 (88 kDa) (Fig. 2b, red). The characteristic rotavirus spikes are formed by protease-sensitive VP4 (Prasad et al. 1990). The VP4 spike has a distinct structure with two distal globular domains, a central body, and an internal globular domain that is buried inside the peripentonal channels, making the total length of the spike 200 Å (Shaw et al. 1993; Yeager et al. 1994). The composition of the spike was first confirmed by cryo-EM studies of TLPs complexed with VP4-specific antibodies (Prasad et al. 1990; Tihova et al. 2001). VP4 is implicated not only in cell attachment and cell penetration but also in hemagglutination, neutralization, and virulence (Estes and Kapikian 2006). It is interesting to note that the VP4 sequence contains a putative fusion domain similar to that seen in the enveloped viruses such as alphaviruses and influenza viruses (Mackow et al. 1988).

2.2 Proteolytic Fragments of VP4

Proteolytic cleavage of VP4 enhances viral infectivity by several fold (Arias et al. 1996; Estes et al. 1981) and facilitates virus entry into cells (Kaljot et al. 1988). During proteolysis, VP4 (88 kDa) is cleaved into VP8* (28 kDa, aa 1 247) and VP5* (60 kDa, residues 248 776), and the cleavage products remain associated in the virion (Fiore et al. 1991). Rotavirus proteolysis is particularly relevant considering that replication takes place in the gut, an environment rich in proteases. In many studies, including structural studies, typically, rotavirus particles grown in the presence of trypsin are used. This is mainly because the yield of virus particles is significantly better when grown in the presence of trypsin. In cryo-EM reconstructions of rotavirus particles grown in the presence of trypsin, the spikes are well defined and appear dimeric, as was convincingly shown by antibody labeling studies (Prasad et al. 1990; Tihova et al. 2001). In contrast, as shown by biochemical and structural studies, the spikes are disordered in cryo-EM reconstructions of rotavirus particles grown in the absence of trypsin, and these particles are less infectious (Crawford et al. 2001). These results thus indicate that trypsin cleavage imparts structural rigidity to the VP4 spikes in de novo synthesized virus particles and that these ordered spikes make virus entry into cells more efficient.

2.3 VP8* Structure

Structures of the sialic acid binding domain VP8* (residues 60 224) from various group A rotavirus strains have been determined (Blanchard et al. 2007; Dormitzer et al. 2002b; Monnier et al. 2006). The polypeptide fold of VP8* consisting of a five-stranded and a six-stranded β-sheet, a β-hairpin, and a lone C-terminal α-helix

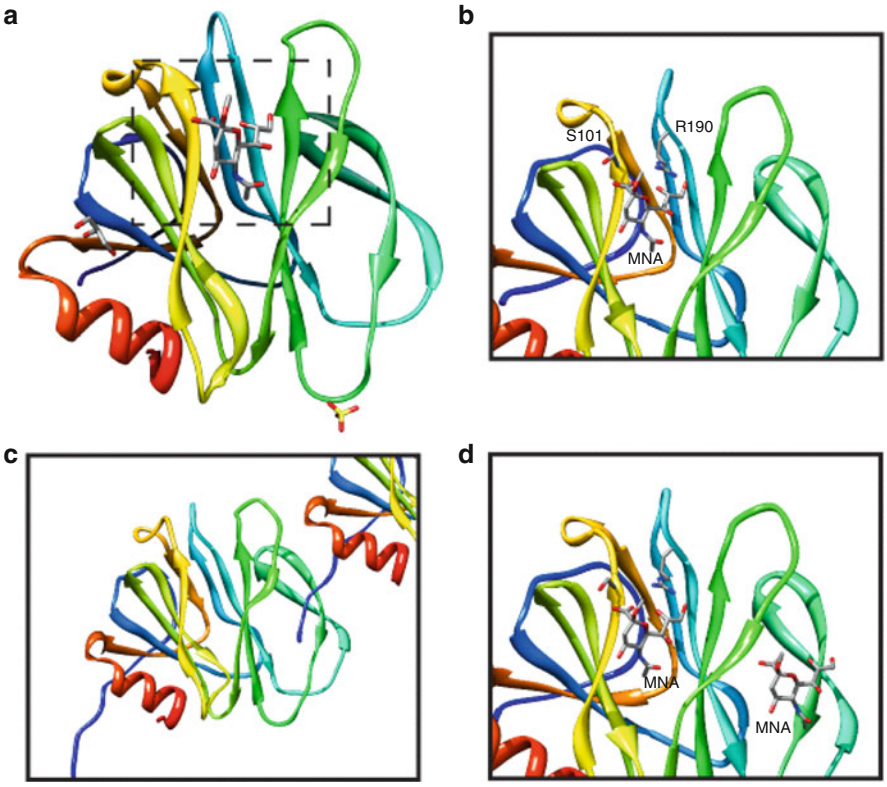

Fig. 3 VP8* SA interactions. The structure of VP8*(PDB ID: 1KQR) complexed with 2 O methyl alpha D *N* acetyl neuraminic acid (MNA) is shown in (**a**). The ribbon diagram is colored from the N terminus (*red*, residue 65) to the C terminus (*blue*, residue 225). The 2 O methyl alpha D *N* acetyl neuraminic acid is shown in the *boxed region*. A zoomed in view of this region is shown in (**b**). In the DS 1 VP8* structure (**c**, PDB ID: 2AEN), an N terminal tail from the neighboring VP8* molecules interacts with another groove that is close to the cleft implicated in the SA binding for Neu sensitive VP8* (Monnier et al. 2006). In the CRW 8 VP8* (Neu sensitive) structure (**d**, PDB ID: 2I2S), an additional SA molecule is seen binding to the same peptide binding groove in DS 1 (Blanchard et al. 2007)

resembles that observed in galectins, a family of lectins (Dormitzer et al. 2002b) (Fig. 3). The crystallographic structure of VP8* fits very well into the distal globular heads of the spikes, confirming that they are made of VP8* (Li et al. 2009; Monnier et al. 2006).

2.4 VP5 Structure*

Each of the globular VP8* domains is attached to a central body of the VP4 spike that extends outwards from the virion surface. This central body of the spike that

further extends into the inner capsid layers is that of VP5*. X-ray structures of C-terminal-truncated VP5* (VP5*-t) in both dimeric (2B4H, aa 250 476) and trimeric (1SLQ, aa 253 522) states have been reported (Dormitzer et al. 2004; Yoder and Dormitzer 2006) (Fig. 4). The overall subunit structure, in both, is similar, except for a long C-terminal α-helix in the trimeric structure. The subunit contacts, however, are different in these crystal structures. Two VP5*-t subunits fit into the central body of the spike in the cryo-EM map. The orientations of the fitted subunits, although with a slight twist, are similar to that observed in the dimeric structure (Li et al. 2009). The rest of the VP4 density, including the internal buried density not accounted by the VP5*-t structure, is likely due to the remaining C-terminal portion of VP5*.

2.5 Oligomeric State of VP4

In the cryo-EM reconstructions of rotavirus particles grown in the absence of trypsin, VP4 spikes are not seen, suggesting that VP4 may be disordered (Fig. 4, row 1), while in the presence of trypsin, the VP4 spike clearly adopts a dimeric appearance above the capsid surface (Fig. 4, row 2). This is further substantiated by antibody labeling studies that show two VP4-specifc Fab molecules attached to VP4 spikes (Prasad et al. 1990; Tihova et al. 2001). However, based on the crystallographic observation that VP5*-t forms a trimer, Dormitzer et al. (2004) proposed a possibility that spikes could indeed be formed by three VP4 molecules, but only two of them form the visible spike observed in the icosahedrally averaged reconstructions of rotavirus particles grown in the presence of trypsin. Other recent observations appear to substantiate this notion. First, in the recent cryo-EM reconstruction of the intact rotavirus at ∼9.5 Å resolution, the buried internal VP4 domain, consisting predominantly α-helices, shows distinct threefold symmetry (Fig. 2, inset) (Li et al. 2009). Second, cryo-EM studies of pH-treated particles show that at elevated pH, the spike undergoes a drastic irreversible conformational change and becomes stunted with a pronounced trilobed appearance (Pesavento et al. 2005) (Fig. 4, row 3). Biochemical analysis of pH-treated particles indicates that VP4 is present in the same amount as in native particles. Further, cryo-EM studies showed that three Fab fragments of the VP5*-specific neutralizing monoclonal antibody bind to the altered spike structure. Thus, these studies underscore the unique nature of the VP4 spike and its ability to undergo drastic structural alterations.

2.6 VP7 Capsid Layer

The outer 35 Å-thick capsid layer of rotavirus is formed by 260 triangular plate-like VP7 trimers (Fig. 2b, yellow). Cryo-EM has shown that the VP7 subunit

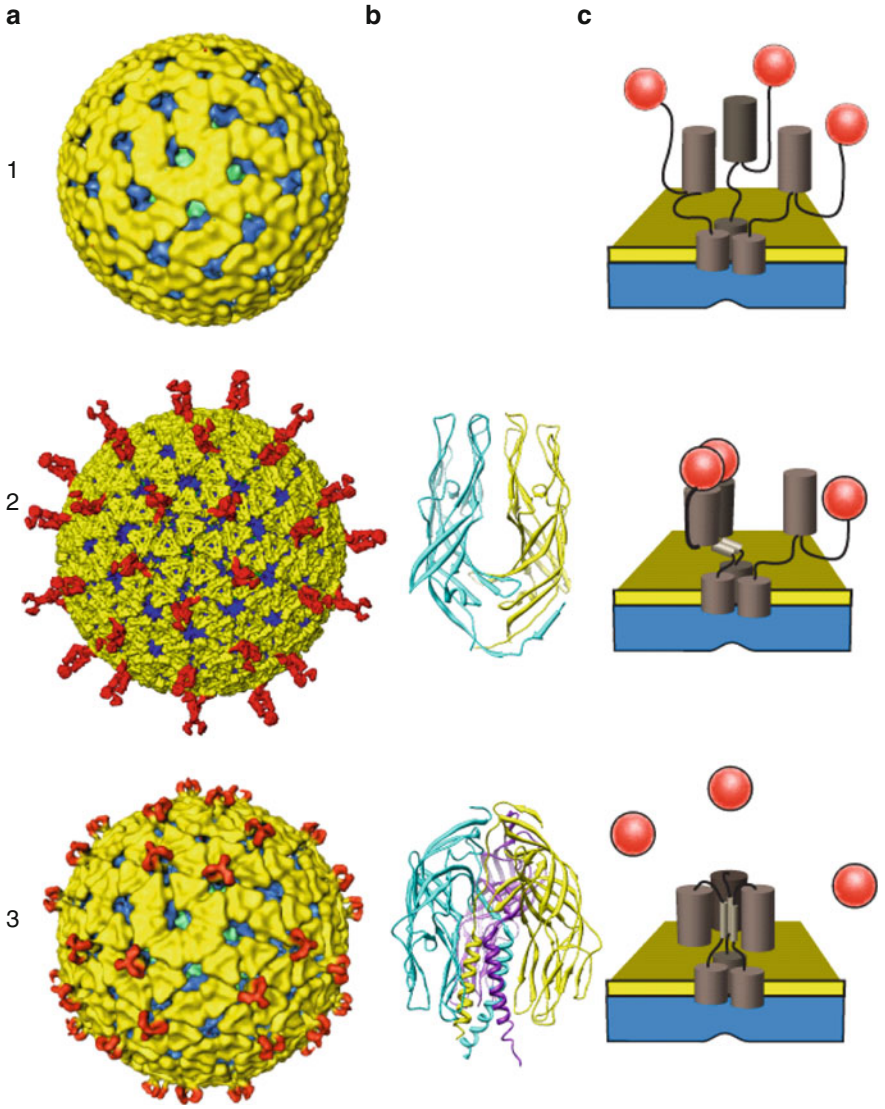

Fig. 4 Structural states of VP4 spike. In the first row, untrypsizined rotavirus is shown in *column A* with a corresponding schematic diagram of the arrangement of VP4 shown in *column C*. In row 2, the cryo EM density map of trypsinized rotavirus (*column A*) and the corresponding crystal structure of the dimeric VP5* t (PDB ID: 2B4H, *column B*) are shown. A schematic of VP4 arrangement of this state is shown in *column C*. In this model, VP8* from one monomer rests atop the neighboring VP5* monomer. A long loop extends from the C terminus of the VP8* molecule down the side of the adjacent VP5* molecule, forming a strand in a β sheet. Near the capsid surface, this loop crosses over and links the N terminus of its VP5* molecule, likely imparting stability to the VP4 dimer in trypsinized rotavirus. A pH treated rotavirus reconstruction appears to indicate the presence of three VP4 molecules (*column A*); a trimeric VP5* t crystal structure is shown in row 3, *column B* (PDB ID: 1SLQ). A schematic view is seen in *column C*. In the schematic views, VP8* is represented in *red*, VP5* is shown in *gray*, the VP7 layer is *yellow* and the VP6 layer is *blue*

consists of well defined α-helical segments along with β-sheets (Li et al. 2009). Recent X-ray crystallographic structure of VP7 with VP7 specific Fab confirmed these observed secondary structural elements and indicated that VP7 has a distinct Rossmann fold and a β-barrel domain (Aoki et al. 2009).

VP7 is a calcium binding protein and calcium stabilizes the outer capsid (Gajardo et al. 1997). The VP7 layer along with the VP4 spikes can be removed by treating the TLPs with calcium chelating agents such as EDTA. The resulting particles with now exposed VP6 are referred to as DLPs. Removal of the layer, which happens during the rotavirus cell entry processes, renders the resulting DLPs transcriptionally competent; the TLPs are transcriptionally inactive (Cohen 1977; Lawton et al. 1999). DLPs are not infectious unless lipofected into permissive cells, suggesting that the outer layer proteins are necessary for cell binding and internalization (Bass et al. 1992; Feng et al. 2002). Based on the X-ray structure of VP7, Aoki et al. (2009) have proposed that neutralizing antibodies against VP7 stabilize the VP7 trimer and inhibit the VP4 rearrangement.

2.7 VP6 Layer

Directly beneath the VP7 layer, 260 trimers of VP6 form an intermediate capsid layer and are arranged on a $T = 13$ icosahedral lattice (Fig. 2b, blue). The VP6 trimers are located directly below the VP7 trimers in such a way that the aqueous channels in the two layers are in register. VP6 is the major component of the rotavirus capsid comprising ~51% of the total capsid mass. A crystallographic structure of the VP6 shows that it has two domains (Mathieu et al. 2001). VP6 subunits wind around each other to make a compact trimer with an overall structure similar to VP7 of bluetongue virus (BTV) (Grimes et al. 1997, 1998) and to the μ1 protein of orthoreovirus (Liemann et al. 2002). Fitting of the crystallographic structure of VP6 trimers into cryo-EM reconstruction has provided details of how VP6 trimers interact with one another to form $T = 13$ VP6 layer and the regions that interact with VP7, VP4, and VP2 (Li et al. 2009; Mathieu et al. 2001). The distal domain that exhibits an eight-stranded β-barrel fold makes contacts with the VP7 and VP4, while the proximal domain with a cluster of α-helices makes contact with the VP2 layer. Many of these structural details are substantiated further by a recent cryo-EM reconstruction of the rotavirus DLP to near atomic resolution (Zhang et al. 2008).

Through its interactions with VP7 layer at the top and with the inner most VP2 layer at the bottom, VP6 plays an important role in stabilizing the entire rotavirus capsid and integrating the two essential functions of the TLP: cell entry and endogenous transcription. Structural integrity of the DLP is an essential requirement for endogenous transcription. Cryo-EM studies of actively transcribing particles have shown that dsRNA segments are transcribed within the confines of the DLP and capped transcript exit through aqueous channels at the fivefold axes (Lawton et al. 1997a).

2.8 VP2 Layer and the Transcription Enzyme Complex

The innermost capsid layer deviates significantly from the outer two layers, exhibiting $T = 1$ icosahedral symmetry. Composed of 120 copies of VP2 (Fig. 2b, green and purple) in two structural isoforms, this layer encapsidates the viral genome and RNA-polymerase complex comprising of VP1, the RNA-dependent RNA polymerase, and VP3, the capping enzyme (Lawton et al. 1997b; Li et al. 2009; Prasad et al. 1996). Twelve copies of VP1 and VP3 are incorporated as heterodimers anchored to the inside surface of the VP2 layer, surrounded by partially ordered genome, at each of the 12 fivefold vertices (Li et al. 2009; Prasad et al. 1996) (Fig. 2b, orange). Similar structural localization of the innermost layer and enzymes, particularly the polymerase, is found in other members of Reoviridae such as BTV (Gouet et al. 1999; Nason et al. 2004), rice dwarf virus (Nakagawa et al. 2003; Zhang et al. 2008), aquareovirus (Nason et al. 2000), orthoreoviruses (Zhang et al. 2003), and cypovirus (Zhang et al. 1999). Such structural conservation is not surprising, given that in all these viruses endogenous transcription of multiple segments is a common and necessary phenomenon. However, a contrasting feature is the location of the capping enzyme. In viruses such as rotavirus, BTV, and rice dwarf virus, the capping enzyme is located inside the core layer, whereas in viruses such as the orthoreovirus, aquareovirus, and cypovirus, the capping enzyme forms a distinctive turret structure with a central hole localized at the fivefold axis of the virion (Hill et al. 1999).

3 Cell Entry

Unlike in the case of several enveloped viruses, mechanisms that allow internalization of nonenveloped viruses in general and rotavirus in particular remain poorly understood. Lack of a well established reverse genetics for rotavirus has made this effort even more challenging. However, based on several studies, a general outline has emerged for rotavirus cell entry that includes proteolytic priming, cell attachment, solubilization of the outer capsid, and internalization of the rotavirus DLP into the cytoplasm.

3.1 Proteolytic Priming

As mentioned in the previous section, a critical step for efficient entry of rotavirus into cells is the proteolytic priming that results in the cleavage of the spike protein into N-terminal VP8* and C-terminal VP5*, which remain associated with the virion (Arias et al. 1996; Estes et al. 1981; Konno et al. 1993; Ludert et al. 1996). Each of these fragments has a distinct role in facilitating the entry process

(Estes and Kapikian 2006). Interestingly, involvement of such a priming step in the entry pathway has been observed in both enveloped viruses, such as influenza virus and HIV, and nonenveloped viruses, such as poliovirus and orthoreovirus (Hogle 2002; Skehel and Wiley 2000). Despite being a nonenveloped virus, rotavirus exhibits striking parallels with influenza virus in regard to proteolytic priming. As in rotaviruses, proteolytic cleavage is an essential step for influenza virus cell entry (Skehel and Wiley 2000). In the case of influenza viruses, proteolysis primes the HA (hemagglutinin) protein for an ensuing irreversible conformational change, which occurs in the low-pH environment of endosomes prior to membrane fusion. The rotavirus VP5* and VP8* are analogous to the proteolytically cleaved fragments of the influenza virus hemagglutinin, HA1 and HA2. Much like rotavirus VP8*, the HA1 subunit plays an accessory role by providing initial binding to the cell via sialic acid containing receptors. HA2 functions more like VP5*, as it is required and sufficient on its own for cell fusion.

3.2 Endocytosis or Direct Penetration

How does rotavirus enter the cell? Two principal ways by which a virus can gain entry into cells are by an endocytic pathway or by direct penetration of the plasma membrane (Dimitrov 2004). Both ultrastructural (Ludert et al. 1987; Petrie et al. 1981; Quan and Doane 1983; Suzuki et al. 1985) and biochemical studies (Bass et al. 1995; Cuadras et al. 1997; Fukuhara et al. 1988; Kaljot et al. 1988; Keljo et al. 1988a) using model cell lines such as MA104 (epithelial monkey kidney cell line) and Caco2 (human colon carcinoma cell line) with cell culture adapted rotavirus strains have been pursued to decipher rotavirus cell entry pathways. Despite some conflicting results, the prevailing notion from these studies has been that trypsin-activated rotavirus, which shows rapid kinetics of internalization, enters through direct penetration, leading to a productive infection, whereas nontrypsinized particles enter with much slower kinetics through an endocytic pathway, and infectivity is considerably reduced. Several of these studies using specific inhibitors have categorically ruled out the classical endocytic mechanism for trypsin-primed rotavirus. Infectivity is not blocked by drugs such as cytochalasin D or dansylcadaverine that inhibit the intracellular trafficking of endosomal vesicles (Bass et al. 1995; Cuadras et al. 1997). Preventing acidification of endosomes by lysosomotropic agents does not inhibit rotavirus infection (Kaljot et al. 1988). Similarly, agents that inhibit clathrin-mediated endocytosis, such as hypertonic sucrose, chlorpromazine, and those that inhibit caveolin-mediated endocytosis, such as filipin and nyastatin, also do not block rotavirus infectivity (Sanchez-San Martin et al. 2004). These results are consistent with that data in cells overexpressing dominant-negative mutants of proteins such as Eps15, crucial for clathrin coated pit assembly, and caveolin-1 and caveolin-3, critical for caveolin-dependent pathway. However, interestingly, rotavirus infectivity is blocked in cells treated by methyl-B-cyclodextrin, a drug that sequesters cholesterol specifically, and in cells

overexpressing a dominant-negative dynK44A mutant, suggesting that cholesterol and dynamin may play a role in rotavirus cell entry (Sanchez-San Martin et al. 2004). Involvement of cholesterol and dynamin, a GTPase implicated in various intracellular vesicular activities, including membrane dynamics (Conner and Schmid 2003), suggests that rotavirus entry may be through a novel glycolipid-raft driven and dynamin-dependent vesicular pathway (Sanchez-San Martin et al. 2004). The role of dynamin in the entry pathway, however, has to be reconciled with the results of earlier biochemical experiments, which showed that energy inhibitors such as sodium azide and dinitrophenol do not affect rotavirus infectivity (Kaljot et al. 1988). Involvement of cholesterol is particularly interesting as cholesterol is known to function as a "glue" to keep the lipid rafts or lipid microdomains together at the plasma membrane (Simons and Ehehalt 2002). While the role of the lipid rafts in rotavirus entry remains to be firmly established, they potentially can provide an optimal environment for facilitating rotavirus interactions with its multiple receptors (Isa et al. 2004) and a mechanism for any necessary signal transduction. Involvement of lipid rafts has also been suggested for other stages in the morphogenesis of rotavirus (Cuadras and Greenberg 2003; Sapin et al. 2002). Mature rotavirus particles as well as purified VP4 have been shown to interact with cholesterol and sphingolipid-enriched model lipid membranes (Sapin et al. 2002).

3.3 Membrane Permeabilization

Irrespective of the entry pathway, the rotavirus entry mechanism should incorporate two aspects namely, membrane permeabilization and uncoating of the outer capsid layer. The molecular mechanisms underlying these two processes are not fully understood. Various studies using variety of techniques including chromium release, ethidium bromide entry, and α-sarcin coentry have clearly shown that rotaviruses permeabilize cells (Cuadras et al. 1997; Falconer et al. 1995; Kaljot et al. 1988; Liprandi et al. 1997). Fluorescence dequenching experiments, by monitoring release of vesicle-entrapped fluorophores in artificial liposomes as well as suspended MA104 cells, have shown that only trypsin-activated rotavirus particles, not DLPs or nontrypsinized particles, permeabilize membranes, this activity is optimal at neutral pH, and is considerably reduced at acidic pH providing further support for the direct penetration model (Nandi et al. 1992; Ruiz et al. 1994). The trypsin-activated particles also induce cell cell fusion from without in suspended MA104 cells, and both the outer layer proteins, VP4 and VP7, are required for this activity (Falconer et al. 1995). Neutralizing monoclonal antibodies to VP4 domains and VP7 inhibit membrane-associated activities of rotavirus particles. Consistent with these observations, solubilized forms of VP5* and VP7 exhibit membrane permeabilizing activity (Charpilienne et al. 1997; Denisova et al. 1999; Ruiz et al. 1997). A sequence motif in VP5* that bears similarities with the fusion peptide sequence observed in enveloped viruses such as alphaviruses and influenza viruses has been implicated in the membrane permeabilization activity of

recombinant VP5* (Denisova et al. 1999). In the context of rotavirus cell entry, how the membrane permeabilizing activities of the outer capsid components, VP7 and VP5*, are coordinated remains to be elucidated.

3.4 Uncoating

A persistent notion in the rotavirus field, primarily based on two early studies, is that uncoating of the outer capsid layer of rotavirus occurs during the cell entry processes. Ultrastructural EM studies of rotavirus infected cells revealed that intact trypsinized TLPs undergo significant morphological changes immediately following penetration of the cell membrane (Suzuki et al. 1985). A biochemical analysis of the rotavirus components during an infection with trypsin-treated particles suggested that the outer layer proteins are localized to the plasma membrane and the DLP components are internalized (Fukuhara et al. 1988). However, the precise mechanism of uncoating and where it occurs during an infection still remains unclear. As mentioned earlier, *in vitro* studies indicate that the stability of the outer capsid layer is calcium-dependent as this layer can be removed by the addition of calcium chelating agents such as EDTA (Cohen 1977; Gajardo et al. 1997). Biochemical studies on recombinant VP7 have shown that formation of VP7 trimers is calcium-dependent and the disassociation of VP7 trimers following calcium chelation may be the biochemical basis for *in vitro* uncoating (Dormitzer et al. 2000). The possible role of calcium in uncoating the outer capsid layer during rotavirus infection has been examined by inducing alterations in the extracellular and intracellular calcium levels by using calcium ionophores (Chemello et al. 2002; Cuadras et al. 1997). These studies have produced conflicting results. In one study, it was shown that virus entry/infectivity was not affected by drugs such as calcium ionophore A23187, ionomycin, and endoplasmic reticulum calcium-ATPase inhibitor thapsigargin, which elevate intracellular calcium levels (Cuadras et al. 1997). In another study, rotavirus infectivity was significantly reduced in the presence CaEGTA and the endosomal H+-ATPase inhibitor bafilomycin A1, prompting the authors to suggest a calcium-dependent endocytosis model in which the drop in endosomal calcium levels driven by the electrical gradient generated by the proton pump results in uncoating (Chemello et al. 2002). On the other hand, studies with VP4-specfic neutralizing antibodies suggest an attractive alternate mechanism in which conformational changes in VP4, perhaps caused by its interactions with receptor molecules, may trigger uncoating (Dormitzer 2008). One of the neutralizing antibodies that targets VP8* causes uncoating of the outer capsid layer, indicating the possibility that VP4-receptor interactions may also induce conformational changes propagating from VP8* to the VP7 layer leading to uncoating (Dormitzer 2008; Zhou et al. 1994). As separately discussed below, structural studies have indicated the possibility of large conformational changes in the VP4 spike during cell entry process. In support of this possibility a VP5*-specific neutralizing antibody protects the VP4 spike from undergoing conformational changes, suggesting that

this particular antibody neutralizes the virus by preventing conformational changes in VP4 that may be required for internalization (Pesavento et al. 2005).

3.5 Rotavirus Receptors

A consensus model for receptor-mediated cell entry of rotaviruses that has emerged from a variety of studies is that entry involves sequential and coordinated interactions of the outer layer proteins with multiple receptors including sialic acid (SA) receptors in the initial attachment step followed by postattachment sequential interactions with integrins, such as α2β1, α4β1, αxβ2, αvβ3, and heat shock cognate protein 70 (Hsc70) (Fig. 1). In this process, the VP8* domain is implicated in the interactions with SA-containing receptors, VP5* with α2β1 and Hsc70, and VP7 with αxβ2 and αvβ3 (Ciarlet and Estes 2001; Dormitzer 2008; Lopez and Arias 2004, 2006).

SA receptor : SA is the first and perhaps the best characterized rotavirus receptor that is implicated in the initial cell attachment through its interactions with VP8* domain (see review by (Isa et al. 2006). Prompted by the initial observations that several rotavirus strains agglutinate erythrocytes, subsequent studies showed that infectivity in cultured cells by certain rotavirus strains, particularly of animal origin, was sensitive to neuraminidase treatment and that sialylated glycoproteins of various origins significantly inhibited infection with these strains (Fukudome et al. 1989; Keljo et al. 1988b; Willoughby et al. 1990; Yolken et al. 1987). It was also noticed that other rotavirus strains, especially human strains, did not agglutinate red blood cells and infectivity was not sensitive to neuraminidase treatment of cells. This observation led to the classification of rotaviruses as neuraminidase (Neu)-sensitive and Neu-insensitive and the suggestion that the entry pathway for the former class is SA-dependent and for the latter is SA-independent (Ciarlet and Estes 1999; Mendez et al. 1993). However, such a simple classification of rotaviruses has been called into question by several studies, indicating that insensitivity to neuraminidase treatment does not necessarily mean that the entry pathway is SA-independent (Delorme et al. 2001; Haselhorst et al. 2009). These studies have shown that while the Neu-sensitive strains recognize the terminal SA moieties, the Neu-insensitive rotavirus stains bind to internal SA moieties in gangliosides that are resistant to neuraminidase treatment. Although most of the studies have involved group A rotaviruses, requirement of SA for cell binding is also demonstrated for group C rotaviruses (Svensson 1992). Thus most rotaviruses, if not all, likely require SA for cell attachment, and a more robust classification in terms of ganglioside specificity instead of neuraminidase sensitivity is recommended (Banda et al. 2009; Haselhorst et al. 2009). Pending acceptance of such a refined classification, for simplicity, we will retain the original classification based on neuraminidase sensitivity in the following sections.

Irrespective of neuraminidase sensitivity, it is clear that the VP8* domain of the VP4 spike protein in both classes of rotaviruses is involved in binding to SA moiety

in the gangliosides (Dormitzer et al. 2002b; Fiore et al. 1991; Haselhorst et al. 2009). However, the requirement of SA binding in the cell entry process of Neu-insensitive rotaviruses and whether their cell entry pathway substantially differs from that of Neu-sensitive rotaviruses remains controversial and requires further studies. Ciarlet et al. (2002) showed that in polarized epithelial cells, the Neu-sensitive rotavirus strains enter at the apical surface, whereas the Neu-insensitive strains enter through both apical and basolateral surface. In addition to the their differences in binding to external or internal SA, other available data including noticeable differences in the SA binding pocket of VP8*, as discussed separately later, with these two classes of rotaviruses raise the possibility of subtle or even significant differences in their cell entry pathways. The distribution of escape mutations selected with VP8*- and VP5*-specific neutralization antibodies in the two rotavirus classes is significantly different (Mackow et al. 1988; Mendez et al. 1993). For the Neu-sensitive rotaviruses, such antibodies predominantly map to the VP8* in contrast to other class, in which they map largely to VP5* with fewer mutations in VP8*. In one study, while the infectivity of Neu-sensitive RRV was blocked by both VP8*- and VP5*-specific neutralizing antibodies, the infectivity of a mutant of this strain, selected by the passage of wt RRV on neuraminidase treated cells, was only blocked by VP5* antibody (Zarate et al. 2000b). In addition, these studies showed that recombinant VP5* exhibits cell binding and that it can block not only the cell binding of both the wt and the mutant rotaviruses but their infectivity as well. Taken together, these studies thus suggest, first, the involvement of VP5* in cell entry, second, the possibility of additional cell attachment factors, and third Neu-sensitive and Neu-insensitive strains may exhibit some differences in their cell entry pathways.

A complex entry pathway involving multiple receptors is becoming a norm rather than an exception with many nonenveloped and enveloped viruses (Lopez and Arias 2004). Examples include nonenveloped viruses such as picornaviruses, adenoviruses, polyomaviruses, and orthoreoviruses (discussed elsewhere in this book) and enveloped viruses such as herpesviruses, respiratory synctial viruses, and HIV. Interestingly as with rotaviruses, many of these viruses utilize integrins (Stewart and Nemerow 2007; Triantafilou et al. 2001).

Integrins: Several integrins, such as $\alpha 2\beta 1$, $\alpha 4\beta 1$, $\alpha x\beta 2$, and $\alpha v\beta 3$, have been identified as possible receptors for rotavirus cell entry (Coulson et al. 1997; Graham et al. 2003; Guerrero et al. 2000; Zarate et al. 2000a, 2004). Antibodies to these integrins or synthetic peptides that represent integrin ligand motifs that are found in VP5* or VP7 block the infectivity to noticeable levels. Involvement of these integrins is further supported by the increased binding and infectivity of rotavirus in poorly permissive cell lines upon expression of these integrins through transfection (Ciarlet et al. 2002; Hewish et al. 2000). The level of enhanced permissivity, however, is not to the same extent as that in the fully permissive cell lines, suggesting that these integrins may function as internalization receptors or as coreceptors in establishing productive infection (Ciarlet et al. 2002). The observation that integrins such as $\alpha 2\beta 1$ and $\alpha v\beta 3$ influence cell binding in an additive manner has led to the suggestion that these integrins may play a role in

different stages of the cell entry process (Guerrero et al. 2000). Although the question of precisely at what stage of the cell entry pathway these integrins are involved is not yet answered, the current opinion is that they are involved at a postattachment stage.

Both VP5* and VP7 are implicated in interactions with integrins. Based on antibody and peptide blocking experiments, VP5* interacts with α2β1 through a DGE (aa 308 310) motif (Graham et al. 2003; Zarate et al. 2000a), and VP7 with αxβ2 and α4β1 integrins through its GPR (Graham et al. 2003) and LDV motifs, respectively (Coulson et al. 1997). Based on the recent X-ray structure of VP7, GPR motif (residues 253 255) is on the inner surface of the VP7 trimer and would only be available during uncoating (Aoki et al. 2009). Interaction with αvβ3 is shown to be mediated by VP7 through a novel non-RGD motif (Zarate et al. 2004). None of the outer layer proteins has the classical RGD sequence motif that is well established in recognizing αvβ3. Similar non-RGD interaction has been suggested for hantaviruses (Gavrilovskaya et al. 1999). Based on sequence comparisons with Hantavirus G1G2 protein, a stretch of sequence encompassing a CPN motif in VP7 that is highly conserved among rotavirus strains is implicated in interactions with αvβ3. The CPN peptide has been shown to bind to αvβ3 directly at a different site other than the RGD binding site. This peptide inhibits rotavirus infectivity without blocking cell binding, suggesting postattachment involvement of αvβ3 in rotavirus cell entry.

Hsc70: In addition to SA-containing receptors and integrins, the heat shock cognate protein, hsc70, has been suggested to interact with rotavirus after the initial attachment phase of cell entry. This interaction is mediated by VP5* based on the observations that a synthetic peptide corresponding to amino acid residues 642 658 in VP5* and the recombinant VP5* competed with binding of RRV and nar3 mutant to Hsc70. The synthetic peptide noticeably blocked infection but not cell binding (Guerrero et al. 2002; Zarate et al. 2003). Additionally, an intriguing observation is that DLPs and synthetic peptide from VP6 (aa 280 297) inhibit entry of three different rotavirus strains in two different cell lines in a dose-dependent manner (Gualtero et al. 2007). DLPs appear to directly interact with Hsc70, suggesting that VP6 may also have some indirect role in rotavirus cell entry.

4 Structural Insights into Cell Entry

Unlike other viruses such as picornaviruses and adenoviruses, structural studies of rotavirus particles in complex with cognate receptor molecules have thus far not been possible. The only receptor interaction that has been structurally characterized is that of VP8* (Neu-sensitive) SA interactions (Blanchard et al. 2007; Dormitzer et al. 2002b; Kraschnefski et al. 2009; Monnier et al. 2006). Binding affinity for this interaction is in the low millimolar range (Dormitzer et al. 2002a). Although direct interaction between the α2I domain of integrin and recombinant GST-tagged VP5* has been demonstrated (Graham et al. 2003), a direct quantitative binding analysis between the intact virus and any of the receptors is not available. It is possible that

the rotavirus binds to these receptors such as integrins and Hsc70 with a low affinity and the interactions could be transient during the entry pathway requiring appropriate conformational changes in the VP4 spike structure to expose the required binding sites for the suggested sequential interactions with these receptors. As discussed below, structural studies do indeed underscore such a possibility during cell entry pathway beginning with the obligatory proteolytic event.

4.1 Trypsin-Induced Order-to-Disorder Transformation in VP4

Comparative cryo-EM and biochemical analysis of nontrypsinized and trypsinized rotavirus have shown that the VP4 spike undergoes a disorder-to-order transition upon trypsin priming (Crawford et al. 2001; Pesavento et al. 2006) (Fig. 4). The dimeric configuration of the VP4 spike induced by trypsinization likely confers a more optimal state for the binding of cellular receptors and structural rearrangements required for subsequent cell entry processes including membrane penetration. The idea of a trypsin-induced disorder-to-order transition is indeed unique and has not been documented with any other virus thus far. An interesting question is whether trypsin acts from within or outside of cells. This question in turn relates to where the assembly of VP7 and VP4 takes place during rotavirus infection. VP7 is synthesized on the ribosomes associated with the ER and is cotranslationally associated with the ER membrane, whereas VP4 is synthesized on free cytosolic ribosomes (Estes and Kapikian 2006). Although it is generally agreed upon that the DLPs, which are assembled in the viroplasm, acquire the VP7 layer during their budding into ER, where and how the spike protein VP4 is assembled onto the particles is unclear (Estes and Kapikian 2006). One possibility is that during virus infection, trypsin acts outside the cells on the newly formed VP4 and that this trypsinized VP4 is able to assemble properly onto the rotavirus particles with VP7 layer already assembled (in the ER). Such a hypothesis would be consistent with the finding, using confocal microscopy of virus-infected MA104 cells, that high amounts of VP4 are present at the plasma membrane ~3 h postinfection and that the N-terminal region, that is, VP8* is accessible to antibodies (Nejmeddine et al. 2000). Targeting of VP4 to the plasma membrane appears to be a general phenomenon as it is seen in both polarized and nonpolarized cells (Sapin et al. 2002). Assembly of VP4 at the plasma membrane prompts a question as to how the large proximal globular domain of VP4 is inserted into the peripentonal channels of the particles with the VP7 layer already assembled. It is possible that VP7 trimers undergo conformational changes to facilitate insertion of the VP4 proximal domain into the channels. Alternatively, a more likely scenario is that trypsin acting from outside the cells may impart the rigid "dimeric" configuration to the disordered VP4 subunits that are assembled on the TLPs intracellularly. Such a possibility is consistent with *in vitro* experiments of recoating DLPs with recombinant VP4 and VP7 (Trask and Dormitzer 2006). These experiments show that VP4 must be added before VP7 to the DLPs followed by trypsin priming to produce infective

rotavirus particles. Further structural and biochemical studies are needed to provide a better understanding of how and where trypsin affects spike assembly.

4.2 Initial Cell Attachment: VP8–SA Interactions*

VP8* SA interactions that represent the initial cell attachment stage has been crystallographically characterized (Fig. 3a, b). In addition to the structures of the VP8* core domain from both classes of rotaviruses, Neu-sensitive and Neu-insensitive, X-ray structures of VP8* SA complexes from two of the Neu-sensitive strains have been determined (Blanchard et al. 2007; Dormitzer et al. 2002b; Kraschnefski et al. 2009; Monnier et al. 2006). These structures show that the β-sandwich galectin-like fold, composed of two twisted β-sheets with a shallow cleft in between, remains invariable in the VP8* from both classes of rotaviruses.

The VP8* structure from Neu-sensitive strains (RRV and porcine CRW-8) with bound SA indicate that SA binds above the cleft coordinated by direct- and water-mediated hydrogen bonds with amino acid residues Arg101 and Ser190 in addition to van der Waals interactions involving residues from the cleft (Fig. 3b). Identification of critical residues in SA binding prompted further studies to probe into the functional relevance of the SA binding site in VP8* using site directed mutagenesis (Kraschnefski et al. 2009). These studies showed that mutation of either Arg101 or Ser190 to Ala reduced both the binding of RRV VP8* (Neu-sensitive) to SA and also the ability to compete with RRV infection in untreated MA104 cells, confirming that the same SA binding site is used during rotavirus infection. These studies also found that, except for the Ser190Ala mutant, both the wild-type and Arg101Ala mutant inhibited infection upon sialidase treatment of MA104 cells, suggesting that first VP8* in RRV can recognize uncapped glycan structure remaining after the sialidase treatment through the same binding site, and second, that Ser190 is essential for this interaction.

Whether VP8* from Neu-insensitive strains also utilize the same site, as discussed earlier for the Neu-sensitive VP8*, for SA (internal) binding is unclear. The amino acid residues in the SA binding cleft in Neu-sensitive VP8* are highly conserved but not in the Neu-insensitive strains. Crystallization of VP8* from two of the Neu-insensitive strains (human DS-1 and Wa) yielded crystals of only the unliganded VP8*, despite the presence of SA during crystallization trials (Blanchard et al. 2007; Monnier et al. 2006). Comparative analysis of VP8* from both Neu-sensitive and -insensitive strains indicate significant alterations including a 2.6 Å widening of the cleft in Neu-insensitive VP8* structures (Blanchard et al. 2007). In the DS-1 VP8* structure, an N-terminal tail from the neighboring VP8* molecules interacts with another groove that is close to the cleft implicated in the SA binding for Neu-sensitive VP8* (Monnier et al. 2006) (Fig. 3c). Based on this observation and significant alterations in the cleft, Monnier et al. (2006) have argued that Neu-insensitive strains may not bind to SA but instead bind to a yet unidentified protein receptor. Interestingly, in the CRW-8 VP8* (Neu-sensitive)

structure, an additional SA molecule is seen binding weakly to the same peptide binding groove in the DS-1 VP8* structure (Blanchard et al. 2007) (Fig. 3d). From this observation, Blanchard et al. (2007) have hypothesized that this groove is used for binding to carbohydrates by the VP8* in the Neu-insensitive strains. Further studies are clearly necessary to understand how VP8* in Neu-insensitive strains binds to glycans.

In the context of virion structure, fitting of the VP8* crystal structure into the recently determined high resolution cryo-EM map of the (Neu-sensitive) rotavirus strain clearly shows that VP8* is appropriately oriented with the SA binding cleft exposed at the tips of VP4 spikes (Li et al. 2009). Whether the orientation of the VP8* domain in the VP4 spikes of the Neu-insensitive rotavirus strains is altered or remains unchanged is not known as there are no cryo-EM reconstructions of Neu-insensitive rotavirus strains. This is a particularly relevant question considering that, when the known neutralization escape mutations in Neu-insensitive and Neu-sensitive strains are mapped onto the VP8* structure, they show a distinctly different distribution, perhaps indicating that the conformation of the VP4 spike or the orientation of VP8* in the VP4 spikes recognized by the neutralizing antibodies in each of these two classes of rotaviruses may indeed be different (Monnier et al. 2006).

Does SA binding induce conformational changes in VP8*? Crystallographic structures of VP8*-SA complexes indicate minimal conformational changes in VP8* upon SA binding (Monnier et al. 2006). However, in the context of the entire rotavirus structure during entry, whether this is indeed true remains to be examined. Structural studies of the rotavirus particles with longer glycans with a terminal or an internal SA moiety may provide some answers to this question.

4.3 Entry-Related Structural Gymnastics of the VP4 Spike

Cryo-EM structural analyses of nontrypsinized rotavirus (Neu-sensitive) particles showing disordered spikes, trypsin-primed particles showing dimeric spikes (Crawford et al. 2001), alkaline pH-treated particles showing triskelion arrangement of the VP4 molecules (Pesavento et al. 2005), and the crystallographic analysis of VP5* showing both dimeric and trimeric states (Dormitzer et al. 2004; Yoder and Dormitzer 2006) indicate an intriguing possibility of fascinating structural gymnastics that the VP4 spikes may undergo during the cell entry process (Fig. 4). These studies also indicate that the VP4 spike could indeed be formed by three VP4 molecules instead of a dimeric configuration as previously thought from the cryo-EM studies of just the trypsinized rotavirus (Dormitzer et al. 2004). The possibility that the spike is composed of three molecules is further substantiated by the strong threefold symmetry observed in the foot or the internal globular domain of the VP4 that is tucked inside the peripentonal channels of the VP7 and VP6 capsid layers (Li et al. 2009).

A hypothetical model for the entry-related structural rearrangements from the various structural observations is that upon trypsinization, two of the three VP4 subunits form the visible spike as seen in the cryo-EM reconstruction of the trypsinized rotavirus particles, and during cell entry, by a yet unknown entry associated event, the floppy VP4 subunit associates with the other two molecules to transit from a dimeric configuration to a trimeric state, resembling that observed in one of the crystal structures of VP5* (Dormitzer et al. 2004) (schematically shown in Fig. 4, column C). Such a twofold to threefold transition, involving a 180 rotation of the central VP5* domain about an axis that is roughly perpendicular to the dyad axis of the twofold spike, allows translocation of the DGE motif in VP5*, which is implicated in the interactions with integrins, and the fusion peptide motif, which is implicated in membrane penetration, to the apical surface (Dormitzer 2008; Yoder and Dormitzer 2006). Based on the X-ray structure of VP7, Aoki et al. (2009) have proposed that removal of Ca^{2+} in VP7 could be an uncoating trigger for VP4 rearrangements.

Cryo-EM and biochemical analysis of alkaline pH-treated Neu-sensitive rotavirus provide circumstantial evidence for the proposed structural transformation in the VP4 spikes during cell entry (Pesavento et al. 2005). At alkaline pH, the dimeric spike undergoes a drastic irreversible conformational change and becomes stunted with a pronounced trilobed appearance (Fig. 4, row 3). Despite the loss of infectivity and the ability to hemagglutinate, the high pH-treated particles surprisingly exhibit specific binding to neuraminidase-treated cells, in contrast to native virions, which exhibit SA-dependent cell binding. Furthermore, the pH induced structural changes in the spikes, SA-dependent cell binding, and hemagglutinating functions of the virion are completely prevented when the virions are bound by Fab fragments of VP5*-specific monoclonal antibody (2G4) prior to pH treatment. However, when 2G4 is bound to the pH-altered particles, cell binding is completely lost. A hypothesis that is proposed from these studies is that high pH treatment triggers the twofold to threefold transition in the VP4 spikes that mimics a postattachment step, and the mechanism by which the 2G4 antibody neutralizes infectivity is by preventing this conformational change necessary for a productive entry (Pesavento et al. 2006).

5 Conclusions

As previously compared to an intricately choreographed dance (Lopez and Arias 2004), rotavirus utilizes a complex set of interactions in nearly every aspect of the viral lifecycle. Mitigated mainly by VP4, viral entry appears to occur through large structural rearrangements that facilitate the optimal interface between viral and host components. While the current model appears to capture the basics of viral entry, many questions remain. Do the receptors identified thus far adequately explain tissue tropism, host range, and age dependence? What is the interrelationship with disease induction and disease progression with entry processes? How and where

does the assembly of VP4 take place in infected cells? How does trypsin facilitate proper assembly of the VP4 spike during rotavirus replication? What is the role of ganglioside binding in Neu-insensitive strains? How is SA binding coordinated with binding of other receptors? What triggers twofold to threefold transition in the spike structure that appears to be required for the suggested sequential interactions with various receptors and membrane penetration? Involvement of integrins raises questions such as whether rotavirus entry involves cellular modifications such as tight junction disruption to access these integrins, and whether interactions with integrins affect signaling pathways. Further investigations using genetic, molecular, and structural approaches besides answering some of these questions are likely to unravel additional novel insights into the fascinating process of rotavirus cell entry. A detailed molecular understanding of the critical process of cell entry undoubtedly will be useful in designing more efficacious treatment and control of rotavirus infection.

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Structures and Functions of Parvovirus Capsids and the Process of Cell Infection

Colin R. Parrish

Contents

Abstract To infect a cell, the parvovirus or adeno-associated virus (AAV) genome must be delivered from outside the plasma membrane to the nucleus, and in the process, the capsid must follow a series of binding and trafficking steps and also undergo necessary changes that result in exposure or release the ssDNA genome at the appropriate time and place within the cell. The 25 nm parvovirus capsid is comprised of two or three forms of a single protein, and although it is robust and stable, it is still sufficiently flexible to allow the exposure of several internal components at appropriate times during cell infection. The capsid can also accommodate insertion of peptides into surface loops, and capsid proteins from different viral serotypes can be shuffled to create novel functional variants. The capsids of

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the different viruses bind to one or more cell receptors, and for at least some viruses, the insertion of additional or alternative receptor binding sequences or structures into the capsid can expand or redirect its tropism. The infection process after cell binding involves receptor-mediated endocytosis followed by viral trafficking through the endosomal systems. That endosomal trafficking may be complex and prolonged for hours or be relatively brief. Generally only a small proportion of the particles taken up enter the cytoplasm after altering the endosomal membrane through the activity of a VP1-encoded phospholipase A2 domain that becomes released to the outside of the viral particle. Modifications to the capsid that can occur within the endosome or cytoplasm include structural changes to expose internal components, ubiquination and proteosomal processing, and possible trafficking of particles on molecular motors. It is still not clear how the genomes enter the nucleus, but nuclear pore-dependent entry of particles or permeabilization of nuclear membranes have been proposed. Those processes control the infection, pathogenesis, and host ranges of the autonomous viruses and determine the effectiveness of gene therapy using AAV capsids.

1 Parvoviruses: Capsid Structures, Assembly and DNA Packaging

There are many different parvoviruses that infect a wide variety of animal hosts, ranging from crustaceans to mammals, and they cause diseases that range in severity from subclinical to severe and fatal infections, depending on the virus and on the various host factors. The related adeno-associated viruses (AAV) can enter host cells and establish a latent infection that involves chromosomal integration of the viral genomic DNA, but the AAV are nonpathogenic and replicate their DNA when the cell is co-infected with a helper adenovirus or herpesvirus. There are many strains of parvoviruses, including several serotypes or genetically distinct strains in humans, and a great number of AAV types have been identified on the basis of their antibody reactivity and by genetic comparisons. Many AAV types and variants are being developed for human gene therapy applications, targeting a variety of tissues (Coura Rdos and Nardi 2007; Mueller and Flotte 2008), while some autonomous parvoviruses are being tested for their ability to target tumors or to express genes in cells.

The parvovirus capsid is a $T = 1$ icosahedron that is about 25 nm in diameter, with the different viral capsids varying from a relatively smooth surface (in invertebrate-infecting densoviruses) to those having distinct surface spikes (most viruses infecting vertebrates) (Fig. 1). The capsid is initially assembled from either two (VP1 (~10%) and VP2 (~90%)) or three proteins (VP1 (~10%), VP2 (~10%), and VP3 (~90%)). The VP1 is the largest protein and ranges in size from ~65 to 85 kDa in different viruses, while the other proteins are essentially smaller versions of VP1, which share their C-terminal sequences and range in size from ~60 to 75 kDa. In many parvoviruses, five loops form a cylinder that surrounds the fivefold

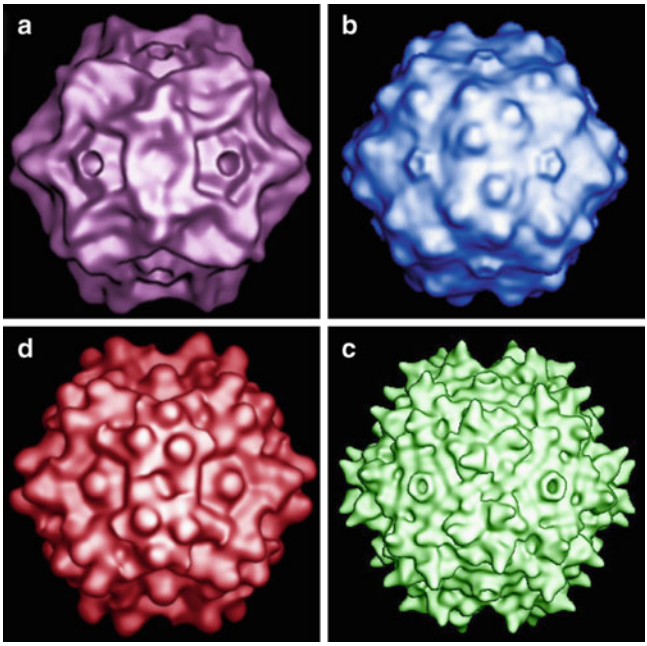

Fig. 1 Shaded surface representations of the capsids of CPV (**a**), AAV5 (**b**), ADV (**c**), and AAV2 (**d**), all viewed down a twofold axis of symmetry. The structures of the capsids are all show at around 21 Å resolution to show the major surface features. From (Walters et al. 2004) with permission

axes, which shows an ~10 Å diameter pore in the high resolution capsid structures, although the loops appear flexible and that pore likely expands under some conditions (Chapman and Rossmann 1993; Farr et al. 2006; Farr and Tattersall 2004). The ssDNA is packaged into the partially or fully assembled capsid through the helicase activity of the major nonstructural proteins (NS1 in the autonomous viruses or Reps in the AAVs) (James et al. 2003; Mansilla-Soto et al. 2009; Yoon-Robarts et al. 2004). It appears that the 12-fold helicase engages the fivefold axis of the virus by an unknown mechanism and threads the ssDNA genome through one of the pores at that position of the capsid (Bleker et al. 2005; Cotmore and Tattersall 2005; Farr and Tattersall 2004). After the genome is packaged 20 30 bases of the 5′-end remain on the outside of the capsid with an NS1 or Rep molecule covalently attached, and that DNA can be cleaved off without affecting viral infectivity (Cotmore and Tattersall 1989; Prasad and Trempe 1995). The capsid has a limited capacity for DNA, with increased lengths >110% of those found in the wildtype viruses being inefficiently or incompletely packaged (Cotmore and Tattersall 2005; Grieger and Samulski 2005). In many viruses, the genomic DNA also associates with the interior surface of the capsid, with the bases of the interacting sequences facing towards the protein (Chapman and Rossmann 1995). While no single sequence is associated with the ssDNA capsid interaction, there are low level motifs associated with many of the 60

interactions (Chapman and Rossmann 1995). That specific association of the viral sequences may result in alternative sequences replacing the viral sequences in the viral genome not being packaged as efficiently and can also result in the particles being less infectious (Lang et al. 2005). The dependence on specific genomic sequences may differ between the autonomous parvoviruses and the AAVs, since the latter appear to be more tolerant of replacement of viral sequences by those from other sources (Kestler et al. 1999). Although the DNA-containing (full) and empty particles appear very similar in X-ray crystal structures (Wu and Rossmann 1993), they do have some distinct properties. The N-terminal 18 20 residues of some copies of the VP2 of the autonomous parvoviruses are displayed on the outside of full capsids where they can be cleaved to VP3, while for the AAVs, the empty and full capsids have different surface charges (Brument et al. 2002; Okada et al. 2009; Qu et al. 2007).

The shared region of the capsid proteins contains a core eight-stranded β-barrel that comprises a main structural domain of the protein, and large insertions between some of the β-strands form most of the exposed surface of the capsid (Chapman 1998). Some capsid proteins assemble into capsids when expressed alone in mammalian or insect cells, although the VP1 may not assemble when expressed alone, likely because of interference from the large N-terminal domains. Only the major capsid protein (VP2 or VP3) is required to make a capsid and package the viral ssDNA. However, those particles are noninfectious when lacking the functional N-terminal unique sequence of the VP1, as that is necessary for cell infection, likely because of its role in the escape of the capsid from the endosomal compartments and nuclear trafficking. The interactions between the capsid proteins involve both residues in the β-barrel and in the inter-strand loops (Reguera et al. 2004). There are clearly conserved assembly structures that are shared between viruses of different AAV serotypes, as mosaic capsids that contain the capsid proteins of multiple serotypes (which differ by up to 40% in protein sequence) can be prepared by co-expressing multiple capsid proteins together in the same cell (Rabinowitz et al. 2004). Similarly, shuffled capsid protein sequences comprised of varying portions of the capsid proteins of two or more AAV serotypes have been generated, and many of those can assemble and form infectious capsids (Grimm et al. 2008; Li et al. 2008) (Fig. 2).

Additional sequences can be inserted into exposed surface loops of the capsids or added to either the N- and C-termini of the capsid proteins (Muller et al. 2003). The positions of insertion of the peptides may need to be quite specific to allow assembly into capsids. Short peptide sequences inserted may contain antigenic or receptor-binding peptides, while others may be as large as green fluorescent protein or antibody binding domains (e.g., Girod et al. 1999; Grifman et al. 2001; Michelfelder et al. 2007; White et al. 2007). In many cases, the capsids with insertions are infections, indicating that for some viruses, particularly for AAVs, a variety of receptors that show no relationship to the normal host cell receptor can be used for cell infection. Sequences added to the N or C termini of the AAV capsid proteins may still allow capsid assembly, and those can also be displayed on the surface of capsids that retain infectivity (Lux et al. 2005; Zhang et al. 2002). It is not

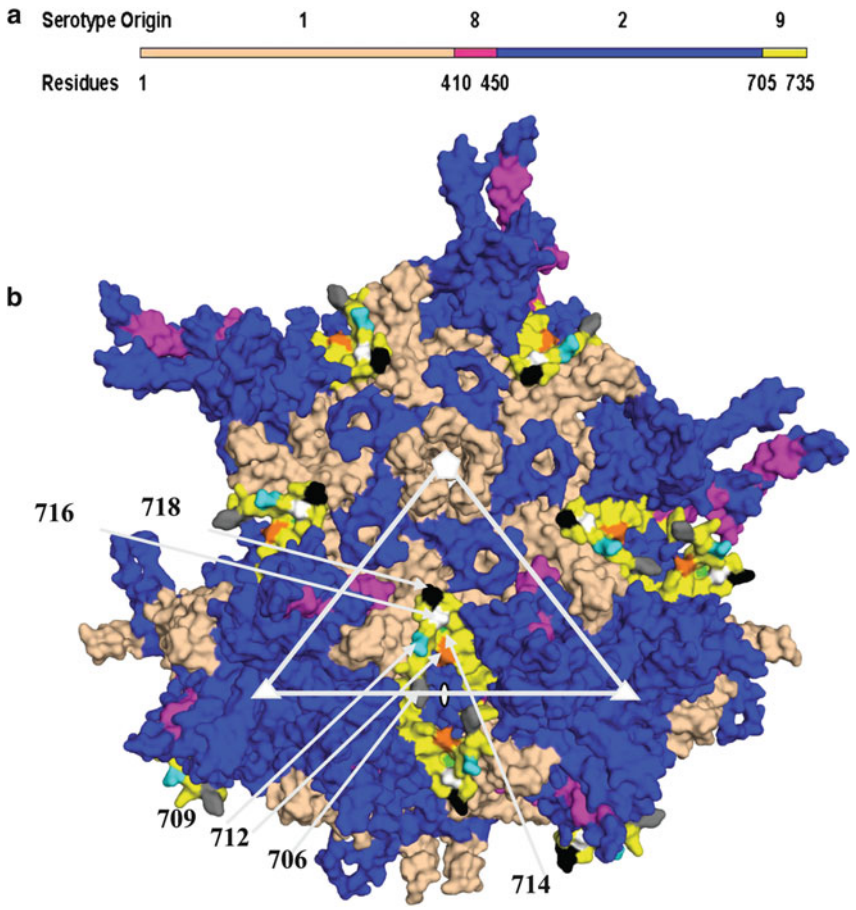

Fig. 2 An example of a chimeric AAV capsid (Chimeric 1829) prepared by shuffling portions of the AAV2, AAV8, and AAV9 protein encoding genes to make a complete gene, and then selecting for the versions that are functional and that can package DNA and transduce CS1 cells. (**a**) Primary structure of novel adeno associated virus (AAV) variant selected from CS1cell. (**b**) Three dimensional model showing VP3 subunits of AAV1829 in relation to the fivefold (*white penta gon*); threefold (*white triangle*) and twofold (*white oval*) axes of symmetry. AAV1 derived residues are colored salmon, AAV2 in *blue*, AAV8 in *dark pink*, and AAV9 in *yellow*. From (Li et al. 2008) with permission

clear from the capsid structures how those sequences become displayed on the capsid surface, as the C-terminus of the protein is on the interior of the particle as are the N-termini of most of the proteins. The N-terminal unique extension of VP1 (120 200 residues) is essential for infectivity of the capsids (Tullis et al. 1993), and that contains motifs that include basic amino acids that likely function in nuclear transport of the proteins, as well as the Ca^{++}-dependent PLA2 enzyme activity that is required for infection of cells (Farr et al. 2005; Grieger et al. 2006; Zadori et al. 2001). That VP1 unique domain is sequestered inside the capsid after assembly of

AAV or most autonomous parvovirus capsids, and it is displayed on the outside only after relatively robust treatments or during cell entry within endosomes, although those treatments leave the capsids largely intact (Kronenberg et al. 2005; Sonntag et al. 2006).

There may be a different arrangement of the VP1 and VP2 proteins in the human parvovirus B19 capsid. When examined by cryoEM analysis, the VP2 N-termini appeared to be displayed on the surface of intact particles adjacent to the fivefold axes, but appeared not to pass through the pore (Kaufmann et al. 2008) (Fig. 3). In

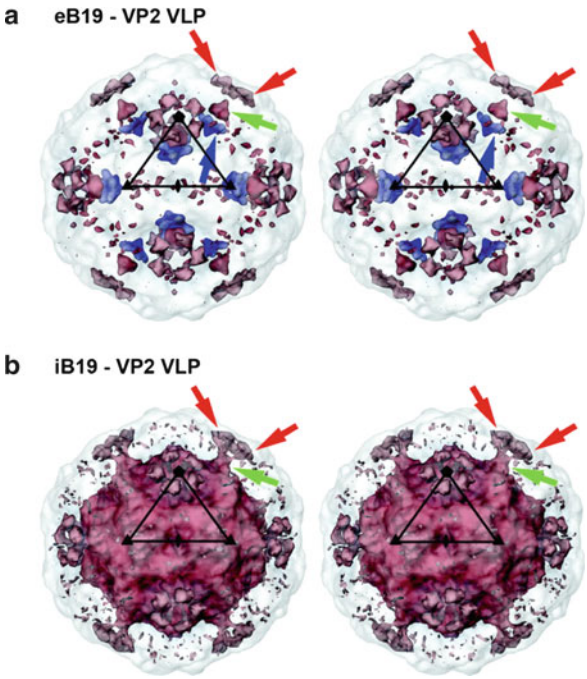

Fig. 3 Significant differences between wild type B19 (empty or containing DNA, but also containing VP1) and recombinant VP2 particles (lacking VP1) cluster at and around the icosahedral fivefold axes. (**a**) Stereoview of a surface rendering of a difference map between empty B19 and B19 VP2 VLPs at 11.3 Å resolution, viewed down an icosahedral twofold axis. Positive densities are rendered in *red*, and negative densities in *blue*. The difference densities are superpositioned onto a semitransparent VP2 VLP. The *black triangle* marks an icosahedral asymmetric unit. The most significant positive differences (matter present in empty B19 particles but not VLPs) are located around the fivefold cylindrical structure at the outer viral surface, labeled for one fivefold axis by *red arrows*, and within the fivefold channel (*green arrows*). Significant negative difference densities (matter present in VLPs but not empty B19 capsids containing VP1) can be identified next to the fivefold axes at the inner viral surface (*blue arrows*). (**b**) A difference map between infectious (DNA containing) B19 and B19 VP2 VLPs at 7.7 Å resolution, as described for panel A. The largest positive differences besides the central DNA density are located around the fivefold cylinder at the outer viral surface, indicated by *red arrows*, and at the base of the fivefold channel, highlighted by *green arrows*. Connecting density diverges off the central fivefold axis halfway up the pore. From Fig. 3 of (Kaufmann et al. 2008) with permission

addition, the unique region of VP1 in B19 particles appears to be more readily exposed than those of other parvoviruses, and it displays neutralizing epitopes accessible to antibodies outside the cell (Ros et al. 2006; Rosenfeld et al. 1992; Saikawa et al. 1993). It is possible that the VP1 N-terminal domain is on the outside of the capsid although sequestered, and that it becomes exposed under relatively mild conditions (Ros et al. 2006).

2 Dynamic Properties of the Parvovirus Capsid Structure and Cell Infection

Parvovirus capsids are robust structures, and most remain infectious after heating to temperatures between 50 and 65°C for many minutes, as well as after exposure to high or low pH, or to salts or detergents (Mani et al. 2007; Nelson et al. 2008; Yunoki et al. 2003). However, the capsids are highly multifunctional and it is now clear that their structures can undergo various controlled modifications. A proportion of the N-terminal sequences of the VP2 molecules of the autonomous parvoviruses are exposed on the surface of the full (DNA containing) capsids, and may be cleaved off by various proteinases between about 18 and 24 residues from the N-termini (Clinton and Hayashi 1976; Nelson et al. 2008). As those N-termini are likely exposed through the pores at the fivefold axes of symmetry, and each pore has the capacity to display only one peptide at a time, then up to 11 VP2 N-terminal peptides would be exposed simultaneously (one pore being occupied by the 5′-proximal sequences of the viral DNA). As up to 90% of the ~55 VP2 N-termini can be cleaved off upon protease treatment, the four or five VP2/VP3 N-termini (depending on the presence of VP1) must exchange through the pores that are close to the positions they occupy. Those four or five VP2 N-termini surrounding the pore that is occupied by the DNA may not be able to exchange, leaving ~10% of the VP2 uncleaved after extended treatment. VP2 terminal exposure appears to be a dynamic process that relies on the opening and closing of the pore, and it is also temperature dependent, and VP2 and DNA exposure can both be modified by mutations of residues within the pore (Farr et al. 2006; Farr and Tattersall 2004; Grieger et al. 2007). VP2 to VP3 cleavage is also associated with a shift in buoyant density of the capsids on CsCl gradients (Clinton and Hayashi 1976). However, comparison of the high resolution structures of particles does not show any significant differences that explain the density difference (Kontou et al. 2005; Wu and Rossmann 1993), and it may result from changes in cesium accessibility of the capsid or to other small or reversible conformational changes.

The exposed N-terminal peptide of VP2 also influenced the nuclear export of newly produced full capsids of MVM, in a process controlled by phosphorylation of Ser residues within that sequence (Maroto et al. 2004). In the capsid structures of AAV, the VP3 protein (the major capsid protein) does not have its N-terminus exposed on full particles, but the N-terminus of VP2 (an intermediate sized protein

in AAV) may be exposed after relatively mild treatments, and fusing additional protein to that sequence gives capsids with the extra domain exposed on the outside (Warrington et al. 2004). Transient differences in structure and flexibility of capsid surface loops also occur, and those have been identified in the atomic structures of the capsids due to variation in structures determined under different conditions, by differences in temperature factors (Simpson et al. 2000), and also by changes in proteinase sensitivity (Nelson et al. 2008; Van Vliet et al. 2006) (Fig. 4). Those transient changes are likely important for many of the viral functions; for CPV alterations in the flexibility of surface loops influences the virus binding to receptors and to type-specific antibodies, even though the structures appear to be otherwise very similar (Govindasamy et al. 2003; Hafenstein et al. 2009; Hueffer et al. 2003a).

The capsid stability and protease susceptibility of different viruses also likely depend on the environments they encounter in their normal life cycles, and would therefore be influenced both by their routes of transmission and sites of replication. For example, canine parvovirus (CPV) is produced in the intestinal epithelial cells and shed in the feces of infected animals and is resistant to most proteinases. However, a small proportion (5 15%) of the VP2 or VP3 in the capsids can be cleaved at one or two positions within the surface loops and still be retained within the capsid (Nelson et al. 2008) (Fig. 4). Some of those cleaved sites are within binding sites of the TfR and of antibodies and those would likely prevent or favor binding of those ligands (Hafenstein et al. 2007, 2009). Other parvoviruses and many AAVs appear to be transmitted by respiratory routes, and their capsids can be more susceptible to digestion of surface loops by proteases such as trypsin, and those cleavages then reduce binding of the capsid to heparin sulfate proteoglycan (HSPG) (Van Vliet et al. 2006). The capsids of Aleutian mink disease virus purified from tissues of mink with chronic infections are digested at several positions (Aasted et al. 1984).

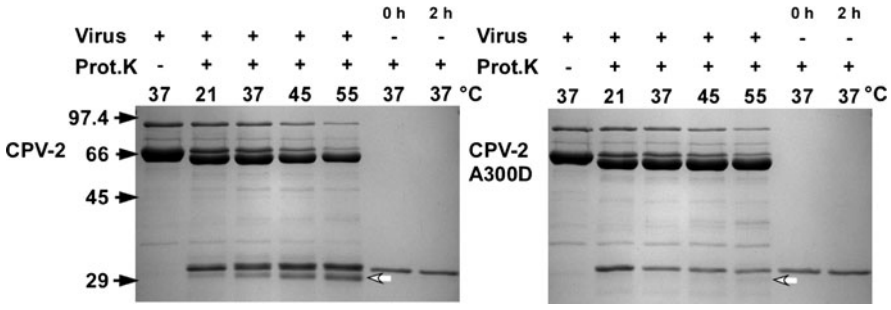

Fig. 4 Small changes in the sensitivity of the full (DNA containing) canine parvovirus capsids to treatment with proteinases K, due to the presence of altered residues within surface loops. Here CPV full capsids are incubated with proteinase K, as indicated. While all digested virus samples show conversion of VP2 to VP3, a difference in the susceptibility to protease cleavage is determined by whether VP2 residue 300 is an Ala (CPV 2 left) or Asp (CPV 2 A300D). Adapted from Fig. 3 of (Nelson et al. 2008) with permission

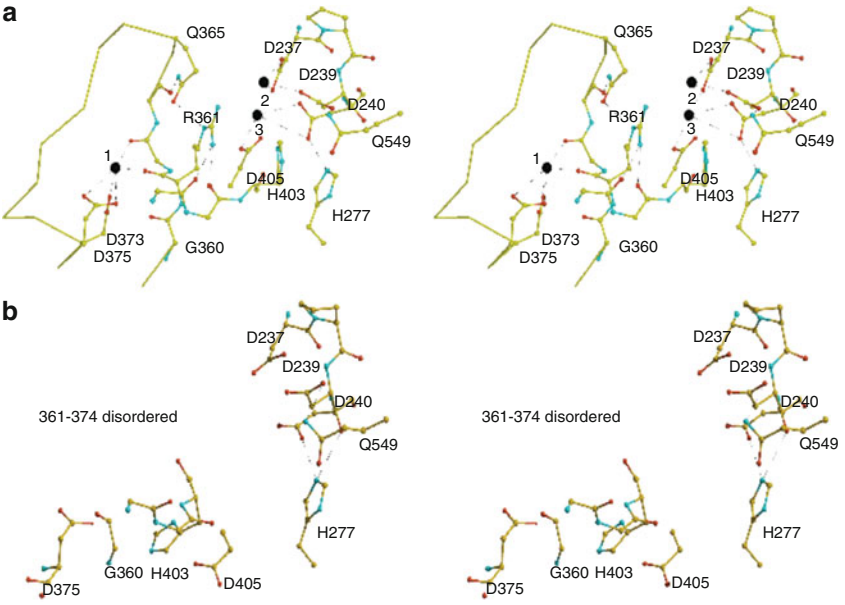

Fig. 5 The locations of divalent ions in the capsids of FPV crystallized at pH 6.2 (**a**) or 7.4 (**b**). The images are stereoviews of FPV capsids loops that coordinate divalent ions (*black dots*), likely Ca^{2+} under the conditions of these studies. Residues that are involved in coordinating the ions within the structure are identified. At pH 7.5 there are three ions coordinated into each capsid subunit, while there are two at pH 6.2. Further ions are removed at lower pHs, or by treating with EDTA (not shown). From Fig. 5 of (Simpson et al. 2000) with permission

Other capsid structural flexibility derives from the binding and release of ions, including Ca^{++} or Mg^{++} (Simpson et al. 2000). In the case of CPV and FPV capsids, 120 (CPV) or 180 (FPV) (2 or 3 per subunit in the $T = 1$ capsid) divalent ions are coordinated into the capsid by a cluster of Asp and His residues. Treatment of the capsids with low pH or EDTA could remove those ions, with intermediate forms containing one or two ions bound per subunit, resulting in changes in the position and flexibility of surface loops (Simpson et al. 2000) (Fig. 5). Similar divalent ion-coordinating structures have been described in MVM, where removal of those ions resulted in more ready exposure of the viral DNA (Cotmore et al. 2009), and it is likely that ions are bound into the capsids of other parvoviruses.

An important structural change essential for cell infection is the release of part or all of the viral DNA. Complete disintegration of the viral capsid does not appear to occur except under harsh conditions (such as heating to >70°C), but more subtle changes can allow the release of 3′-end of the viral DNA so that it can act as a template for DNA polymerase (Cotmore et al. 1999; Farr et al. 2006). Conditions that allow that release include heating to non-physiological temperatures (>45°C), treatment at very high or low pHs, or removal of capsid-bound ions, and some of those conditions also release the VP1 N-terminal domain (Cotmore et al. 1999,

2009; Farr et al. 2006; Nelson et al. 2008). The natural processes that cause the viral capsid to release the internal structures or DNA during cell infection are not yet fully defined, although the low pH of the endosomes (perhaps along with bound receptor) and the low Ca^{++} concentration of the cytoplasm are likely candidates. Other modifications in the endosome or cytoplasm include proteolysis by cathepsins B or L, caspase cleavage, or conjugation with mono- or poly-ubiquitin, all of which may lead to further modifications or digestion (see also below).

3 Receptor Binding, Structural Effects, and Internalization from the Cell Surface

Parvovirus infection initiates with virion binding to one or more cell surface receptors, followed by receptor-mediated endocytosis. Many different types of molecules have been identified as receptors or co-receptors for different viral capsids, including glycoproteins, glycans, and glycolipids (Table 1). Some viruses can bind to multiple cell surface receptors, although it is not always clear what the role of each receptor is in the infectious process or how they interact with the capsid or with each other.

For example, CPV and the closely related FPV use the feline transferrin receptor-1 (TfR) as the primary receptor for binding and uptake, but many strains of those viruses also bind to sialic acids. However, the binding of virus to sialic acid on cells at neutral pHs appears to decrease virus infection, and prolonged growth of a virus in a cell expressing a sialic acid that it can bind selects for viral mutants that no longer bind that sialic acid, so a direct beneficial role in cell infection is unlikely (Barbis et al. 1992; Hueffer et al. 2003b; Palermo et al. 2003). The sialic acid

Table 1 The receptors that have been defined as binding to parvoviruses, which in most cases also mediate the process of cell infection

Virus	Cell surface receptors and binding molecules	Host(s)
Minute virus of mice	Sialic acids	Rodents
Human B19 virus	Globotetraosylceramide or globocide erythrocyte P Antigen	Humans (primates)
FPV and CPV	Transferrin receptor 1 Sialic acid in some breeds	Cats, dogs, related carnivores (host ranges may differ)
AAV2	Heparan sufate proteoglycan, αVβ5 integrin, fibroblast growth factor receptor 1, hepatocyte growth factor receptor 2	Human
AAV4	*O* linked α2 3 sialic acid	Human
AAV5	*N* linked α2 3 sialic acid Platelet derived growth factor receptor	Human
AAV6	*N* linked α2 3 and α2 6 sialic acid	Human
AAV8	37/67 kDa laminin receptor	Human
Bovine AAV	Gangliosides	Bovine

binding of those viruses varies in its pH dependence, but all wild-type viruses can attach at pH < 6.5, and only some strains bind at pH > 7.0. The form of the sialic acid is also important, and the viruses can bind N-glycolyl neuraminic acid (Neu5Gc) but not N-acetyl neuraminic acid (Neu5Ac). FPV only binds sialic acid at pH < 6.5, and hence would not bind the Neu5Gc expressed by cats at the neutral pH of the tissues or blood. As many dogs only express Neu5Ac, they would not bind CPV under any condition. It is therefore possible that the sialic acid binding activity of the capsids is conserved because it is useful at some other stage of the life cycle, such as transmission via the intestinal contents or in feces, which have a pH of 6.5 or less, and where binding might protect the virus in the environment.

Many strains of AAV2 are reported to bind to HSPG, αVβ5 integrin, fibroblast growth factor receptor 1, hepatocyte growth factor receptor, and the 37/67-kDa laminin receptor. While HSPG is a key functional receptor for cultured cells, some AAV2 variants do not bind that receptor, and mutations that prevent HSPG binding leave the virus infectious (Shi et al. 2001, 2006). HSPG binding is associated with surface-exposed patches of positively charged residues, and binding induces small changes in the capsid structure, which are detectable by cryoEM analysis (Levy et al. 2009; O'Donnell et al. 2009) (Fig. 6). As mentioned, proteinase cleavage within the HSPG binding site prevents binding to the cleaved capsid site (Van Vliet et al. 2006). AAV5 binds to both α2-3- and α2-6-linked sialic acids and also to the platelet-derived growth factor receptor (PDGFR-α-polypeptide), and those can independently mediate virus infection (Kaludov et al. 2001; Pasquale et al. 2003; Walters et al. 2001). How and why these viruses retain the necessary binding sites and structures for so many receptors is unclear, but given the small size of the capsids, there must be significant overlap between the different binding sites.

While the feline and canine TfRs both bind to CPV capsids, those show markedly different affinities of attachment, as well as distinct patterns of attachment and endocytosis on cells in culture. At early times after virus addition, CPV capsids bound over the entire surface of the feline cells, but attached primarily to filopodia of canine cells, although the later stages of the infection pathways converged on the same endosomal compartments (Harbison et al. 2009).

3.1 Binding Site Insertion and Retargeting

In studies aimed at improving gene therapy vectors, AAVs have been experimentally modified to introduce different cell or tissue tropisms. Those have included the insertion of a variety of different peptides and protein domains into surface loops of the capsids, allowing them to bind alternative receptors (Stachler and Bartlett 2006). In other cases, randomized sequences were introduced into the capsids, and after transfection into cells, those that were most efficiently infecting were selected (e.g., Grifman et al. 2001; Michelfelder et al. 2007; Ried et al. 2002; Shi et al. 2001; Yu et al. 2009). Those modified capsids still assembled and packaged the viral genome, but they transduced cells using alternative receptors, although in

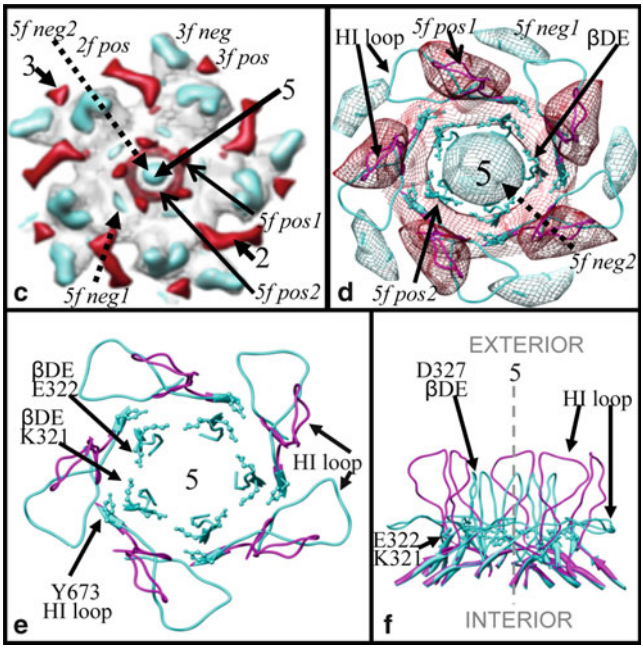

Fig. 6 The cryoEM structures of AAV2 capsids (virus like particles VLPs) that are either free of ligand, or that are bound to heparin, showing the changes in the outer capsid structure around the fivefold axis that occurs upon receptor binding. (**a**) Difference map of AAV2 Heparin minus AAV2 VLP), with positive (*red*) and negative (*cyan*) differences superimposed onto a shaded surface density map (*gray mesh*) of the AAV2 crystal structure close to the fivefold axis. Positive and negative density differences are labeled (e.g. *2f pos positive density close to the twofold axis*). (**b**) Close up of fivefold channel and HI loop difference densities. HI loop is shown as observed in the crystal structure (*cyan coil*) close to the negative (*cyan*) density and modeled (*magenta coil*) into positive (*red*) density. βDE ribbons (*cyan coil*, as in the crystal structure) are indicated with *arrows*. (**c**) Top down view of HI loop model. Positions of residues K321 and E322 at the base of the βDE ribbon, and Y673 at the base of the HI loop are indicated by *arrows* for some of the monomers. The cyan loops and residues indicate the position of these regions in the crystal structure. The *magenta coil* indicates proposed new position of HI loop. (**e**) Side view of HI loop model. The positions of the HI loops when heparin is not bound (*cyan*) and when heparin is bound (*magenta*). Residue D327 at the tip of the βDE ribbon, and the HI loop are indicated with *arrows* for one monomer. A two , three , and fivefold axis are indicated on each panel where appropriate. Adapted from Fig. 3 of (Levy et al. 2009) with permission

some cases with lower efficiency. For CPV and FPV, the binding to the TfR may be necessary for successful infection. However, as long as they bind to some level, those viruses can variously use the feline, canine, or human TfRs for uptake and infection, even though each shows different binding affinities (Palermo et al. 2006). Modification of the capsid to insert an Arg Gly Asp integrin binding motif into the surface allowed transduction of cells by a luciferase-expressing CPV vector (Maxwell et al. 2001). But, CPV was not able to infect cells using a modified

receptor composed of antibody domains linked to the stalk, transmembrane, and cytoplasmic sequences of the feline TfR (Hueffer et al. 2004). MVM capsids use sialic acids as their primary receptors, and mutant capsids with altered sialic acid binding properties resulted in different efficiencies of cell infection and altered tropism (Lopez-Bueno et al. 2006).

3.2 Receptor Binding and Effects on Capsid Structure

An open question is whether the attachment of the receptor to the capsid itself induces changes in the structure, and whether such changes alter cell infection. Analysis of CPV capsids incubated with purified TfR ectodomains did not show significant alterations in the protease susceptibility (Nelson et al. 2008). As mentioned previously, heparin binding to AAV2 capsids induced structural changes that were detectable by cryoEM, including a slight opening of the pore at the fivefold axis and a flattening of the threefold protrusions (Levy et al. 2009; O'Donnell et al. 2009) (Fig. 6). The capsids of CPV were seen to bind to sphingomyelin and induce changes in the structure that were detected by fluorescence methods (Pakkanen et al. 2008).

4 Antibody Binding and Capsid Structures and Functions

All parvoviruses that infect vertebrates induce strong anti-capsid antibody responses, which generally assist in the process of recovery and protect against reinfection. The capsids are potent antigens, and induce strong antibody production, immune selection, and affinity maturation, and the capsid structure would likely have been molded by immune selection. The raised regions on the surface of the parvoviruses infecting vertebrates may specifically favor the production of antibodies, as the capsids of the densoviruses that infect invertebrates have much smoother surfaces (Bruemmer et al. 2005; Simpson et al. 1998). The antibody binding structures of parvoviruses can be defined by identifying surface residues that alter or prevent antibody binding, by identifying capsid peptides that bind to antibodies, and by cryoEM of complexes between the capsids and antibody Fabs (Hafenstein et al. 2009; Kaufmann et al. 2007). Those various approaches give different but overlapping views of the antibody recognition. In the case of CPV and FPV, mutations that altered recognition by monoclonal antibodies (MAb) clustered into two small regions of the capsid surface, described as the A and B sites (Strassheim et al. 1994). When the binding footprints of eight of the MAb were defined on the surface of the capsid using cryoEM, those covered ~70% of the viral surface. Those were primarily focused on the raised region of the capsid, while regions not contacted by the antibodies were in surface depressions or on the cylinder surrounding the fivefold axis of symmetry (Hafenstein et al. 2009) (Fig. 7). An antigenic site on the capsid of MVM recognized by a mouse

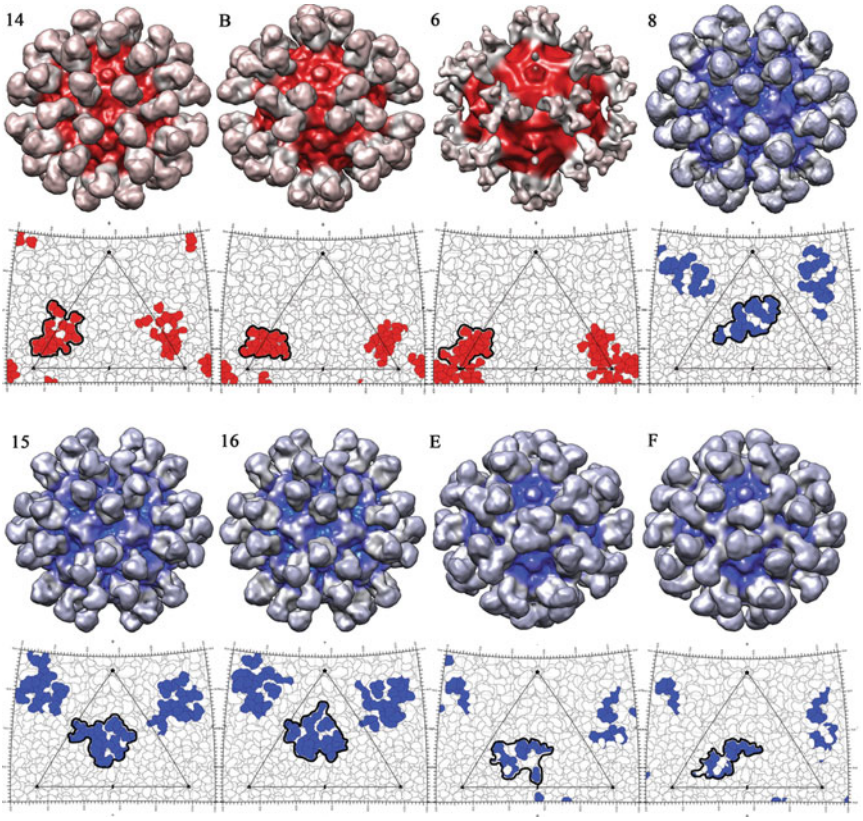

Fig. 7 Surface rendered cryoEM reconstructions of canine parvovirus (CPV) complexed with Fab fragments from eight different anti capsid antibodies. Density further than a 125 Å radius from the center of the virus is shown in *gray*. Shown directly below each reconstruction is the footprint of the Fab in the complex. Two general antigenic sites have been defined on the CPV capsid, identified as A and B, and complexes and footprints are color coded *red* for antibodies binding the A site, and *blue* for antibodies binding the B site. From Fig. 3 of (Hafenstein et al. 2009) with permission

MAb was at the top of the threefold axis of symmetry in a position that allowed only a single Fab to bind per threefold (Kaufmann et al. 2007). Systematic mutational analysis of the AAV2 capsid showed that positions affecting the binding of a mouse MAb and human polyclonal antibodies were clustered on or near prominent protrusions around the threefold axes of the capsid (Lochrie et al. 2006).

Many antibody binding sites directly overlapped receptor binding sites, suggesting a complex interaction between those two functions. This was emphasized by the results obtained for eight MAb against CPV, which all neutralized the virus when tested as IgGs. However, five of the eight Fabs derived from those antibodies were non-neutralizing, even though their binding sites overlapped the TfR

binding site on the capsid, and most showed some competition for binding of the TfR (Nelson et al. 2007).

5 Endosomal Uptake, Signaling, and Infection

All parvoviruses infect cells by endocytosis, but the details of the processes used are still being revealed. The TfR enters cells by clathrin-mediated endocytosis, and under normal circumstances, the capsids of CPV and FPV are also taken up by that process (Parker and Parrish 2000). However, altering or deleting the Tyr Thr Arg Phe (YTRF) AP2 binding signal in the cytoplasmic tail of the TfR delayed uptake but did not significantly reduce virus infection (Hueffer et al. 2004). Also, expressing a dominant negative dynamin-2 in cells delayed uptake and infection by CPV and AAV, but many of those cells still become infected, albeit with delayed kinetics, indicating that various uptake mechanisms can be used (Duan et al. 1999; Hueffer et al. 2004; Parker et al. 2001). Minute virus of mice, AAV2, and AAV5 capsids use sialic acids or HSPG as cell surface receptors and are taken up primarily by clathrin-mediated endocytosis, even though those receptors do not specifically mediate clathrin-mediated uptake (Bantel-Schaal et al. 2002; Bartlett et al. 2000; Linser et al. 1977). Other routes of cell entry by parvoviruses could include nonspecific pinocytosis from the cell surface or less well defined clathrin-independent mechanisms (Marsh and Helenius 2006; Smith and Helenius 2004).

An unresolved question for many parvoviruses is the specific role of receptor binding in inducing cellular signaling pathways to influence uptake or infection. AAV2, for example, binds and clusters αVβ5 integrins, which signal through Notch1 and Rac to enhance internalization by clathrin-mediated endocytosis (Sanlioglu et al. 2000). Receptor clustering and cross-linking may also affect the later intracellular trafficking of the receptor virus complex. Clustering TfR with oligomeric transferrin changes the intracellular trafficking patterns of the TfR-bound ligand, and so it is likely that the uptake and trafficking of multivalent parvoviral capsids is likewise altered. CPV capsids are taken up by the TfR and many enter the Rab11 recycling compartment as expected from the normal trafficking of transferrin, but do not recycle to the cell surface (Harbison et al. 2009). Possible reasons include the clustering and cross-linking of the dimeric receptor by the multivalent capsids, while the size of the capsid may also prevent efficient trafficking within the vesicular-tubular portions of the recycling pathways.

In some cases, the viruses bind the cell receptors and are internalized, but infection does not occur, possibly due to alterations in trafficking that prevents viral release or that delivers the particle to the wrong cellular compartment. For example, transduction of recombinant AAV2 capsids is more efficient from the basolateral surface of polarized human airway epithelia compared to the apical surface, despite similar numbers of particles binding and apparently entering from each surface (Duan et al. 1998).

5.1 Trafficking Within the Endosomal System

Virus particles attached to receptor(s) enter the normal endosomal pathways of the cell and then rapidly traffic through the early endosomes to the later compartments, including the late endosomes, recycling endosomes, or both compartments, and in some cases particles can later be found within lysosomes. The particles can move with different speeds as they pass through the different vesicles or vesicular-tubular portions of the endosomal trafficking pathways. The complexity and dynamic nature of viral movement within the endosomal compartments are becoming increasingly appreciated, particularly by examination of particles in live cells. For CPV, cells fixed after viral uptake and stained with antibodies show capsid accumulation in perinuclear vesicles within 30 min. This normal uptake pattern was disrupted by depolymerization of the microtubule network with nocodazole, exposure of cells to lower temperatures, or by expression of a dynamin-2 K44A dominant negative mutant (Parker and Parrish 2000; Suikkanen et al. 2003a; Vihinen-Ranta et al. 1998).

After uptake, dissecting the dynamic properties and functional roles of the different endosomal compartments the viruses must pass through before escaping into the cytoplasm has proven difficult (Suikkanen et al. 2003b). The particle-to-infectious unit ratio of most parvoviruses appears to be high (100:1 to >10,000:1), so most particles entering the cell do not infect. In the case of CPV, the entering virions infect slowly, and capsids stay associated with the TfR in the endosomal system, since infection can be blocked by injecting an antibody against the cytoplasmic tail of the TfR into cells after viral uptake (Parker et al. 2001). When cells are fixed at different times after uptake, CPV and AAV capsids have been variously co-localized with markers of the early endosome, late endosome, recycling endosome, and lysosome (Bartlett et al. 2000; Ding et al. 2005, 2006; Parker and Parrish 2000; Suikkanen et al. 2002). Live cell analysis of fluorescently labeled particles showed simultaneous localization in various endosomal compartments and overlapping types and rates of particle movement within the vesicular system (Harbison et al. 2009; Seisenberger et al. 2001).

Many of the serotypes of AAV have ~45% and <10% differences in the amino acid sequences of their capsid proteins, and those can show diverse intracellular trafficking patterns. For example, capsids of AAV5, but not other serotypes, were seen to accumulate in the Golgi compartment (Bantel-Schaal et al. 2002). Differences in cell type and capsid concentration may also affect the distribution of AAV2 particles within different endosomes and the efficiency of transduction (Ding et al. 2006; Hansen et al. 2001). When cells are fixed after uptake of low multiplicities of particles, AAV2 capsids localize primarily in Rab7-positive late endosomes, while at higher multiplicities they showed increased localization with Rab11-positive recycling endosomes. When Rab7 or Rab11 are overexpressed, or inhibited by RNAi treatments, the Rab11 pathway allowed more efficient transduction of AAV2 than Rab7-positive vesicles (Ding et al. 2006). However, other studies suggest that AAV2 escapes from an early endosomal compartment, and that trafficking to later

compartments are less important for infection (Bartlett et al. 2000; Mani et al. 2006). The reasons for these different results are unclear.

5.2 Low pH and Virus Infection

Acidification of the endosomes is essential for infection by all parvoviruses examined, although the specific role of the low pH has not been defined. Bafilomycin A_1 inhibits the ATPase responsible for endosomal acidification while NH_4Cl neutralizes the endosomal pH, and both significantly reduce infection if added within 30 min of AAV inoculation (Bartlett et al. 2000) or within 90 min for CPV (Parker and Parrish 2000). Pre-exposure of capsids to low pH does not overcome any of those blocks to infection (Parker and Parrish 2000; Suikkanen et al. 2002). Some reversible changes in capsid structures occur at low pH, including release of bound divalent ions from CPV capsids, and internal capsid components (such as the VP1 unique region) may be more easily released under these conditions (Farr et al. 2005; Mani et al. 2006; Vihinen-Ranta et al. 2002), although in studies of CPV capsids using proteinases as probes, variation in capsid structure was detected at pH 4.5 but not at pH 5.5 (Nelson et al. 2008). Alternatively, an acid-dependent host factor may be required for infection, such as an acid-dependent protease or endosomal trafficking, and vesicular fusion components may be low pH-dependent (Akache et al. 2007; Sollner 2004; van Weert et al. 1995; Vihinen-Ranta et al. 1998).

Cathepsins B and L interact with AAV2 or AAV8 proteins by yeast two hybrid screening, and inhibitors of these proteases decrease transduction in vivo (Akache et al. 2007). Trypsin- or other protease-mediated cleavage of the AAV2 capsid surface may be involved in initiating structural rearrangements that increase capsid flexibility in preparation for uncoating. While the particles remain intact in vitro, differences in negative staining suggests structural rearrangement or flexibility due to the cleavage event (Van Vliet et al. 2006). While the prolonged incubation with proteases reduces the infectivity of the particle due to loss of heparin binding activity, that may also assist in uncoating and release of the viral genome (Van Vliet et al. 2006).

5.3 Capsid Structural Changes and Endosomal Escape

Besides the VP1 release described earlier, there may be virus-specific processing requirements. The parvovirus B19 is sensitive to inactivation at low pH and exposes both the VP1 unique N terminus and the genome under these conditions (see Fig. 3), while CPV and MVM capsids remain intact and infectious (Boschetti et al. 2004; Ros et al. 2006; Simpson et al. 2000). Cleavage of VP2 to VP3 in the

autonomous parvoviruses may enhance the release of the VP1 N terminal sequences (Farr et al. 2006; Farr and Tattersall 2004; Ros et al. 2002). The VP1 unique region appears to be extruded through the fivefold pore of CPV and MVM without capsid disassembly when the virus is in the endosome, and one of those pores also likely forms the site for genome release later in infection (Cotmore et al. 1999; Farr and Tattersall 2004). Capsids may show VP1 release even in the presence of endosomal neutralizing drugs (Mani et al. 2006; Suikkanen et al. 2003b). The PLA2 activity is responsible for endosomal escape of MVM, as point mutants in the PLA2 active site could infect cells when endosomal lysis was induced by addition of polyethylene imine or adenovirus capsids (Farr et al. 2005). Exposure of AAV to acidic conditions also results in conformational changes that activate or expose the PLA2 domain of AAV2 capsids. Mutations in the active site of the PLA2 of AAV result in delay of gene expression, also suggesting a role in viral entry (Girod et al. 2002). Capsid mutations that reduce exposure of the PLA2 domain dramatically decrease infectivity, and mutations of residues around the fivefold axis of symmetry that are predicted to relax the pore can restore transduction activity in mutant particles lacking VP1 (Bleker et al. 2005; Grieger et al. 2007). The viral PLA2 is not directly lytic, but modifies membranes and may induce curvature by altering the lipid head groups to change their packing, and other viral or cellular factors likely cause endosomal escape. Only very transient or limited pore form in the endosomal membrane must occur, as neither α-sarcin nor large dextrans enter the cytoplasm with incoming viral capsids (Parker and Parrish 2000; Suikkanen et al. 2003b).

6 Viral Trafficking in the Cytoplasm

The capsid-associated processes that operate in the cytoplasm are still poorly understood. In general, it is difficult to specifically examine capsids that are within the cytoplasm, as most of the incoming capsids are retained in the endosomal system for many hours after uptake. Capsid-associated cytoplasmic processes may to be examined by microinjecting capsids into the cytoplasm or by plasma membrane permeabilization with digitonin. Capsids may be modified or proteolyzed in the cytoplasm, and inhibitors of proteosome activity reduce MVM infectivity if they are added up to 3 h after infection, late enough to affect a cytoplasmic processing event. Viruses entering cells in the presence of protease inhibitors accumulated at the cytoplasmic side of the nuclear membrane but did not enter the nucleus (Ros et al. 2002; Ros and Kempf 2004). However, most inhibitors used can affect a variety of proteases including those in the endosomes, and they may be generally toxic to the cells, which would nonspecifically reduce viral infection and replication. Proteases play both positive and negative roles in AAV infection. The proteasome appears to inhibit AAV entry, and treatment with proteasome inhibitors enhances AAV transduction, perhaps by altering endosomal

trafficking, processing of capsids, or by decreasing ubiquitin-dependent degradation of cytoplasmic capsids. Intact particles appear not to be highly ubiquinated, and endosomal processing and partial disassembly may be required to prime AAV capsids for ubiquitination (Yan et al. 2002). Microinjection of capsids into cells after they have been treated with low pH or various proteases would show whether those modifications are sufficient to activate the particles. Indeed, some processing steps appear to be required, as AAV2 capsids injected into the cytoplasm did not give a productive infection even in the presence of adenovirus helper (Sonntag et al. 2006). Alternatively, cleavage of capsid proteins could lead directly to DNA uncoating or may prime the capsids for ubiquitination. Ubiquitination and proteasome-mediated degradation of AAV vectors can be modulated by EGFR protein tyrosine kinase activity, and inhibition of that activity blocked degradation of AAV and facilitated nuclear transport and transduction of AAV vectors, and the modification of surface Tyr residues to Phe enhanced AAV2 transduction (Zhong et al. 2008).

7 Nuclear Trafficking and DNA Release

From the site of endosomal release, the capsids must be transported to the vicinity of the nucleus, as particles the size of viral capsids do not normally diffuse efficiently in the cytoplam. However, parvovirus particles within endosomes are efficiently transported to the perinuclear region, and an active mechanism for further transport of the free capsids may not be essential. Treating cells with nocodazole led to a redistribution of microinjected capsids within the cytoplasm (Vihinen-Ranta et al. 2000). Genome release from the capsid occurs slowly for autonomous parvoviruses, as microinjecting anti-capsid antibodies into cells blocked CPV infection even when administered a few hours after inoculation (Vihinen-Ranta et al. 2000). For AAV, the situation is not well resolved. AAV2, 5, and AAVrh10 were seen to interact with microtubule-associated proteins (Kelkar et al. 2006). However, results for nocodazole or other microtubule-directed drug treatments on virus infection or transduction are still unclear, and in some studies, treatments prevented directed motion of viral particles in the cytoplasm and nucleus, while in others they did not (Hirosue et al. 2007; Sanlioglu et al. 2000; Seisenberger et al. 2001). Taxol treatment, which stabilizes microtubules, gave a mild enhancement of transduction by AAV2 (Hirosue et al. 2007).

The 26 nm diameter parvovirus capsids should be able to pass through the NPC, as that has an internal channel diameter of ~10 nm, but which is reported to accommodate complexes up to 39 nm under some circumstances (Feldherr and Akin 1990; Pante and Kann 2002), and newly synthesized capsids appear to leave the nucleus by that route (Bar et al. 2008; Maroto et al. 2004). However, it is still not clear whether the intact viruses enter the nucleus through the NPC, despite the results of some studies (Riolobos et al. 2006; Vihinen-Ranta et al. 2002).

Disruption of the outer nuclear envelope was seen when MVM capsids were microinjected into the cytoplasm of Xenopus oocytes, and blocking the nuclear pore by adding the lectin wheat germ agglutinin that binds to the NPC complex glycoproteins did not inhibit nuclear entry by the injected capsids, suggesting a pore-independent mechanism (Cohen and Pante 2005). When MVM capsids were added to mammalian cells, nuclear envelope breakdown was also seen with changes in the distribution of nuclear Lamin A/C (Cohen et al. 2006). A role for the NLS in these viruses may therefore be to target the capsid to the nuclear membrane and to dock it at the nuclear pore instead of directing transport through the pore. However, further studies are required to define the mechanisms involved in productive infection.

The uncoating mechanism of the parvovirus DNA is also not well understood, but complete capsid disassembly seems unlikely, and the viral DNA is more likely extruded or extracted from relatively intact capsids, with a variety of factors influencing this release, including low pH and changes in levels of capsid-bound divalent ions (Cotmore et al. 1999, 2009) (Fig. 8). A fluorescently labeled probe for the 5′ end of the MVM genome detected the partial exposure of DNA in the cytoplasm, while the 3′ end (which would prime new DNA synthesis) remained inside the capsid (Mani et al. 2006). The capsid form that enters the nucleus is still unresolved and may vary between viruses. Most MVM or CPV capsids taken up by normal routes were not detected in the nucleus (Harbison et al. 2009; Mani et al. 2006). For AAV, there is conflicting evidence whether uncoating occurs before or after nuclear entry. Fluorescently labeled AAV2 capsids were reported within the nucleus within 2 h of uptake(Bartlett et al. 2000), while particles were seen only within membrane invaginations of the nuclear envelope but not within the nucleus 15 min after uptake when utilizing single particle tracking technology (Seisenberger et al. 2001). Adenovirus capsids are reported to enhance cell infection by AAV and on the conversion of the AAV genome from a ssDNA to a dsDNA form, and studies utilizing fluorescently labeled particles and sub-cellular fractionation followed by DNA hybridization suggest that adenovirus can facilitate AAV translocation into the nucleus (Xiao et al. 2002). Furthermore, microinjection of anti-capsid antibodies into the nucleus blocked infection, suggesting that the AAV genome is associated with the capsid after it enters the nucleus, although since the same antibodies would have been present in the cytoplasm after injection, the precise mechanism is not clear (Sonntag et al. 2006). Other studies using GFP-labeled particles suggested that, while adenovirus capsids increased the number of particles in the nucleus, most capsids remained detectable outside the nucleus, while viral genomes were detectable by fluorescence in situ hybridization within the nucleus of cells within 2 h post-transduction, irrespective of Ad5 co-infection. While no co-localization of viral genomes and intact viral capsids was observed within the nucleus, co-localization was detectable in the perinuclear area and within the cytoplasm (Lux et al. 2005). These findings argue in favor of viral uncoating before or during nuclear entry.

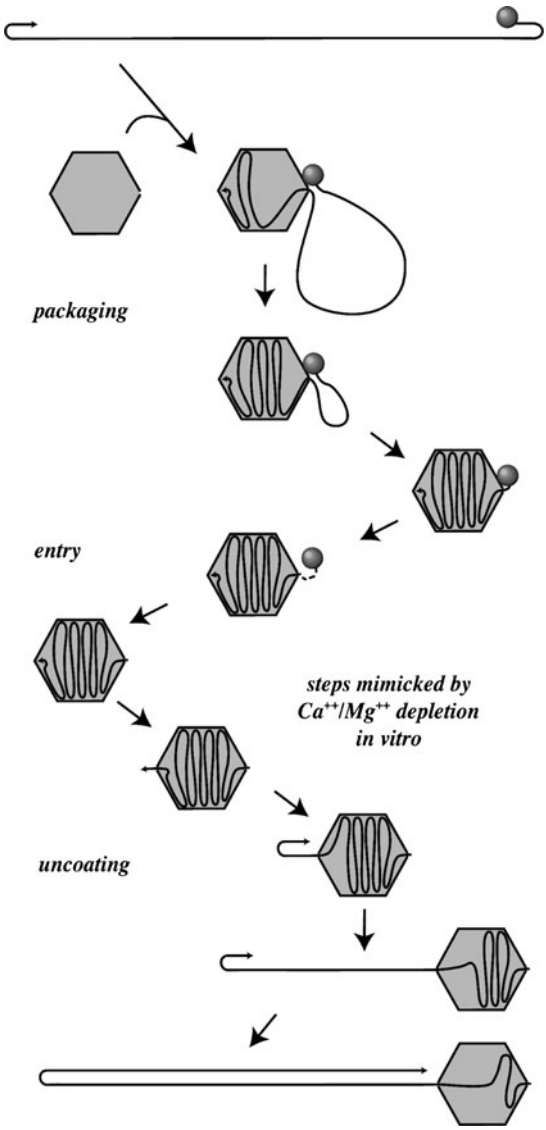

Fig. 8 Diagram showing possible mechanisms of DNA packaging and release from the parvovirus capsid "pass through" or "second portal" model for packaging and uncoating. The newly displaced single stranded progeny genome is depicted 3′ to 5′, left to right, across the page, with a *shaded circle* denoting a covalently bound NS1 molecule. This DNA is introduced into a pre assembled capsid in a 3′ to 5′ direction, but the 3′ end of the genome locates near the capsid shell, proximal to its destined exit pore. The rest of the strand is then pumped into the particle under increasing pressure, adopting a topology that allows it to be subsequently unraveled in a 3′ to 5′ direction. NS1 is removed from the 5′ end of the DNA, during cell entry *in vivo*, or by trypsin digestion. Divalent ions (Ca^{2+} or Mg^{2+}) in the virion are required to stabilize the pressurized structure, but when these are depleted *in vitro*, perhaps mimicked by a specific host factor trigger *in vivo*, the 3′ end of the DNA, followed by the coding sequences, is ejected. This uncoated DNA can support complementary strand synthesis, while the 5′ end of the negative strand genome remains threaded through the capsid. From Fig. 8 of (Cotmore et al. 2009) with permission

8 Summary and Future Challenges

It is now clear that, despite the small size and superficial simplicity of the parvovirus capsid, the various forms of the capsid control a large number of different functions, which in other viruses are the responsibility of separate proteins. It is

so-far difficult to define the exact functions of each protein form, or even the roles of subdomains of the proteins, as many protein components influence multiple functions, and small or transient changes may control important functions. Controlled modifications of the proteins, including phosphorylations of serines, threonines, or tyrosines on the capsid shell or exposed components such as the VP2 N-terminal peptide, can be important. The controlled exposure of components from inside to the outside of the capsid occurs during the infectious cycle. The presence of metal ions in the structures has not been carefully investigated for most parvoviruses, but those may control reversible changes in the capsid protein structures. Finally, asymmetry may be present in the capsids but that is difficult to investigate, and unless very prominent or present in a large proportion of the capsid subunits, would be missed by methods that use 60-fold averaging. However, asymmetries must be present in the single position of DNA exposure from full capsids, in the 5 6 VP1-unique domains inside capsids that become exposed under some conditions, and others are identified by differential protease sensitivity of a small subset of the capsid proteins. Defining the structural controls of the many parvovirus functions will require more sophisticated analyses to detect small changes in the capsids, as well as in varying proportions of the capsid proteins. Some changes are transient or reversible, while others may not affect the overall conformation of the protein, but only alter its flexibility. The interactions with receptors, antibodies, and other host or environmental factors have also been refined by evolutionary selection, resulting in intricate mechanisms and balanced processes.

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Cellular Entry of Polyomaviruses

Billy Tsai and Mengding Qian

Contents

Abstract Polyomaviruses (Pys) are nonenveloped DNA tumor viruses that include the murine polyomavirus (mPy), simian virus 40 (SV40), and the human BK, JC, KI, WU, and Merkel Cell viruses. To cause infection, Pys must enter host cells and navigate through various intracellular compartments, where they undergo sequential conformational changes enabling them to uncoat and deliver the DNA genome into the nucleus. The ensuing transcription and replication of the genome leads to lytic infection or cell transformation. In recent years, a more coherent understanding of how Pys are transported from the plasma membrane to the nucleus is starting to emerge. This review will focus on the decisive steps of Py entry, including engagement of the host cell receptor, targeting to the endoplasmic reticulum (ER), penetration across the ER membrane, nuclear entry, and genome release. Strikingly, a number of these steps resemble the intoxication pathway of the AB_5 bacterial toxins. Thus, as Pys and bacterial toxins hijack similar cellular machineries during infection, a general principle appears to guide their entry into host cells.

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Abbreviation

CT	Cholera toxin
ER	Endoplasmic reticulum
mPy	Murine polyomavirus
PDI	Protein disulfide isomerase
Pys	Polyomaviruses
SV40	Simian virus 40

1 Introduction

The murine polyomavirus (mPy) was the first polyomavirus isolated (Gross 1953). It was shown to induce multiple tumors in mice and was hence given the name "polyoma". Simian virus 40 (SV40) was next discovered in 1960 (Eddy et al. 1958; Sweet and Hilleman 1960), followed by two human Pys in 1971: the BK and JC viruses (Gardner et al. 1971; Padgett et al. 1971). Within the last few years, three new Pys in humans have been isolated, including the KI (Allander et al. 2007), WU (Gaynor et al. 2007), and Merkel cell (Feng et al. 2008) viruses discoveries that have galvanized the study of Pys as human pathogens and carcinogens. Recent advances in biochemical, cell biological, and imaging approaches have slowly unraveled the cellular basis of the Py infection pathway. These insights will undoubtedly be fundamental for developing strategies geared toward disease intervention. Here, we will focus on the intracellular transport of Pys, from the point of cell surface receptor attachment to the arrival of the viral genome into the nucleus. This review will also highlight parallel strategies used by Pys and certain bacterial toxins during infection, which suggest that there is likely a general mechanism used by these pathogens during entry. As the intracellular transport of mPy and SV40 is the best characterized, we will accordingly focus on these two viruses. However, where appropriate, the entry pathways of the human BK and JC viruses will also be discussed.

Structurally, Pys are composed of 72 pentamers of the major structural protein VP1 (Fig. 1a). Each VP1 molecule extends its long C-terminal arm to adjacent pentamers to stabilize interpentamer interactions (Liddington et al. 1991; Stehle et al. 1994). The individual VP1 pentamer also binds to an internal VP2 or VP3 protein through hydrophobic interactions (Fig. 1b) (Chen et al. 1998); the VP2 and VP3 proteins overlap in sequence at the C-terminus and differ only at their N-terminus where the longer VP2 protein contains a myristic acid (Streuli and Griffin 1987). The circular double-stranded viral DNA genome, with bound host-encoded histones, is in turn packaged within the virus.

To infect cells, the mPy, SV40, and BK viruses bind to identified receptors on the plasma membrane of the host cell (Tsai et al. 2003; Gilbert and Benjamin 2004;

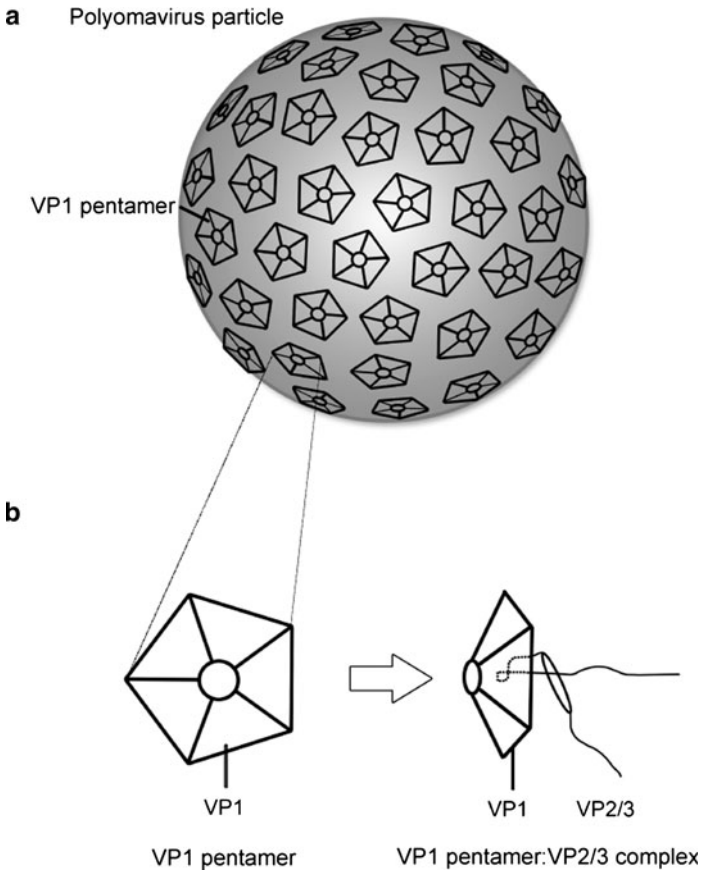

Fig. 1 The General architecture of polyomaviruses. (**a**) The polyomavirus particle is composed of 72 copies of the VP1 pentamer. (**b**) Each VP1 pentamer encloses the internal protein VP2 or VP3. The circular double stranded DNA genome is in turn packaged within the viral particle

Dugan et al. 2005; Low et al. 2006). The viruses are subsequently transported in a retrograde manner to the lumen of the ER (Kartenbeck et al. 1989; Pelkmans et al. 2001; Tsai et al. 2003). Transport to the ER is essential as treatment of cells with brefeldin A (a drug that disrupts retrograde COPI-dependent transport between the ER and the Golgi complex) blocks mPy, SV40, and BK virus infections (Norkin et al. 2002; Richards et al. 2002; Gilbert and Benjamin 2004; Low et al. 2006). From this compartment, these viruses are believed to penetrate the ER membrane in order to reach the cytosol (Norkin et al. 2002; Pelkmans and Helenius 2003; Magnuson et al. 2005; Schelhaas et al. 2007). While it is possible that Py is transported directly from the ER into the nucleus as the ER membrane is contiguous with the nuclear membrane, evidence shows that SV40 is transported into the

cytosol prior to reaching the nucleus (Nakanishi et al. 1996). Regardless of the pathway that delivers the virus into the nucleus, the subsequent transcription and replication of the viral DNA leads to lytic infection or cell transformation.

Within the framework of the aforementioned pathway, Py entry can be divided conceptually into four distinct steps (Fig. 2): (1) receptor binding, (2) targeting to the ER, (3) ER membrane penetration, and (4) nuclear entry. As Py traffics through each of these steps, it experiences a series of programmed conformational changes that serve one purpose to deliver the viral genome into the nucleus without being trapped or disassembled prematurely. Hence, the temporal and spatial organization of the remodeling events imparted on the virus must be orchestrated precisely to achieve productive infection.

2 Engaging the Host Cell Receptor

The first step in the viral entry process is binding of the virus to a host cell factor on the plasma membrane (Fig. 2, Step 1). The primary function of this virus receptor interaction is to promote internalization of the viral particles, which targets the virus towards an infectious route. Those cellular factors that mediate viral binding and endocytosis without facilitating productive infection are not considered as functional receptors, but accordingly are referred to as "decoy" or "pseudo" receptors.

The VP1 protein of Pys is responsible for interacting with the host cell receptor. Earlier studies first implicated a number of diverse host molecules as potential receptors for the Pys. In the case of SV40, the MHC class I molecule was initially considered as a host receptor (Atwood and Norkin 1989; Breau et al. 1992; Stang et al. 1997). However, the observation that MHC class I does not enter with SV40 (Anderson et al. 1998) subsequently raised the possibility that it may only serve as an attachment factor for SV40. Additional studies have also challenged the role of MHC class I molecules in mediating SV40 infection (Basak et al. 1992; Low et al. 2004). For the mPy, early findings indicated that sialic acid is a component of the functional receptor (Fried et al. 1981; Cahan et al. 1983), and that the $\alpha 4\beta 1$ integrin serves as a post-attachment factor for the virus (Caruso et al. 2003). Similar to the characteristic of the mPy receptor, sialic acid is also thought to be a critical part of the human BK virus receptor (Dugan et al. 2005). In addition, a finding suggests that the serotonin receptor is used by the human JC Py to infect cells (Elphick et al. 2004). Juxtaposed to this diversity of molecules implicated as receptors for Pys is the observation that they all share a common fate upon entry: transport from the cell surface to the ER. How then does binding to this myriad of receptors target Pys to the ER?

A unifying theme in the identification of functional receptors for Pys has developed from comparing Py entry to the entry pathways of the AB_5 bacterial toxins. AB_5 toxins, such as cholera toxin (CT) and shiga toxin, are aptly named because they consist of a single catalytic A subunit and a receptor-binding B subunit which is arranged as a pentamer (Merritt and Hol 1995). The pentameric

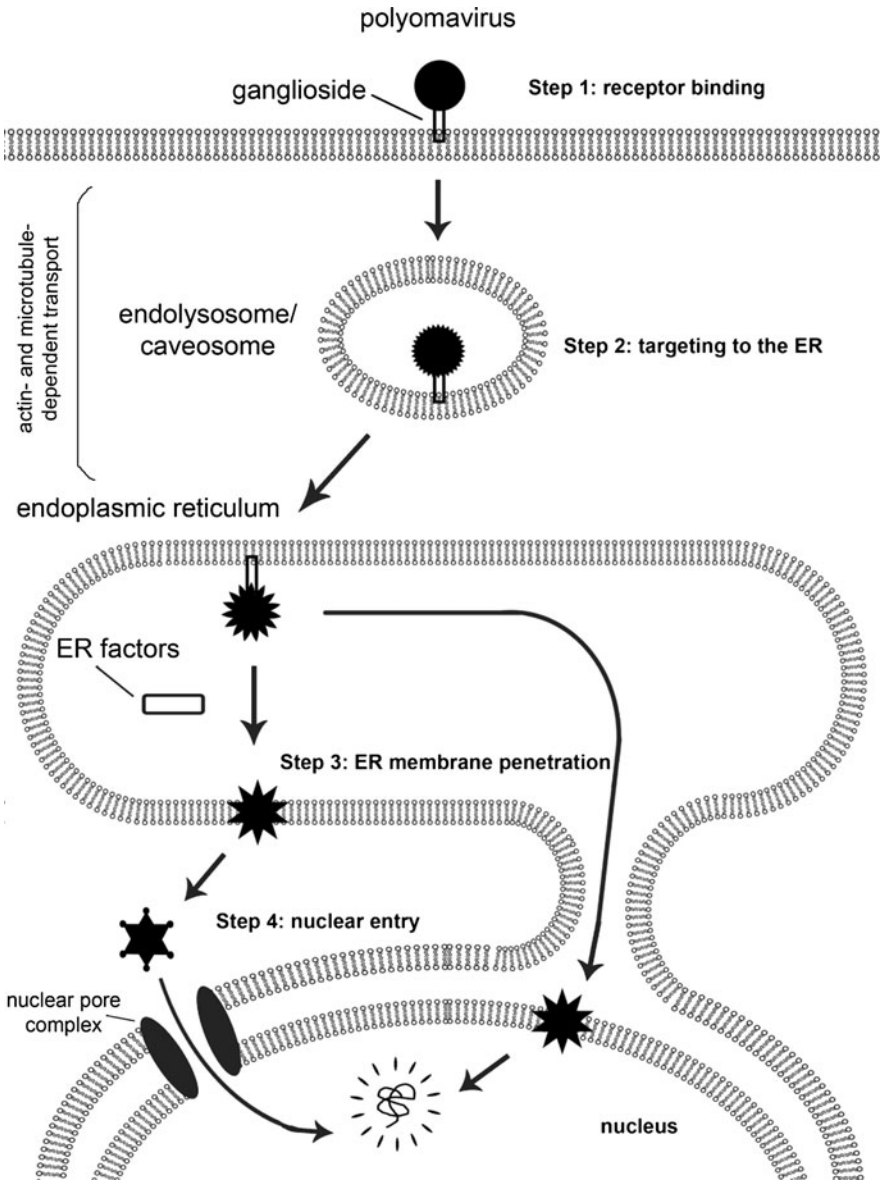

Fig. 2 Intracellular trafficking of polyomaviruses. Polyomavirus entry can be conceptually divided into four distinct steps. In step 1, the virus binds to a receptor on the plasma membrane of the host cell to initiate entry. In step 2, the virus is targeted to the endoplasmic reticulum (ER) after endocytosis. In step 3, the virus penetrates the ER membrane to reach the cytosol. And finally, in step 4, the viral particle is transported into the nucleus to enable infection. As the ER membrane is contiguous with the nuclear membrane, it remains possible that the virus can enter the nucleus without ever reaching the cytoplasm. Viral conformational changes likely occur after receptor binding, during transport to the ER, within the ER, in the cytosol, and upon nuclear entry

B subunits of these toxins bind to glycolipid receptors such as gangliosides to intoxicate cells. Gangliosides are glycolipids with two domains (Fig. 3): a hydrophobic ceramide inserted into the outer leaflet of the plasma membrane and an attached hydrophilic sugar chain that makes contact with the ligand. Upon binding to these glycolipids, the holotoxins are transported in a retrograde fashion to reach the ER lumen, a fate similar to that of Pys (Lencer and Tsai 2003). Subsequent ejection of the toxic A subunit into the cytosol enables the toxins to induce cytotoxicity.

Based on this observation, several groups identified different gangliosides as functional receptors for the mPy, SV40, and BK virus. Specifically, ganglioside GD1a and GT1b were found to be functional receptors for the mPy (Smith et al. 2003; Tsai et al. 2003). The sialic acid galactose moiety of these lipid molecules makes direct contact with VP1 (Stehle et al. 1994; Tsai et al. 2003), and there is evidence that mPy VP1 undergoes conformational changes upon sialic acid binding (Cavaldesi et al. 2004). Ganglioside GM1 was discovered as the functional receptor for SV40 (Tsai et al. 2003; Low et al. 2004), and a recent X-ray structure of the VP1 GM1 complex shows that this interaction also involves sialic acid binding (Neu et al. 2008). Interestingly, *N*-glycolyl GM1, an altered form of GM1, was reported to be a more efficient SV40 receptor than GM1 (Campanero-Rhodes et al. 2007). Gangliosides GD1b and GT1b represent the functional receptors for the BK virus, in which case the di-sialic acid motif of these gangliosides is postulated to interact with the VP1 (Low et al. 2006). Moreover, JC virus coated with ganglioside GT1b blocked infection (Komagome et al. 2002). And finally, a recent study implicates ganglioside GT1b as the host cell receptor for the newly discovered Merkel cell polyomavirus (Erickson et al. 2009). In all of these ganglioside studies, it has yet to be demonstrated that the virus and ganglioside undergo endocytosis simultaneously. Future advances in the synthesis of fluorescently labeled gangliosides coupled with a sophisticated microscopy system will likely enable this process to be visualized directly. Nonetheless, the available evidence collectively implicates gangliosides as functional receptors for the Py family, and raise the possibility that pathogens such as viruses and toxins that are targeted to the ER utilize gangliosides as a common strategy.

3 Trafficking to the ER

After receptor-mediated endocytosis, Pys are directed to the ER to cause infection (Fig. 2, Step 2). In 1989, using electron microscopy, Helenius and coworkers reported the transport of SV40 from the plasma membrane to the ER (Kartenbeck et al. 1989). This was a unique finding as extracellular ligands that were endocytosed had not been found to reach the ER. After this initial discovery, the mPy has also been observed directly to reach the ER (Mannova and Forstova 2003; Tsai et al. 2003; Gilbert and Benjamin 2004). In addition, biochemical and functional studies also implicate transport of the BK virus to this compartment (Low et al. 2006).

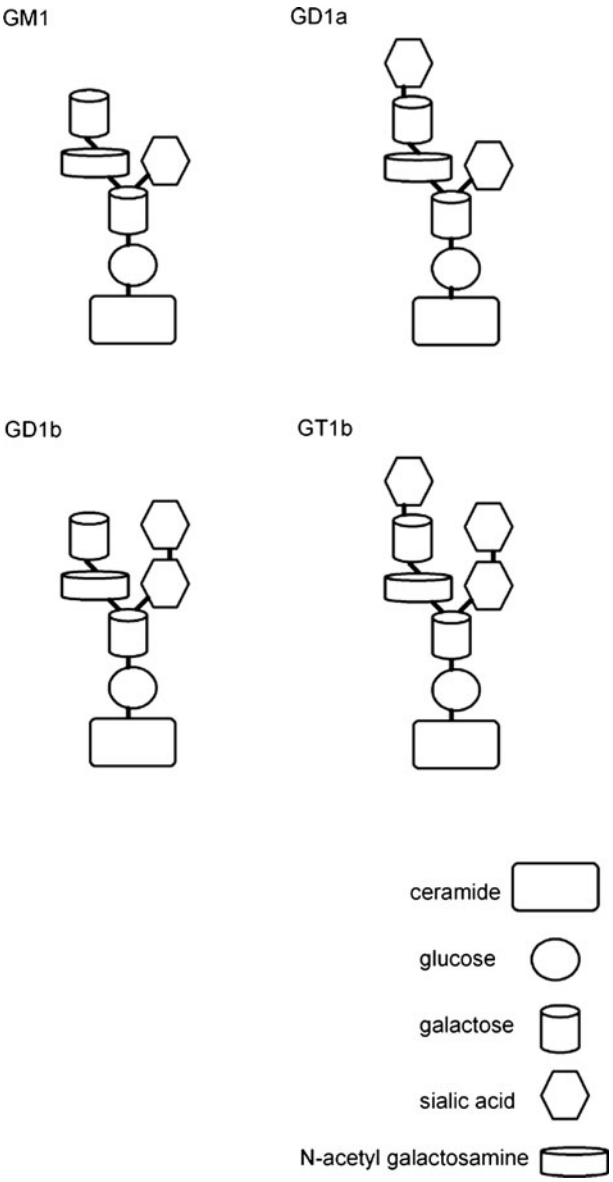

Fig. 3 Structure of gangliosides. Gangliosides are composed of a hydrophobic ceramide domain with an attached hydrophilic sugar chain. The structures of gangliosides GM1, GD1a, GD1b, and GT1b are shown as they have been found to be functional receptors for various polyomavirus members

These studies raise two important questions: what is the molecular mechanism by which Py reaches the ER, and what is the functional significance of transport to the ER? Here, we discuss the nature of the endocytosis machinery and intermediate organelles that target Pys to the ER. We will examine the latter question in the next section.

A stunning diversity of endocytic pathways guides virus entry (Marsh and Helenius 2006). In addition to the classic clathrin-mediated pathway, viruses can enter cells via caveolae, macropinocytosis, clathrin/caveolar-independent pathways, as well as cholesterol-dependent/independent pathways. Moreover, viruses are also capable of inducing the reorganization of cellular factors to further facilitate their entry. While these observations underscore the complexity of the virus entry process, recent findings have nonetheless provided a more defined view of Py entry.

To reach the ER, SV40 primarily uses lipid raft and caveolae-dependent endocytosis. During the initial entry phase, these viral particles are found in small, tight-fitting invaginations called caveolae at the plasma membrane (Anderson et al. 1996; Stang et al. 1997). SV40 in caveolae triggers transient actin breakdown followed by actin recruitment, events that appear to involve activation of tyrosine kinases (Pelkmans et al. 2002). After SV40-induced caveolar endocytosis, the virus then enters a pH-neutral compartment known as the caveosome, from which it is transferred subsequently to the ER in a microtubule-dependent fashion (Pelkmans et al. 2001; Norkin et al. 2002). As actin and microtubules are implicated in SV40, as well as mPy (Gilbert and Benjamin 2004) trafficking, it is tempting to speculate that the molecular motors myosin (which facilitates retrograde vesicular transport on actins) and dynein (which facilitates retrograde transport on microtubules) mediate these movements. Intriguingly, SV40 in caveolar vesicles, in addition to targeting to the caveosome, can be sorted to the early endosomes in a reaction controlled by the small GTPase Rab5 (Pelkmans et al. 2004). The early endosome is considered generally to be derived from clathrin-mediated endocytosis. Sorting to the early endosome, in contrast to the caveosome, represents a nonproductive SV40 infection route (Pelkmans et al. 2004). This finding not only implicates the presence of cross-talk between the caveolar and endosomal systems traditionally viewed as independent, but suggests that regulation of transport between these two pathways is critical for SV40 infection.

Depending on the cell type, both caveolae-dependent (Richterova et al. 2001; Gilbert and Benjamin 2004) and -independent (Gilbert and Benjamin 2000) pathways have been shown to facilitate mPy infection. However, transit through the early endosome is also part of the mPy infectious route (Liebel et al. 2006; Qian et al. 2009), in contrast to SV40 (Pelkmans et al. 2004). Moreover, we found that transport of mPy to the late endosome/lysosome is important for infection as well (Qian et al. 2009). The low pH in these compartments induces conformational changes to the mPy that facilitates the remodeling reactions the virus experiences in the ER (Fig. 2, Step 2) (Qian et al. 2009). Whether the aforementioned receptor-induced conformational change on the mPy (Cavaldesi et al. 2004) influences the efficiency of the low pH and/or ER-dependent structural changes remains to be

determined. Additional support for a role of the endolysosome is the observation that perturbing the low pH of these compartments blocks mPy infection (Liebl et al. 2006; Qian et al. 2009), although another study found that it did not affect infection (Gilbert and Benjamin 2000). Whatever the role of the endolysosome, mPy is sorted from this compartment to the ER by its functional receptor GD1a (Tsai et al. 2003; Qian et al. 2009).

Similar to mPy, the entry of BK virus relies on caveolar endocytosis (Eash et al. 2004; Moriyama et al. 2007) and low pH (Eash et al. 2004; Jiang et al. 2009), again suggesting a critical connection between the caveolar and endosomal systems. Furthermore, the interplay between the caveolar and endosomal systems has also been observed for JC virus entry (Querbes et al. 2006). Elucidation of Py entry has thus yielded the surprising observation that these viruses can use both the caveolar and endosomal systems simultaneously within a given cell type, suggesting that these two transport pathways converge postentry. These studies challenge the traditional view of endocytosis, in which the various individual pathways are considered to operate independently of each other.

Regardless of the nature of the endocytic pathway used during initial entry, Pys must be sorted to the ER. How this process is achieved, while largely enigmatic, is likely to involve their interaction with the ganglioside receptors. This contention is further supported by the observation that bacterial toxins that undergo retrograde transport to the ER, such as CT, also utilize gangliosides as functional receptors (Lencer and Tsai 2003). During normal metabolism, gangliosides are endocytosed and transported to the early and late endosomes, eventually reaching the lysosome where they are hydrolyzed by lysosomal enzymes (Schwarzmann 2001). Although a small fraction of gangliosides can reach the Golgi from the plasma membrane, their retrograde transport to the ER has not been observed. Thus, if gangliosides act as carriers to ferry Pys and bacterial toxins to the ER, transport of gangliosides to the ER is likely to be ligand-activated.

How might the ganglioside virus interaction target Pys to the ER? Because the 360 VP1 molecules of Pys provide 360 ganglioside binding sites, each virus likely binds to multiple gangliosides on the cell surface. In addition, Pys that intersect with the endolysosomal compartment may recruit even more gangliosides during entry, resulting in the clustering of multiple molecules of gangliosides to a single viral particle. Notably, CT also clusters multiple gangliosides on the cell surface via its receptor binding B subunit pentamer (Wolf et al. 2008).

We posit that ganglioside clustering leads to two ER-sorting mechanisms. First, clustering effectively generates a hydrophobic platform within the bilayer, which can trigger transmembrane signaling to recruit cytoplasmic factors that promote budding of virus-containing vesicles from the endosomal membrane. As gangliosides are only inserted into a single leaflet, any potential transmembrane signaling would require interactions with a transmembrane protein. Alternatively, ganglioside clustering may distort the biophysical properties of the membrane underneath the virus, causing membrane invagination followed by budding. This principle was recently demonstrated in the case of shiga toxin, where toxin binding to its ceramide-based glycolipid GB3 receptor induced tubular membrane

invagination (Romer et al. 2007). These two possibilities as potential explanations by which gangliosides sort Pys (and toxins) to the ER have yet to be demonstrated formally.

4 Penetration Across the ER Membrane

Upon reaching the ER lumen, Pys penetrate the ER membrane to access the cytosol (or possibly the nucleoplasm), a process that is poorly characterized (Fig. 2, Step 3). As many viruses penetrate the endosomal membranes during infection (Tsai 2007), it is reasonable to ask why Pys do not penetrate endosomal membranes, but instead, traffic further down the retrograde pathway to reach the ER and subsequently penetrate its membrane. What then is the functional relevance of the ER?

During viral entry, the rate-limiting and often decisive step of infection is the penetration of the limiting membrane. For the enveloped virus family where a lipid bilayer surrounds the viral particle, penetration of the target membrane is well-characterized. Hydrophobic peptides encoded by the virus triggers the fusion between the viral and cell membranes (Poranen et al. 2002). As a consequence of this fusion event, the viral particle is delivered *de facto* into the cytoplasm. By contrast, for the family of nonenveloped viruses in which the virus is not surrounded by a lipid bilayer, their membrane penetration is not as clearly understood as that of enveloped viruses (Tsai 2007). Because Pys belong to this family, there is intense interest in clarifying how these viruses breach their limiting membrane (i.e., ER membrane).

The model of nonenveloped virus membrane penetration posits that a specific cellular factor or condition imparts conformational changes on the virus to initiate the transport process. These factors/conditions, present at the site of membrane penetration, may be the functional receptor, low pH, proteases, or chaperones. The resulting conformational change renders the virus hydrophobic or stimulates the release of a viral lytic factor. In either scenario, the hydrophobic virus or lytic factor binds and perforates the limiting membrane, enabling the core viral particle to cross the membrane.

Hence, in the case of Pys, targeting to the ER is postulated to be critical for infection as only factors present in the ER can specifically induce structural alterations to the virus that initiate its penetration across the ER membrane, enabling the virus to reach the cytosol. This hypothesis gained support by the finding that ERp29, an ER-resident factor that is a member of the protein disulfide isomerase (PDI) family, induces the local unfolding of the VP1 C-terminal arm of the mPy (Magnuson et al. 2005). As the C-terminal arm of Py VP1 invades neighboring VP1 pentamers to stabilize interpentamer interactions (Stehle et al. 1994), conformational changes near this region likely destabilize the virus. More detailed biochemical analysis found that ERp29 exposes VP2, generating a hydrophobic viral particle that binds, integrates into, and perforates the ER membrane (Magnuson et al. 2005; Rainey-Barger et al. 2007). These events are postulated to initiate penetration of the

mPy across the ER membrane. As the ERp29-dependent reaction is most efficient when the disulfide bonds of the mPy are reduced and when the calcium ions that stabilize the virion structure (Brady et al. 1978; Stehle et al. 1996) are removed, reductases and calcium binding proteins are likely additional components of the ER machinery that act on the virus to promote membrane penetration. In this context, siRNA knockdown of PDI, the primary ER-resident protein that facilitates oxidation reduction reactions, attenuates mPy infection (Gilbert et al. 2006), raising the possibility that PDI directly reduces the disulfide bonds present on the mPy to promote infection.

Similarly, ER oxido-reductases have also been shown to be crucial for ER membrane penetration of SV40. Specifically, siRNA-mediated knockdown of PDI and ERp57 (a PDI-like protein) blocked SV40 infection, while knockdown of ERp72 (another PDI-like protein) increased infection (Schelhaas et al. 2007). These observations suggest that PDI and ERp57 facilitate SV40 infection, while ERp72 attenuates this process. Interestingly, the opposing effects of PDI and ERp72 were initially reported in the transport of CT across the ER membrane (Forster et al. 2006). Further biochemical analysis demonstrated that, by promoting the rearrangement of a disulfide bond in SV40 from an inter- to an intrapentamer position, ERp57 uncouples 12 of the 72 pentamers to initiate SV40 uncoating (Schelhaas et al. 2007). The isomerization and reduction of disulfide bonds in the BK virus has also been reported to initiate its uncoating (Jiang et al. 2009), although the identities of the cellular factors that mediate these reactions are not known.

Subsequent to the conformational changes imparted by the PDI-like proteins, Pys must physically cross the ER membrane. Whether this penetration event occurs through the lipid bilayer or across a protein-conducting channel is not clear. In the case of SV40, the internal proteins VP2 and VP3 were found to insert into the ER membrane in a VP1-regulated manner (Daniels et al. 2006). These inserted proteins are proposed to create a pore in the ER membrane through which the DNA genome is injected. Similarly, VP2 and VP3 of the mPy have also been shown to bind and integrate into the ER membrane (Rainey-Barger et al. 2007). In fact, integration of these viral components into the lipid bilayer results in the perforation of the ER membrane. That a limiting membrane is compromised due to interaction with an activated virus (or a viral component) has also been demonstrated for other viral systems (reviewed in Tsai 2007). The disruption of the ER membrane integrity by components of mPy suggests that the viral (or subviral) particle crosses the lipid bilayer of this membrane. Interestingly, the idea that Pys escape the ER by breaching the lipid bilayer (via the "lipid droplet") was also postulated recently (Ploegh 2007).

In contrast to the lipid bilayer, there is also evidence that protein-conducting channels on the ER membrane that are components of the ER-associated degradation (ERAD) machinery are involved in viral penetration. ERAD is a quality control process in which misfolded ER proteins are recognized and ejected into the cytosol for proteasomal degradation (Tsai et al. 2002; Vembar and Brodsky 2008). The misfolded substrates are thought to cross an ER membrane channel in this process. While there is debate regarding the identity of this retro-translocon,

the ER membrane proteins Sec61 and the Derlin protein family appear to be the best candidates. In this context, down-regulation of Derlin-1 blocked SV40 infection (Schelhaas et al. 2007), over-expression of dominant-negative Derlin-1 attenuated BK virus infection (Jiang et al. 2009), and down-regulation of Derlin-2 and over-expression of dominant-negative Derlin-2 decreased mPy infection (Lilley et al. 2006). These studies indicate that the Derlin proteins promote transport of Pys across the ER membrane, raising the possibility that a protein-conducting channel may guide this penetration process. Should this be the case, the relatively large size of the Py particle (typically 400 500 Å in diameter) in comparison to a misfolded cellular substrate suggests that the virus must uncoat dramatically in the ER prior to translocation into the cytoplasm. Alternatively, oligomerization of the Derlin proteins may be required to form a pore size that can accommodate the native viral particle. Establishment of a reconstituted *in vitro* membrane transport system will help to pinpoint the precise nature of the viral ER membrane penetration process.

Co-opting of ER oxido-reductases and Derlin proteins by Pys is similar to the hijacking of ER machineries by CT during its transport from the ER into the cytosol. Upon reaching the ER lumen from the cell surface, CT is targeted to Derlin-1, where the Derlin-1-bound PDI unfolds the catalytic A1 peptide of CT to transfer this toxic peptide into the cytosol (Tsai et al. 2001; Forster et al. 2006; Bernardi et al. 2008). Thus, while Pys and CT are clearly evolutionary distinct, that these pathogenic agents use the same class of functional receptors to reach the ER, as well as usurp similar ER machineries to penetrate the ER membrane, demonstrates a striking conservation in the infection mechanism.

Because the ER membrane is continuous with the nuclear membrane, it is possible that Pys penetrate the inner nuclear membrane and arrive into the nucleus from the ER without ever reaching the cytosol. However, at least for SV40, transport to the cytosol appears crucial for infection as injection of an antibody against VP1 or VP3 blocks infection (Nakanishi et al. 1996). Assuming that Pys are first transferred to the cytosol prior to reaching the nucleus, the viral particles may be subjected to degradation by the cytosolic proteasome, a fate observed for ERAD substrates. In fact, pharmacological inactivation of the proteasome attenuates SV40 (Schelhaas et al. 2007) and BK virus (Jiang et al. 2009) infection, demonstrating that the action of the proteasome on these viruses normally facilitates infection. These findings suggest that proteasomal degradation of these two viruses generates a viral conformation that promotes nuclear entry. Biochemical and structural studies will be required to clarify the composition of the proteasome-degraded viral particles.

In contrast, blocking the proteasome activity stimulates mPy infection (our unpublished results), implicating proteasome-mediated degradation of this virus in the protection of cells against infection. Although the manner by which the proteasome renders mPy inactive is not known, the observation that the mPy infection can occur without inactivating the proteasome suggest that the mPy can partially evade the degradative machinery to reach the nucleus.

5 Nuclear Entry and Genome Release

Nuclear entry of Pys represents the last decisive step of early infection (Fig. 2, Step 4), a process that is best characterized for SV40. During normal transport of cellular cargos across the nuclear pore complex (NPC) on the nuclear membrane, the nuclear localization signal (NLS) present in the cargo interacts with importin α, one component of the heterodimeric nuclear-import receptor complex composed of importin α and β (Stewart 2007). Through interactions with proteins in the NPC, importin β then shuttles the cargo through the pore and into the nucleoplasm.

Early studies implicate a role of the NPC in SV40 infection (Clever et al. 1991; Yamada and Kasamatsu 1993). Subsequent mutational analysis demonstrated that the NLS of SV40's VP2 and VP3 interact with the importin α/β heterodimer to promote viral entry into the nucleus (Nakanishi et al. 2002, 2007). Although SV40 VP1 also contains NLSs which bind to the importin receptors *in vitro*, they do not appear to play a role in nuclear entry (Nakanishi et al. 2002). By contrast, using virus-like particles (VLPs), the NLS of JC virus VP1 was implicated in its nuclear entry (Qu et al. 2004). As the VP2 and VP3 NLS is not exposed in the native SV40 structure (Nakanishi et al. 2002), conformational changes imparted to the viral particle prior to nuclear entry are necessary to reveal this signal. In this context, degradation of SV40 by the proteasome may provide these structural alterations (Schelhaas et al. 2007). Additionally, as the Hsp70 chaperone has been shown to disassemble the mPy *in vitro* (Chromy et al. 2006), this cytosolic chaperone may act similarly on SV40 to promote exposure of its VP2/3 NLS.

What subviral components of Pys reach the nucleus along with the viral genome? For SV40, at least VP3 accompanies the DNA into the nucleus (Nakanishi et al. 1996). That JC virus VP1-VLP reaches the nucleus (Qu et al. 2004) suggests that, in the native virion, VP1 is likely transported into the nucleus. The mPy VP1 was shown to colocalize with nuclear membrane markers as early as 3 h postinfection (Mannova and Forstova 2003), indicating that this protein may target to the nucleus as well. Thus, while it is unlikely that the entire viral particle enters the nucleus, available evidence suggests that a combination of VP1 and VP2/VP3 delivers the DNA genome into the nucleus. Elucidating the virus composition after the series of conformational changes it experiences en route to and in the cytoplasm will define the nature of the subviral particle that enters the nucleus.

Once in the nucleoplasm, the viral genome must be released from any bound viral proteins to initiate transcription and replication. The poly (ADP-ribose) polymerase-1, a nuclear protein involved in reprogramming the structural/functional roles of chromatin proteins, was shown to bind to the mPy VP1, displacing VP1 from the viral DNA to regulate early gene expression (Carbone et al. 2006). Aside from this information, very little is known regarding the manner by which the Py genome disengages from the structural proteins. Imaging studies in which both the genome and structural proteins are fluorescently labeled within a single viral particle might be able to clarify the kinetics and cellular location in which the genome is uncoupled from the structural proteins. This approach, in fact, was used elegantly to demonstrate release of the RNA genome from the nonenveloped poliovirus (Brandenburg et al. 2007).

6 Perspectives

To cause infection, Pys must bind to receptors at the plasma membrane, undergo endocytosis, and maneuver through the complex membranous systems to reach the nucleus. To accomplish this feat, these viruses face considerable challenges, including the potential to become trapped and/or degraded. Not surprisingly, only a small portion of Pys that enters the cells reaches the nucleus. However, as the arrival of only one viral particle into the nucleus is sufficient to trigger infection (Diacumakos and Gershey 1977), Pys infection succeeds despite these obstacles. In each entry step (Fig. 2), including engagement of the ganglioside receptor, transport to the endolysosome, ER, and cytosol, Pys have been shown to undergo conformational changes. These structural changes must be orchestrated precisely with its intracellular transport so that the viral particle can uncoat properly and not prematurely.

Several surprising findings have been discovered in studying the entry pathways of Pys. First, in the initial entry step, glycolipids but not glycoproteins appear to serve as the functional receptor. Second, during endocytosis, Pys use elements of both the caveolar and clathrin-endosomal systems, suggesting that these two pathways considered traditionally to be separate converge postentry. Third, in contrast to most extracellular ligands, Pys are capable of undergoing retrograde transport to the ER. Finally, that Pys hijack ERAD machineries indicates that the manner by which they are ejected into the cytosol may be similar to that of ERAD substrates, although the precise membrane penetration step is likely to be different. The connection between Pys and ERAD substrates was not suspected previously.

Some of the remaining major questions regarding Pys entry are: How does binding to ganglioside receptors sort Pys to the ER? What is the mechanism by which Pys penetrate the ER membrane? Where is the virus uncoated, and what is the physical composition of the virus prior to nuclear entry? And lastly, how and when is the genome disengaged from the viral proteins? Coupling advanced microscopy approaches with fundamental biochemical methods will undoubtedly illuminate fascinating answers to these questions in the future.

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Adenovirus

Jason G. Smith, Christopher M. Wiethoff, Phoebe L. Stewart, and Glen R. Nemerow

Contents

Abstract Of the 53 different human adenovirus (HAdV) serotypes belonging to species A-G, a significant number are associated with acute respiratory, gastrointestinal and ocular infections. Replication-defective HAdV-5-based vectors also

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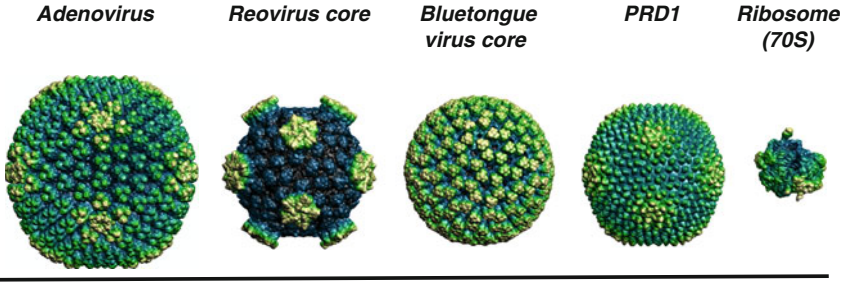
continue to play a significant role in gene transfer trials and in vaccine delivery efforts in the clinic. Although significant progress has been made from studies of AdV biology, we still have an incomplete understanding of AdV's structure as well as its multifactorial interactions with the host. Continuing efforts to improve knowledge in these areas, as discussed in this chapter, will be crucial for revealing the mechanisms of AdV pathogenesis and for allowing optimal use of AdV vectors for biomedical applications.

1 Structural Features of Adenovirus

AdV is a relatively large nonenveloped dsDNA virus possessing a molecular weight of ~150 MDa and a diameter (dia) of ~950 Å, excluding its elongated fiber proteins. AdV is one of the largest and most complex nonenveloped viruses whose structures have been analyzed by cryoelectron microscopy (cryoEM) or X-ray diffraction (Fig. 1). The ~36 kb AdV genome encodes more than 40 different proteins; however, only 12 of these have been shown to be constituents of the virus particle (Lehmberg et al. 1999; Stewart et al. 1993). Crystal structures of individual AdV proteins including the fiber knob (Bewley et al. 1999; Burmeister et al. 2004) and shaft (van Raaij et al. 1999) domains, penton base (Zubieta et al. 2005), hexon (Rux et al. 2003), and cysteine protease (McGrath et al. 2003) have been reported. The major AdV capsid protein (hexon) possess a β-barrel structural motif found in



	Adenovirus	Reovirus core	Bluetongue virus core	PRD1	Ribosome (70S)
Dia. (Å):	950	754	708	702	310
aa in IAU	15,155	4,370	6,267	4,915	3,394
Genome: (Kbp)	35.9	23.5	20.4	14.9	3.3

Fig. 1 Relative dimensions of nonenveloped viruses whose structures have been studied by X ray diffraction or cryoelectron microscopy (cryoEM). The adenovirus structure is from Chiu et al. (2001). The structures of reovirus, bluetongue virus, and PRD1 were retrieved from VIPER (Virus Particle Explorer, http://viperdb.scripps.edu). The diameter (dia) of the virions, the number of amino acids in their icosahedral asymmetric unit (IAU) as well as their genome size (KBp) are indicated

the icosahedral capsids of diverse DNA viruses that infect a wide range of host species ranging from metazoans to archaea (Benson et al. 1999). Although X-ray diffraction data of an entire AdV particle has not yet been reported, "quasi-atomic" models of the complete AdV particle at 7 10 Å resolution have been generated from the crystal structures of the hexon and penton base capsid proteins combined with cryoEM analysis of intact virus particles (Fabry et al. 2005; Saban et al. 2006). These analyses have shown that the AdV particle is composed of two major structural elements, the outer capsid and the core. Within the core, the viral genome is condensed in association with proteins V, VII, and X (or μ), and the 5′ termini of the AdV DNA are covalently linked to the terminal protein (TP). Surrounding the core is a capsid with icosahedral symmetry constructed by noncovalent interactions between seven proteins (II, III, IIIa, IV, VI, VIII, and IX). The $T = 25$ lattice of the icosahedron is comprised largely of 240 trimers of hexon (II). Anchoring the 20 facets at each of the 12 icosahedral vertices is the penton complex composed of the pentameric penton base and trimeric fiber proteins (III and IV, respectively). Several additional proteins are also directly or indirectly associated with the hexons and penton, including proteins IIIa, VI, VIII, and IX, each of which has been proposed to help stabilize (cement) the virion capsid (Saban et al. 2006). Upon assembly of the new virions during the late stage of virus infection, immature particles containing unprocessed (preprotein) molecules are formed, including IIIa, VI, VII, VIII, X, and TP (Gustin and Imperiale 1998). Subsequently, mature virions are formed upon cleavage of these preproteins by the 23 K cysteine protease, which is also encapsidated, and by packaging of the AdV DNA.

Recent cryoEM studies of AdV have provided insight into the architecture of the capsid, including inter-capsomer interactions and assignments for the locations of several cement proteins (Fabry et al. 2005; Saban et al. 2006) (Fig. 2). The copy

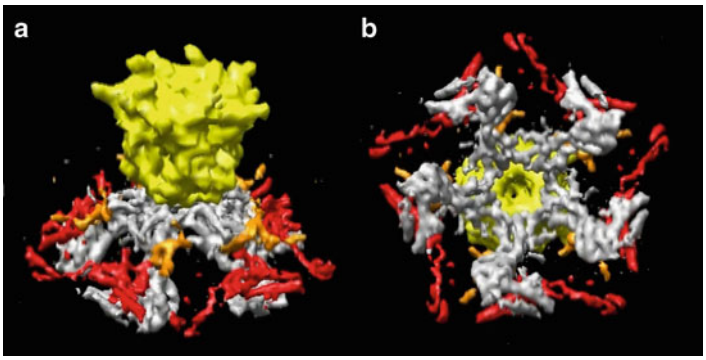


Fig. 2 The adenovirus vertex region as visualized in a cryoEM structure. (**a**) The location and associations of the penton base (*yellow*), protein IIIa (*white*), protein VIII (*red*), and protein VI (*orange*) are shown based on a recent cryoEM structural analysis of an Ad5F35 vector at 6.9 Å resolution (Saban et al. 2006). The peripentonal hexons, which in the intact virion would surround the penton base, are not shown to reveal the inner capsid proteins. (**b**) A view from the interior of the capsid

numbers of the AdV structural proteins are known from a biochemical analysis (van Oostrum and Burnett 1985) as well as from mass spectrometry (Lehmberg et al. 1999). Protein IIIa, present in 60 copies per virion, is located below the penton base and the surrounding peripentonal hexons. This assignment is based on the observation of a cluster of α-helices in agreement with secondary structure predictions for protein IIIa (Saban et al. 2006). A study of HAdV-5 virions with peptide extensions at the N-terminus of protein IIIa confirms this assignment (San Martin et al. 2008). Protein VI, present in 342 369 copies per virion, was originally thought to exist as a "trimer of dimers" based on a cryoEM structure at ~25 Å resolution (Stewart et al. 1993). There is also biochemical evidence suggesting that protein VI forms dimers and interacts with the core (Chatterjee et al. 1985; Everitt and Philipson 1974). In a recent cryoEM analysis, weak density was observed inside the cavity of every hexon trimer, and this was assigned to protein VI on the basis of molecular genetics information (Saban et al. 2006; Wodrich et al. 2003). Present in 120 copies per virion, protein VIII has been assigned to two positions within the asymmetric unit (Fabry et al. 2005; Saban et al. 2006). Both positions are on the inner surface of the capsid, with one position near the vertex and in proximity to protein IIIa and the other closer to the threefold symmetry axis of the capsid. Protein IX, present in 240 copies per virion, was originally assigned on the basis of a two-dimensional scanning transmission electron microscopy analysis to four trimers within the group of nine hexons that comprise the center of an icosahedral facet (Furcinitti et al. 1989). The first cryoEM difference map analysis of AdV showed that the protein IX density sits atop the β-barrels of adjacent hexons on the outer surface of the capsid (Stewart et al. 1993). The position of protein IX at hexon hexon interfaces might explain why AdV mutants lacking protein IX can still assemble, although they possess reduced stability (Caravokyri and Leppard 1995). Recent cryoEM studies at subnanometer resolution indicate that the stabilizing trimers are most likely comprised of only the N-terminal domains of protein IX (Saban et al. 2005, 2006). The C-terminal domain is predicted to have a long α-helix with a strong propensity for coiled coil formation. A coiled coil composed of four α-helices was observed in a cryoEM structure of AdV at 6.9 Å resolution and assigned to the C-terminal domain of protein IX (Saban et al. 2006). This assignment has been confirmed by two additional cryoEM studies of AdV with modified forms of protein IX (Fabry et al. 2009; Marsh et al. 2006). The most tentative of the cement protein assignments is that of protein VI. Determination of the fold and precise interactions of all cement proteins will require additional structural studies.

2 Adenovirus Receptor Interactions

AdV infection involves interactions with multiple host cell receptors that promote sequential steps in cell entry, including attachment and internalization (Wickham et al. 1993). AdV receptor interactions also influence important disease processes,

including inflammation. AdV attachment/entry mechanisms and the consequences of receptor interactions for the host are the subjects of intense investigation, in particular for the evaluation of AdV-mediated gene transfer to treat cardiovascular diseases and cancer. The death of a subject who received a large dose of an AdV vector in a clinical trial (Raper et al. 2003), as well as the potent proinflammatory responses frequently observed upon systemic administration of AdV vectors (National Institutes of Health (NIH) Recombinant DNA Advisory Committee 2002), emphasizes the need to improve our understanding of AdV cell interactions. The following sections highlight research findings in this rapidly developing field and are not intended to be a comprehensive review of these topics.

2.1 Interactions with the Coxsackie and Adenovirus Receptor

The majority of early cell-binding studies were performed with HAdV-2 and HAdV-5, two closely related HAdV-C strains. Philipson et al. first demonstrated that the elongated fiber protein of HAdV-C particles mediates high-affinity binding of the virus to host cells (Philipson et al. 1968). Subsequently, Bergelson et al. and Tomko and Philipson discovered that a 46-kDa host cell membrane protein, known as Coxsackie and Adenovirus Receptor (CAR), mediates cell attachment of most but not all human adenovirus (HAdV) types (Bergelson et al. 1997; Roelvink et al. 1998; Tomko et al. 1997). CAR is a member of the immunoglobulin (Ig) superfamily with two Ig-like domains, a typical transmembrane anchor, and a cytoplasmic tail of 107 amino acids. This latter domain does not appear to be required for AdV infection (Bergelson et al. 1998). Interestingly, CAR is expressed on the basolateral surface of polarized epithelial cells just below tight junctions (Cohen et al. 2001a, b; Walters et al. 1999, 2002). Homotypic associations of CAR may support the formation or enhance the integrity of tight junctions, a function that may be critical for normal tissue homeostasis (Lisewski et al. 2008). The topology of CAR on polarized epithelial cells raises the question of how AdV gains access to the receptor during a natural infection or during AdV-mediated gene transfer. This question is underscored by the failure of one of the earliest clinical trials using AdV vectors to treat patients with cystic fibrosis due to an inability of nonreplicating AdV vectors to efficiently transduce human airway epithelial cells *in vivo* (Stonebraker et al. 2004). A transient break in the cell monolayer may allow initial virus binding and infection through CAR. Subsequent spread of progeny virus to adjacent cells has been proposed to occur via disruption of tight junctions and associated CAR CAR homodimers by an excess of soluble AdV fiber protein or virus particles (Walters et al. 2002).

In addition to the localized expression pattern of CAR, structural features of the AdV fiber protein also impact CAR utilization by different HAdV serotypes. In particular, both the length and the flexibility of the fiber play a substantial role in CAR association. The HAdV-2 fiber protein is a homotrimer extending more than 300 Å from the penton base (van Raaij et al. 1999; Zubieta et al. 2005). The crystal

structure of the fiber C-terminal knob domain in a complex with the first Ig domain of CAR revealed the ability of the fiber to support multimeric associations with CAR (Bewley et al. 1999). Multivalent association results in a relatively high fiber CAR avidity ($K_D \approx 1$ nM) (Lortat-Jacob et al. 2001). Unexpectedly, CAR binding was found to occur on the lateral surfaces of the fiber knob domain rather than at the extreme distal portion of the knob (Bewley et al. 1999). Amino acid residues comprising several exposed loops on the fiber knob mediate CAR association, and these residues are also highly conserved among the different CAR-utilizing AdV types (Law and Davidson 2005; Roelvink et al. 1999; Wu and Nemerow 2004). A second region of the fiber that indirectly influences CAR association is the central shaft domain. The shaft is comprised of a variable number of repeating structural motifs that form a unique triple β-spiral (van Raaij et al. 1999). Within the shaft domain are several distinct segments that likely confer flexibility to the fiber protein, a structural feature that is essential for optimal engagement of the lateral surfaces of the knob domain with CAR (Wu et al. 2003). In addition, the overall length of the fiber shaft domain influences CAR association. HAdV fibers range in size from 5.5 to 22.5 β-repeat elements (Chroboczek et al. 1995). Decreasing (Wu et al. 2003) or increasing (Seki et al. 2002; Shayakhmetov and Lieber 2000) the normal number of β-repeat elements (21.5) in the HAdV-5 shaft dramatically reduces CAR association.

Based on the increasing knowledge of CAR fiber association, numerous attempts have been made to extend the range of host tissues transduced by AdV vectors *in vivo*. In general, the main approach used has been the removal of key CAR-binding residues in the fiber protein by mutagenesis and the insertion of new sequences that confer AdV attachment to different cell receptors (Nicklin et al. 2005). While these studies have demonstrated a proof of principle in cell culture and in preclinical animal models, fiber-retargeted AdV vectors have yet to be fully evaluated in the clinic.

2.2 Interactions with CD46

Previous investigations have demonstrated that not all HAdV types utilize CAR for attachment and infection (Bergelson et al. 1997). More recently, HAdV-B types including HAdV-3, -11, and -35 have been shown to recognize CD46 (also known as membrane cofactor protein, MCP) (Gaggar et al. 2003; Segerman et al. 2003; Sirena et al. 2004). CD46, a member of the family of complement regulatory proteins, is widely distributed on the surface of many different host cell types including hematopoietic cells and, unlike CAR, is not restricted below tight junctions. The extracellular domain of CD46 contains four short consensus repeats (SCR1-4). Interestingly, these SCR domains serve as binding sites for diverse viruses and bacteria, suggesting that this receptor may provide a distinct advantage for cell entry by microbial pathogens (Cattaneo 2004). Recent structural and functional studies have revealed the molecular basis of HAdV-B fiber knob interactions

with the extracellular domain of CD46 (Pache et al. 2008; Persson et al. 2007; Wang et al. 2007). AdV binding requires the interactions of the N-terminal SCR1 and SCR2 domains of the receptor with two fiber knob monomers. Fiber binding is also accompanied by a conformational change in the angle between SCR1 and SCR2 of CD46. This type of receptor engagement, in principle, would allow three molecules of CD46 to bind to each AdV fiber, thereby enhancing avidity.

2.3 Interactions with Sialic Acid

HAdVs-8, -19a, and -37, ocular pathogens belonging to HAdV-D, have been reported to use sialic acid as a receptor rather than CAR (Arnberg et al. 2002b). Although the HAdV-37 fiber knob retains CAR-binding sequences, the virus cannot efficiently use CAR on host cells due to its short (7.5 β-repeat elements) and inflexible shaft domain (Wu et al. 2003; Wu and Nemerow 2004). Instead, the positively charged HAdV-37 fiber knob supports low affinity (mM) interactions with sialic acid (Arnberg et al. 2002a; Burmeister et al. 2004). HAdV-37 is also capable of binding to CD46 on host cells and can use this receptor for infection (Wu et al. 2004). The use of sialic acid residues on host cells does not readily explain the restricted tropism of HAdV-37 for ocular cell types *in vivo* and associated severe eye infections, suggesting that other host cell factors may be involved in ocular tropism (Lukashok and Horwitz 1998).

2.4 Alternative Routes of Adenovirus Attachment to Cells

An unexpected and disappointing observation arose from *in vivo* studies of AdV vectors in which attempts to ablate CAR-binding sequences failed to significantly diminish AdV-mediated gene delivery *in vivo* following systemic administration even though these modifications significantly reduced infectivity *in vitro* (Alemany and Curiel 2001; Smith et al. 2002). This observation strongly suggested that alternative routes for AdV entry are present in the host. An earlier study suggested that heparin sulfate proteoglycans (HSPG) could mediate infection by HAdV-C types 2 and 5 (Dechecchi et al. 2000). AdV-HSPG association was proposed to occur via a basic motif (KKTK) present in the third β-repeat of the fiber shaft. In keeping with this possibility, mutation of the KKTK motif to GAGA in the HAdV-5 fiber substantially reduced AdV-mediated gene delivery *in vivo* (Smith et al. 2003). Since direct binding of the KKTK motif to HSPG has not been formally demonstrated, it is also possible that mutations in this region impact the flexibility of the fiber shaft rather than alter HSPG binding.

Surprisingly, nonfiber-mediated entry mechanisms have also been shown to impact AdV infection *in vivo*. Recent studies have shown that AdV association

with certain soluble blood coagulation factors such as factor X (FX) can also influence tissue tropism (Kalyuzhniy et al. 2008; Waddington et al. 2008). FX binds to the hexon subunit of most, but not all, AdV types, and thereby enables indirect binding to HPSG on liver hepatocytes in animal models *in vivo*. This mode of binding facilitates substantial gene delivery to the liver following systemic administration of HAdV-5-based vectors. Association of AdV vectors with other blood factors including complement components, such as C3 or C4Bp, or noncomplement factors, such as lactoferrin or clotting factors (FIX), may also enhance AdV-mediated gene transfer to the liver (Shayakhmetov et al. 2005; Zinn et al. 2004). The precise binding sites on the AdV particle or the exact cell receptors for these components are yet to be fully characterized. HSPG may serve as the receptor for virus-bound lactoferrin, since treatment with heparinase abrogates increased gene transfer (Shayakhmetov et al. 2005).

3 Adenovirus Internalization

Shortly after binding to high affinity receptors on the cell surface, AdV enters the cell. There is evidence from a number of sources that AdV is first internalized into an endosomal compartment and that the virus does not directly penetrate the plasma membrane. First, virus particles can be visualized in membrane vesicles by EM (FitzGerald et al. 1983; Svensson 1985; Varga et al. 1991). Second, endosomal localization is enhanced when virus is treated with neutralizing serum or in cells treated with inhibitors of uncoating (e.g., DTT) (Svensson and Persson 1984; Wohlfart et al. 1985). Third, fixed virus cross-linked by glutaraldehyde is trapped in endocytic vesicles (Svensson 1985). Fourth, a mutant virus, HAdV-2*ts1*, with a known defect in uncoating, is also trapped in endosomes (Gastaldelli et al. 2008; Miles et al. 1980; Nakano and Greber 2000; Suomalainen et al. 1999).

Although both specific cell-associated receptors and soluble host factors described above promote AdV binding to cells, these molecules generally do not foster rapid uptake of the virus into cells. Instead, association ($K_D \approx 50$ 80 nM) of the AdV penton base protein with the vitronectin-binding integrins αvβ3, αvβ5, and αvβ1 enhance AdV entry (Bai et al. 1993; Li et al. 2001; Wickham et al. 1993). Integrins are heterodimeric proteins comprised of noncovalently associated α and β subunits that often recognize the RGD motif on extracellular matrix proteins. An RGD sequence is also present on each of the five loops protruding from the top of the pentameric penton base complex, and these RGD loops serve as binding sites for αv integrins (Chiu et al. 1999; Stewart et al. 1997). Interestingly, the RGD sequence is conserved in the penton base protein of most but not all AdV serotypes (Albinsson and Kidd 1999; Mathias et al. 1994). It is possible that certain non-RGD containing AdV serotypes (e.g., HAdV-40 and -41) either do not use the vitronectin-binding integrins for infection or that they use distinct receptors, as has been suggested from earlier studies (Chillon and Kremer 2001). Recently, an integrin-binding RGD sequence was identified in the fiber protein

of MAdV-1, further underscoring the importance of integrin interactions in AdV entry (Raman et al. 2009). Deletion or mutation of the RGD sequence in recombinant HAdV-5 vectors results in diminished virus infection *in vitro* as well as *in vivo* (Bai et al. 1993; Einfeld et al. 2001; Goldman and Wilson 1995). The lack of integrin expression on the apical surface of polarized epithelial cells also limits AdV-mediated gene delivery (Goldman et al. 1996). Integrins have also been usurped by other enveloped and nonenveloped viruses; however, it is not clear in all cases whether these receptors mediate virus attachment or entry or both (Stewart and Nemerow 2007).

Association of multiple integrin heterodimers with the penton base protein induces the activation of several cell signaling molecules, including phosphatidyl inositol 3-OH kinase (PI3K), p130CAS, and the Rho family of small GTPases (Li et al. 1998a, b, 2000). These signaling molecules induce the polymerization of actin filaments, a process needed for the rapid internalization of the virus into clathrin-coated pits and vesicles (Wang et al. 1998, 2000). AdV infection also stabilizes the host cell microtubule network in a RhoA- and Rac1-dependent manner, which may contribute to the efficiency of AdV nuclear localization (Warren et al. 2006; Warren and Cassimeris 2007). Integrin signaling may be facilitated through the spacing of the RGD loops on the penton base (i.e., ~60 Å apart), which allows up to 4 5 integrins to be bound to each pentamer (Stewart and Nemerow 2007).

Interestingly, integrin αvβ5 can also facilitate AdV entry events occurring postinternalization. Previous studies showed that expression of integrin αvβ5 on host cells selectively allows AdV-mediated membrane penetration (Wickham et al. 1994). Deletions or specific amino acid substitutions created near the C-terminus of the β5 integrin cytoplasmic tail allow virus internalization but restrict subsequent escape from the early endosome (Wang et al. 2000). Furthermore, the expression of the transcription factor PU.1 in alveolar macrophages results in reduced expression of β5 integrin, which was correlated with a failure of internalized AdV to escape from the endosome (Carey et al. 2007). Although the precise molecular mechanism for β5-integrin enhancement of AdV entry is still unresolved, one possibility is that this receptor participates in AdV disassembly, a process that occurs in the low pH environment of the early endosome. In support of this possibility, integrin αvβ5 but not αvβ3 is capable of binding to the penton base at reduced pH values (Wickham et al. 1994).

Integrin binding by HAdV has important consequences beyond virus internalization and cell membrane penetration. AdV activation of cell signaling molecules appears to be associated with an increase in macropinocytosis (Meier et al. 2002). Although macropinocytosis does not directly mediate AdV uptake into cells, it may facilitate AdV-mediated endosome disruption in a manner that has yet to be fully elucidated (Amstutz et al. 2008; Xie et al. 2006). Activation of PI3K also modulates several other important host cell functions including increasing cell survival (Rajala et al. 2005). Extended cell survival could be advantageous for the virus by allowing adequate time for the production of progeny virions before cell lysis. PI3K activation can also have detrimental consequences for the host. For example, PI3K activation by AdV vectors leads to the production of proinflammatory cytokines

such as TNF-α (Philpott et al. 2004). Such events may contribute to AdV pathogenesis as well as limit the utility of AdV vectors for gene transfer.

4 Uncoating of Adenovirus Particles

Following integrin-mediated endocytosis, AdV is still topologically outside of the cell. As such, there are three distinct barriers impeding delivery of the viral genome to the nucleus: the protein capsid of the virus particle, the endosomal membrane, and the nuclear envelope. The dissolution of the first barrier, imposed by the virus protein capsid, is the conceptual definition of "uncoating." The term "uncoating" has also been defined functionally or phenotypically in various studies to describe changes in the virus either *in vivo* or *in vitro*. Assays for uncoating include (1) alterations in the accessibility of the viral DNA to enzymes (e.g., DNase) or DNA-sensitive dyes (Lawrence and Ginsberg 1967; Lonberg-Holm and Philipson 1969; Philipson 1967; Russell et al. 1967; Svensson and Persson 1984; Wiethoff et al. 2005); (2) alterations in the density of viral protein/DNA complexes (Lawrence and Ginsberg 1967; Lonberg-Holm and Philipson 1969; Philipson 1967; Russell et al. 1967); (3) measurement of the association of capsid proteins with, or dissociation from, the nucleic acid-containing core (Laver et al. 1969; Prage et al. 1968; Russell et al. 1967; Smith and Nemerow 2008; Wiethoff et al. 2005); and (4) morphologic changes observed by microscopy including EM, immunofluorescence, and other fluorescence-based assays such as FRET (Chardonnet and Dales 1970; Greber et al. 1996; Laver et al. 1969; Martin-Fernandez et al. 2004; Morgan et al. 1969; Russell et al. 1967). Although each of these assays measures changes in the virus capsid from the state of the mature, extracellular form of the virus, the alterations in the capsid architecture that result in these phenotypes have not always been precisely defined.

AdV uncoating during cell entry occurs in stages. Although it is conceptually possible that the virus capsid simply falls apart within endosomes or is proteolytically digested to release the virus genome, this was shown not to be the case from the earliest studies of uncoating (Greber et al. 1993; Lawrence and Ginsberg 1967; Sussenbach 1967). Furthermore, the integrity and overall architecture of mature virions are also resistant to lysosomal enzymes (Boulanger et al. 1970). Rather, the virus appears to undergo specific transitions with measurable intermediates, suggesting that particular subsets of proteins are lost sequentially during virus entry and that the viral genome remains associated with a significant portion of the capsid during translocation through the cytoplasm (Lawrence and Ginsberg 1967; Philipson 1967; Russell et al. 1967; Sussenbach 1967). As described later, subsequent studies identified the capsid components that dissociate from the core and established the timeline of uncoating for the entry of HAdV-C. It is important to note that the model for AdV uncoating described herein is based primarily on studies of HAdV-C types. Although this model may be generally true for other HAdV species, emerging evidence indicates that HAdV-B types that use CD46 as a

primary attachment receptor differ substantially in the kinetics and subcellular localization of uncoating (Miyazawa et al. 2001; Shayakhmetov et al. 2003).

4.1 Vertex Dissociation from the Adenovirus Capsid

One widely used assay for the first step in uncoating is accessibility of the genomic DNA to DNase. DNase sensitivity has been shown *in vitro* to correlate with vertex removal, and the vertices are structurally weak parts of the capsid, which can be dissociated biochemically (Laver et al. 1969; Prage et al. 1970). A key concept, established early, is that the penton-less virus is a stable intermediate (Laver et al. 1969; Prage et al. 1970; Russell et al. 1967) and that the DNA is associated with a significant amount of viral protein under conditions in which it becomes sensitive to DNase (Lawrence and Ginsberg 1967; Philipson 1967; Russell et al. 1967; Sussenbach 1967). Therefore, the virus is assembled in such a manner that the association between penton and the peripentonal hexons is labile. Thus, assembly or postassembly maturational processing primes the virus for disassembly in stages.

Vertex removal is temperature-dependent (Lawrence and Ginsberg 1967) and likely occurs at or near the plasma membrane on the basis of several lines of evidence. First, DNase sensitivity occurs rapidly, with a $t_{1/2}$ of 10 20 min after attachment (Boulanger and Hennache 1973; Lawrence and Ginsberg 1967; Lonberg-Holm and Philipson 1969; Philipson 1967). This change closely follows the kinetics of virus internalization defined by loss of sensitivity to neutralizing serum or by protection of hexon from exogenous trypsin cleavage and lags 4 5 min behind the kinetics of attachment (Greber et al. 1993; Lawrence and Ginsberg 1967; Lonberg-Holm and Philipson 1969; Svensson 1985). Second, AdV internalization can be almost completely inhibited by 50 mM sodium azide (Svensson and Persson 1984; Svensson 1985); however, uncoating as measured by DNase sensitivity is reduced by only approximately 25% under these conditions (Svensson and Persson 1984). More recent studies using inhibitors of F-actin (jasplakinolide, Jas) are consistent with fiber loss occurring independently of internalization (Nakano et al. 2000), but the pleitropic effects of Jas on the infected cell make these studies somewhat less compelling. Third, Greber et al. showed that fiber is found only on extracellular virus, but not on virus in intracellular vesicles or in the cytoplasm (Greber et al. 1993). Despite the evidence that vertex removal occurs at the cell surface, isolated cell membranes alone are unable to mediate uncoating, suggesting a need for intact cells to mediate this process (Brown and Burlingham 1973; Svensson and Persson 1984). Although the molecular basis for vertex release is not known, there is some evidence that it is dependent upon integrin engagement, as RGD peptides that block integrin binding by penton base also block vertex release (Nakano et al. 2000).

Which component of the vertex region initially dissociates remains an open question. Is fiber alone removed, or does the entire penton (fiber plus penton base)

dissociate? No study has convincingly resolved this issue. Sussenbach observed that, shortly after internalization, virus is converted to a less buoyant form (1.35 g/cm^3 compared to 1.34 g/cm^3 for intact virus) (Sussenbach 1967). This form was DNase sensitive and, based on mass calculations, missed 5 6% of the virus protein (Sussenbach 1967), consistent with the loss of penton base and fiber (Prage et al. 1970). These particles were also shown to be completely noninfectious, poorly hemagglutinating, and lacking the penton base and fiber antigens (Prage et al. 1968; Sussenbach 1967). In vitro studies demonstrated that DNase sensitivity correlated with loss of fiber, pentons, and peripentonal hexons upon heating of the virus (Russell et al. 1967), providing a link between *in vitro* assays of uncoating and intermediates observed during virus infection *in vivo*. In more recent studies in which virus was first bound to cells at 4°C, hexon or labeled genomic DNA that immunoprecipitated with anti-fiber antibodies was quantified as a function of time postwarming to 37°C (Greber et al. 1993; Nakano et al. 2000). Both studies concluded that fiber release was extensive (80 90% dissociated) and occurred within the first 30 60 min after warming, with a $t_{1/2}$ of 12 min (Greber et al. 1993; Nakano et al. 2000). Neither study was able to distinguish between loss of fiber alone and loss of the entire penton. These studies were further confounded by unequal recovery of fiber at each time point, perhaps indicative of fiber degradation. Penton base undergoes a conformational change when bound to fiber, and the association between fiber and penton base, once established *in vitro*, is basically irreversible (Zubieta et al. 2006). This observation supports the notion that the penton dissociates as a unit rather than as individual components. On the other hand, there is evidence that fiber stabilizes the capsid (Von Seggern et al. 1999). Although fiber-less particles can be produced, these particles are, not surprisingly, less infectious than wild-type particles due to their inability to bind to a high affinity, primary receptor. Rather, their entry pathway is likely solely integrin-dependent (Legrand et al. 1999; Von Seggern et al. 1999). In addition, increased amounts of extra-viral DNA were observed upon EM analysis of these particles compared to wild type (Von Seggern et al. 1999). Given the small surface area on the virus capsid occupied by the fiber protein and the nature of its association with penton base (Von Seggern et al. 1999), it is unlikely that a hole or a channel in the capsid created by removal of fiber alone is large enough for the genome to become accessible to DNase. However, release of the fiber from the penton base could lead to destabilization of the entire vertex region. In support of this notion, anti-fiber antibodies, but not antisera against the other major capsid proteins, have been shown to sensitize virus to DNase (Everitt et al. 1992; Wohlfart et al. 1985), indicating that an interaction with the fiber alone could be a trigger for subsequent uncoating steps, and suggesting that the physiologic interaction between virus and the primary receptor may play a similar role. Further studies using purified components are needed to establish the roles of primary receptor, integrin interactions, and other host cell-derived triggers (e.g., endosomal acidification) in this first step in uncoating. Moreover, conclusive evidence for whether fiber alone or the entire penton is released and the relationship between this first step and subsequent uncoating events remains to be established.

4.2 Adenovirus Uncoating in the Endosome

After vertex removal, the virus undergoes further uncoating in the endosomal pathway. These changes include loss of the peripentonal hexons, IIIa, VIII, IX, and release of protein VI. Most of the evidence for these dissociation events is from the studies by Greber et al. (1993). Specifically, dissociation of the minor capsid proteins from hexon was studied through quantifying the amount of each protein bound to hexon after immunoprecipitation with anti-hexon antibodies as a function of time postinfection. One drawback of this approach is that the values obtained were normalized to the amount of hexon recovered, which was shown to degrade over time. Nonetheless, these studies indicated that IIIa ($t_{1/2} = 11$ min) and VIII ($t_{1/2} = 10$ min) were lost in the same time frame as fiber, whereas protein IX dissociated much later ($t_{1/2} = 30$ min), roughly coincident with DNA dissociation from hexon ($t_{1/2} = 35$ min). Penton base dissociation was reportedly difficult to quantify, but in samples incubated with monensin, it occurred with slightly slower kinetics than that for fiber dissociation ($t_{1/2} = 15$ min). Although these results suggest that fiber and penton base dissociation do not occur simultaneously, from recent structural studies it is unclear how IIIa and VIII could dissociate without prior release of the penton base from the capsid (Saban et al. 2006). In separate publications, it was suggested that protein VI is degraded shortly after endosome disruption ($t_{1/2} = 20$ min) (Greber et al. 1993, 1996). Both VI degradation and penton base dissociation were interpreted to be independent of endosome penetration because they were not inhibited by monensin. However, it is unclear if monensin inhibited infection in these samples, as this was not studied in parallel, and the role of pH in triggering uncoating is unclear (see below). Moreover, these studies could not distinguish loss of protein VI due to membrane association vs. proteolytic degradation.

A crucial finding is that uncoating in the endosome is linked to endosomal penetration. Studies using inhibitors of endosomal acidification (see section on the role of pH below) suggest that HAdV-C escapes from the early endosome approximately 5 min after endocytic uptake (Greber et al. 1993). In EM studies, particles have been observed in the cytoplasm as early as 5 min after virus/cell complexes that were formed at 4°C were warmed to 37°C (Brown and Burlingham 1973; Chardonnet and Dales 1970; FitzGerald et al. 1983). Early EM studies identified particles in the cytoplasm that appeared rounded and that had lost their pentons, similar to penton-less particles produced *in vitro* (Brown and Burlingham 1973; Morgan et al. 1969; Prage et al. 1970). These observations suggested that the association between the peripentonal hexons and the rest of the facet is different than the association between hexons within the facets, allowing an intact capsid remnant to remain after the endosomalytic machinery (e.g., VI) is deployed (Prage et al. 1970). The "minor" capsid proteins were implicated in these cementing roles, a model that has been substantiated by structural studies of AdV at increasing resolutions (Saban et al. 2006); however, the molecular details of the interactions between capsid proteins remain to be elucidated (see above). No particle in the

process of translocating through an endosomal vesicle has been observed, likely due to the rapid nature of such an event and to the fact that such an intermediate would likely be fragile and not preserved during sample preparation, although some suggestive images of particles in proximity to what appear to be disrupted endosomal vesicles have been observed (FitzGerald et al. 1983). Rather, evidence for endosomal penetration has largely been indirect. One widely used class of assays for this process has been the co-entry of macromolecules. In these studies, macromolecules including toxins, DNA complexes, and enzymes, which are unable to penetrate the cell on their own, are shown to gain access to the cytoplasm upon co-entry with AdV particles (Cotten et al. 1992; FitzGerald et al. 1983; Guy et al. 1995). The ability of these molecules to reach cytosolic targets during AdV infection is taken as proof that the virus has mediated disruption of the cellular endosomal membrane. In support of this conclusion, AdV particles and macromolecules have been directly observed in the same endosomal compartment (FitzGerald et al. 1983). These assays provide an experimental system to study the roles of viral and host cell factors on AdV-mediated endosomalysis.

4.3 Identification of Membrane Lytic Factors of Adenovirus

While a prerequisite for partial uncoating of the AdV capsid within endosomes to mediate membrane penetration has been known for roughly two decades (Greber et al. 1993; Svensson and Persson 1984; Svensson 1985), the proteins involved in this step were not clearly defined. Previous attempts to identify components of the AdV capsid involved in membrane penetration implicated the penton base (Seth et al. 1984a, c, 1985b). Most of these studies were done prior to our understanding of the role of penton base integrin interactions in AdV endocytosis, a necessary step occurring prior to virus uncoating and penetration of cellular membranes. In none of these studies was the penton base found to directly interact with and permeabilize membranes, however.

The first study to implicate the penton base in HAdV-2-mediated penetration of endosomal membranes monitored membrane penetration biochemically by assaying the ability of HAdV-2 to facilitate the cytosolic translocation of an otherwise membrane-impermeable toxin, a pseudomonas exotoxin-epidermal growth factor conjugate (Seth et al. 1984a). In the cytoplasm, this toxin conjugate inhibits protein synthesis. In these studies, the authors demonstrated that transcriptionally inactive, UV-treated AdV enhanced toxin-dependent inhibition of protein synthesis. Enhancement was inhibited by the presence of anti-penton base antiserum, which was significantly more effective at blocking than hexon antiserum, although neither interfered with virus attachment or internalization. This led the authors to conclude that penton base disrupts endosomal membranes to permit the virus access to the cytoplasm. More recent studies have demonstrated that RGD peptides or antibodies against penton base can inhibit penton binding to integrins, thus preventing virus endocytosis, subsequent capsid uncoating in endosomes, and permeablization of endosomal

membranes (Greber et al. 1996; Wickham et al. 1993). So, these results cannot directly implicate the penton base in the permeablization of endosomal membranes.

Additional studies have demonstrated that incubation with anti-penton antiserum also prevents AdV-dependent release of chromium or choline ions from cells (Seth et al. 1984c) or plasma membrane vesicles (Seth 1994). Although this approach might be a more direct assessment of the ability of AdV to permeabilize membranes, alternative conclusions are possible. For example, these results could be explained by a requirement for penton integrin interactions that are necessary to facilitate conformational changes in the capsid that would render the virus membrane-lytic. In fact, using identical assays, Wickham et al. demonstrated that the efficiency of AdV-mediated permeabilization of cell membranes to choline was dependent on the type of integrin present on the cell surface (Wickham et al. 1994). In these same studies, incubation of cells with 1 mg/ml of purified penton base prevented HAdV-2-mediated release of choline, suggesting that penton base alone at extremely high concentrations was unable to permeabilize cell membranes and consistent with a model in which soluble penton base competed with virus for integrin binding. Finally, the precise mechanism and physiological relevance of these assays is still poorly understood, especially considering that AdV does not directly penetrate the plasma membrane as a major route of entry.

Protein VI, another AdV protein that is present within the capsid underneath the hexon proteins and not exposed on the capsid surface, was recently shown to be membrane lytic and has been implicated in the AdV-dependent disruption of endosomal membranes during cell entry (Wiethoff et al. 2005). As mentioned earlier, partial uncoating of AdV capsids, either in the endosome during cell entry or *in vitro*, results in the release of penton base, peripentonal hexons, fiber, protein IIIa, and protein VI from the remaining capsid and nucleic acid core. In vitro studies demonstrated that AdV-dependent liposome disruption requires partial uncoating and that the membrane lytic activity is primarily a property of the proteins released from the capsid and not of the remaining capsid components associated with the core (Wiethoff et al. 2005). Immunodepletion of the penton base from these capsid-dissociated proteins had no effect, while immunodepletion of protein VI removed virtually all of the membrane lytic activity. Recombinant purified protein VI was also shown to have pH-independent membrane lytic activity similar to that of heat-disrupted capsids, a property never observed for purified penton base. Taken together, these findings suggest that protein VI is a major membrane-lytic factor of AdV, that the reported pH-dependence of AdV-dependent membrane lysis is related to capsid uncoating (Blumenthal et al. 1986; Seth et al. 1984c), and that the membrane lytic activity of AdV per se is a pH-independent process.

Protein VI is expressed as a 250-residue precursor protein (preVI) during assembly of virions in the nucleus (Fig. 3). It plays a key role in viral assembly by facilitating nuclear import of the hexon protein (Wodrich et al. 2003), and the preVI C-terminal 11 residues serve as an activator of the virally encoded 23 K proteinase, adenain (Ding et al. 1996; Webster et al. 1993). Mature virions contain protein VI that has undergone cleavage by adenain before residue 34 and after residue 239. Secondary structure analysis of the mature form of protein VI (residues

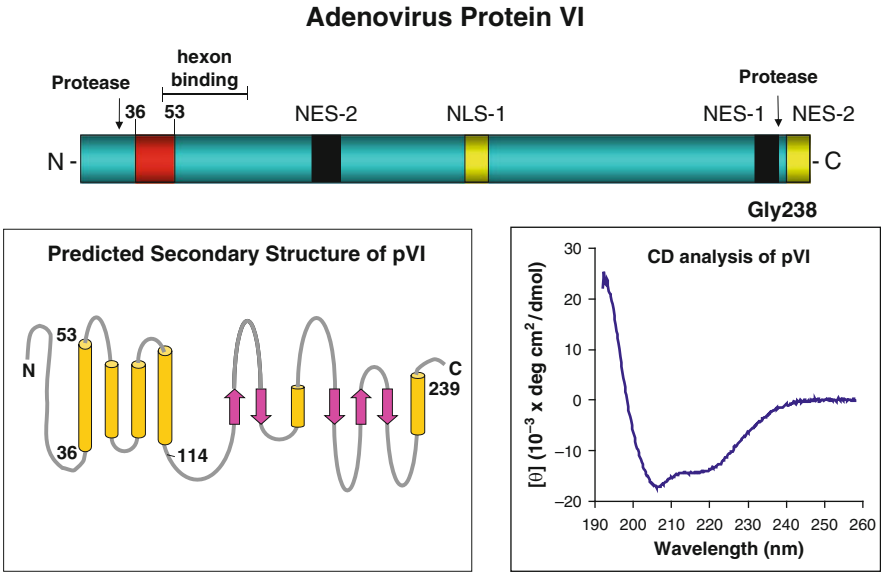


Fig. 3 The secondary structure and functional domains of adenovirus protein VI. A schematic representation of preprotein VI and its nuclear import (NLS1, 2) and nuclear export (NES 1, 2) sites, protease cleavage sites, and hexon binding domains are shown. Depicted in red (residues 36 53) is the predicted amphipathic α helical domain that mediates membrane disruption. The predicted secondary structure of preprotein VI is shown below (*left*). Note that residues 34 114 are likely to form four α helical segments. Consistent with this possibility (*right panel*), the CD spectra of a recombinant $pVI_{34\ 114}$ protein indicates that its secondary structure is estimated to be 85% α helix and15% random coil by analysis of the spectrum with the CONTIN software package

34-239) using a variety of prediction algorithms indicates that the N-terminal 80 residues comprise a domain predicted to possess four α-helices, one of which (20 residues in length) is the new N-terminus of protein VI that is generated upon proteolysis of preVI by adenain. The α-helical structure of this N-terminal 80-residues has been confirmed by circular dichroism spectrapolarimetry (Fig. 3). The primary sequence and physicochemical properties of this α-helix are highly conserved among sequenced strains of AdV, including its amphipathicity, net charge, and the presence of three glycine residues spaced roughly equidistant along the length of the helix (Wiethoff et al. 2005). This helix is a critical determinant of protein VI membrane lytic activity *in vitro*. The remaining 115 C-terminal residues of VI are likely largely disordered, with 85% predicted to be random coil. Although initial data from *in vitro* studies supports the notion that protein VI possesses >95% of the membrane lytic activity of the capsid, contributions from other capsid or cellular proteins during cell entry cannot be completely excluded.

Although a definitive role for protein VI in AdV-mediated membrane penetration has yet to be obtained, it is compelling to consider how a role for protein VI in AdV-cell entry compares with emerging paradigms for membrane penetration by

other nonenveloped viruses. Like AdV protein VI, the reovirus μ1 protein, which is responsible for reovirus penetration of endosomal membranes, is located directly underneath the major capsid protein σ3. Upon digestion of σ3 by intestinal or endosomal proteases, μ1 becomes exposed, and a myristoylated N-terminal μ1 peptide is released and binds membranes, leading to the formation of size selective pores (Agosto et al. 2006; Ivanovic et al. 2008). Polyomaviruses such as SV40 and mouse polyomavirus are endocytosed and undergo retrograde transport to the endoplasmic reticulum where members of the oxidoreductase family of ER proteins facilitate a conformational change in the capsid that exposes internal capsid proteins VP2 and VP3, which are involved in penetration of the ER membrane (Daniels et al. 2006; Magnuson et al. 2005). Similar paradigms in which a conformational change in external capsid proteins leads to exposure or release of internal capsid proteins or peptides that facilitate penetration of cellular membranes during entry have also been observed for parvoviruses (Farr et al. 2005; Mani et al. 2006), picornaviruses (Belnap et al. 2000), and nodaviruses (Janshoff et al. 1999). Further analyses will ultimately be required to understand how similar these mechanisms of nonenveloped virus membrane penetration actually are as well as any potential basis for the evolution of similar mechanisms by seemingly disparate viruses.

4.4 Role of Acidic pH in Adenovirus Entry

One major unresolved question in AdV entry is the nature of the trigger that leads to uncoating and endosomal penetration. While it is widely held that the acidic pH of the endosome plays a role in this process, the literature provides conflicting evidence for exposure to acidic pH per se in triggering uncoating. These studies focus primarily on the effects of inhibitors of endosomal acidification including weak bases (ammonium chloride, amantadine, chloroquine, and methylamine), ionophores (monensin), and inhibitors of the vacuolar H^+-ATPase (bafilomycin A1) on various measures of virus uncoating and infection including DNase sensitivity, macromolecule co-entry, production of viral transcripts, viral protein production, transgene expression, and plaque formation. As each of these compounds can have pleiotropic effects on cells and may function at different concentrations in different cell types, careful monitoring of toxicity and positive controls for the effects of the drugs on known pH-dependent processes in parallel are vital to the interpretation of experiments using these inhibitors.

With these considerations in mind, the following studies provide support for an acidic pH-dependent step in AdV uncoating and infection. Infection at low moi is inhibited approximately sevenfold by 25 mM ammonium chloride and 25-fold by 20 μM monensin (Greber et al. 1993). This assay was dependent upon a relatively brief exposure to the inhibitors (150 min) followed by prolonged incubation in the absence of inhibitors (5 6 days) and measured plaque formation. This study suggests that virus, which fails to penetrate the early endosome during the window of exposure to drug (150 min), is no longer capable of infecting the cell when the

drugs are washed out. In a macromolecule co-entry assay using an epidermal growth factor-Pseudomonas toxin fusion protein (PE-EGF), 40 μM chloroquine, 10 mM ammonium chloride, 5 mM methylamine, 100 μM *N*-hexylamine, and 1 μM monensin were required for half maximal inhibition of the enhancement of toxicity of PE-EGF by AdV (Seth et al. 1984b, c). These treatments did not affect virus attachment or internalization, supporting the conclusion that exposure to acidic pH is required for AdV-dependent endosomalysis, although none of the agents were able to completely block the AdV-dependent enhancement at any nontoxic concentration. By a similar approach, fluorescent dextran internalization was enhanced by AdV, but not by heat-inactivated virus, and led to cytosolic distribution of the label compared to a localization in pinocytic vesicles that was observed in control cells (Yoshimura 1985). The kinetics of enhancement were similar to those of AdV internalization, and this effect was blocked by 20 mM sodium azide and also partially blocked by 500 μM chloroquine, 50 mM ammonium chloride, and 50 μM monensin. The effects of these inhibitors correlated with an incomplete inhibition of virus nuclear localization and a modest (<50%) inhibition in DNase sensitivity as a measure of uncoating. In a separate study, cyclic D,L-α-peptides inhibited virus infection and blocked AdV-dependent macromolecule co-entry (using α-sarcin), and in cells exposed to these inhibitors, the block in infection correlated with the exposure of virus to a higher endosomal pH than that in control cells (Horne et al. 2005). Similarly, we have also observed that AdV-dependent macromolecule co-entry (α-sarcin) is sensitive to 0.3 μM bafilomycin A1 (Wiethoff et al. 2005). In addition to assays of infection or endosome penetration, direct AdV-dependent release of chromium or other small molecules from cells has also been shown to be pH-dependent (Seth et al. 1984c, 1985a). However, the physiologic relevance of these assays for AdV entry is unclear. Similarly, Triton X-114 association was used as a measure of changes in the hydrophobicity of the HAdV-2 capsid or the major virus capsid proteins. In this assay, it was shown that the hydrophobicity of the virus is enhanced at pH 5.0 and that penton base is the capsid protein most highly associated with detergent at this pH, although hexon and fiber also show increased detergent association under acidic conditions (Seth et al. 1985b). Taken together, these studies provide substantial evidence for a requirement of exposure to acidic pH in AdV infection; however, in no case is inhibition by the lysosomotropic agent complete.

Evidence against an obligate role for acidic pH in AdV infection has also been reported. In one study, 40 mM ammonium chloride, 400 μM chloroquine, or 40 mM methylamine had no effect on internalization or uncoating (Svensson and Persson 1984). A subsequent study from the same group using synchronized infections showed a modest effect of these reagents on the distribution of HAdV-2 in infected cells but only a small enhancement in the vesicular localization of HAdV-2 (Svensson 1985). In another study, 100 μM chloroquine was shown to have no effect on the enhancement of α-sarcin cytotoxicity by AdV, although this concentration of inhibitor was shown in parallel experiments to block enhancement by vesicular stomatitis virus and Semliki Forest virus, two known pH-dependent viruses (Otero and Carrasco 1987). In perhaps the most careful study of the role of endosomal

acidification in AdV entry and uncoating, four different lysosomotropic inhibitors at both nontoxic and moderately cytotoxic concentrations (5 and 10 mM ammonium chloride, 100 and 500 μM amantadine, 2.5 and 5 μM chloroquine, and 5 and 10 mM methylamine) had no effect on AdV uncoating or viral gene transcription (Rodriguez and Everitt 1996). Late effects on hexon production were observed for several of the inhibitors, but they were irrespective of the time of addition during infection, suggesting that the effects were not on entry. Similarly, 1 μM bafilomycin A1, 100 μM chloroquine, or 50 mM monensin did not affect AdV protein synthesis under conditions in which Semliki Forest virus was inhibited (Perez and Carrasco 1994), and 100 μM chloroquine, 5 μM monensin, or 10 mM ammonium chloride did not block AdV infection or AdV-dependent enhancement of DNA transfection when endosome neutralization was monitored by acridine orange staining (Meunier-Durmort et al. 1997). These studies provide strong evidence that a low pH-dependent step is not required for AdV entry and suggest that previous studies using higher concentrations of inhibitors and relying on downstream indicators of infection (e.g., plaque formation or hexon production) may have been confounded by toxicity or pleiotropic effects of the inhibitors on the cell.

Biochemical studies of the effects of low pH buffers on mature AdV particles have been equally conflicted. Incubation of AdV in low pH buffers has been described to result in vertex removal, although this is also dependent upon ionic strength (Everitt et al. 1988; Prage et al. 1970). While the thermostability of AdV can, in some instances, be significantly reduced at acidic pH (Wiethoff et al. 2005), other studies have found no effect of pH on the DNase sensitivity of virus (Svensson 1985) or even an enhancement of the stability of particles at low pH (Rexroad et al. 2006). The pH-dependence of purified AdV lysis of liposomes was biphasic with a pH optimum of pH 6 at 2°C and low ionic strength (Blumenthal et al. 1986). However, the pH requirement for liposome lysis at room temperature was drastically different than that at low temperature, presumably due to the aggregation of the virus at the high concentrations necessary for these experiments. These discrepancies make it difficult to reach any conclusions about the role of pH in the exposure of a membrane lytic factor from AdV during virus infection based on these experiments. Moreover, subsequent studies of recombinant protein VI, described earlier, suggest that this protein mediates pH-independent membrane lysis (Wiethoff et al. 2005).

Although some of these studies support a requirement for exposure to acidic pH during AdV entry, a hypothesis to explain the conflicting data is that the proton gradient itself is more important than the presence of a higher concentration of protons (Carrasco 1994). Alternatively, the pH requirements for AdV-mediated toxin entry may differ from those of AdV-cell entry. For example, while acidic pH may not be required for AdV penetration of endosomal membranes, there may be pH-dependent release of the toxins from association with the endosomal membrane, or in the case of toxin conjugates (e.g., PE-EGF) with specific receptors (Seth et al. 1984b). Another possible role for pH is to activate endosomal proteases; however, there is currently no evidence that lysosomal proteases, such as cathepsins, play a role in AdV uncoating, and lysosomal enzymes alone were unable to render the

virus DNase sensitive (Boulanger et al. 1970). Finally, AdV may use both pH-dependent and -independent entry pathways. Clearly, further studies are needed to define the precise role for pH in AdV uncoating and infection and to elucidate in molecular detail the process of capsid disassembly.

4.5 Role of the Encapsidated Adenovirus Protease in Entry

AdV encodes a 23 K cysteine protease (also known as adenain) that is required for maturational cleavage of six capsid proteins: IIIa, VI, VII, VIII, μ(X), and TP (McGrath et al. 1996; Weber 1995; Webster et al. 1989). A role for the 23 K cysteine protease has also been implicated in entry. Greber et al. found that reduction and alkylation of virus reduced infectivity, presumably without disrupting particle integrity (Greber et al. 1996). Although a number of viral capsid proteins were labeled by NEM in these experiments, the 23 K cysteine protease was the most strongly labeled, suggesting that inhibition of the protease was reducing infection (Greber et al. 1996). Similarly, another protease inhibitor, copper chloride, also blocked AdV-mediated transgene expression (Cotten and Weber 1995). Viruses bearing alkylated proteases were internalized normally and translocated to the nuclear membrane but failed to disassemble at the nuclear pore complex, the final step in uncoating (Greber et al. 1996). Loss of TCA-precipitable protein VI, either due to degradation or membrane association, was blocked by protease alkylation, and protein VI loss was dependent upon integrin engagement. The authors proposed that integrin engagement exposes sites in protein VI that become cleaved once the encapsidated protease is activated by the reducing environment of the endosome, an alternative trigger for uncoating that is not mutually exclusive of acidic pH (although disulphide bond rearrangement required to activate the 23 K cysteine protease would be inhibited by cysteine protonation) (Greber et al. 1996; Greber 1998). Protein VI degradation is then proposed to be required for subsequent dissociation of DNA from the capsid at the nuclear pore complex. However, an equally plausible event is that release of VI from the capsid during uncoating could render this molecule susceptible to host cell proteases.

4.6 Uncoating at the Nuclear Pore Complex

The final destination of the virus genome is the nucleus. After dynein-dependent translocation along microtubules to the microtubule-organizing center, the HAdV-2 is actively rescued by a CRM1-dependent process and associates with the nuclear pore complex (Bailey et al. 2003; Chardonnet and Dales 1970; Dales and Chardonnet 1973; Leopold et al. 2000; Strunze et al. 2005). There, the virus undergoes further uncoating indicated by increased reactivity with nonneutralizing anti-hexon or anti-VII antibodies (Greber et al. 1996). HAdV-2 and HAdV-7 at the

nuclear pore complex have been observed by EM to be "markedly altered or actually stripped off" or to consist of empty shells, suggesting that the viral genome, but not the bulk of the partially uncoated capsid, is translocated into the nucleus (Chardonnet and Dales 1970; Morgan et al. 1969).

4.7 The Impact of the Immune System on Uncoating

Effectors of both the innate and adaptive immune system have been shown to neutralize AdV infection by blocking virus entry. Although neutralizing antibodies (NAbs) against hexon, penton base, and fiber have been described, the major target for neutralization appears to be hexon (Roy et al. 2005; Sumida et al. 2005). Fiber and penton base antisera act primarily by blocking receptor interactions (Philipson et al. 1968; Stewart et al. 1997; Wohlfart et al. 1985). In contrast, anti-hexon NAbs have been shown to have multiple modes of action including blocking receptor interactions and preventing uncoating and endosome escape by effectively cross-linking the capsid (Toogood et al. 1992; Wohlfart 1988; Wohlfart et al. 1985). In addition, both anti-hexon and anti-fiber NAbs can aggregate AdV. Recently, we have described the activity of an anti-hexon NAb, called 9C12, which blocks HAdV-5 infection by a novel mechanism (Smith et al. 2008; Varghese et al. 2004). Rather than disrupting an early stage in entry, this NAb appears to block a step between endosome penetration and translocation to the microtubule-organizing center. Although the mechanism of inhibition is not known in molecular detail, inhibition of infection at such a late stage in entry by a NAb was unanticipated.

Less is known about the role of the innate immune response in blocking early entry events in nonenveloped virus infection; however, emerging studies suggest that antimicrobial peptides, one effector arm of the innate immune response, may be potent natural anti-virals. We have recently described the mechanism by which one family of antimicrobial peptides, human α-defensins, neutralize HAdV infection (Smith and Nemerow 2008). These small, amphipathic peptides were found to bind directly to the virus capsid, thereby preventing uncoating and endosome penetration. Studies of human papillomavirus suggest that these peptides may inhibit this nonenveloped virus at a similar stage of infection (Buck et al. 2006). Further studies are needed to determine which interactions between these peptides and the virus capsid are critical for inhibition, and to determine the role of these peptides as natural anti-virals *in vivo*.

5 Conclusions

The role of specific cell receptors and other host factors in AdV attachment and internalization are now fairly well defined, although further *in vivo* analyses are needed to fully understand the earliest events in AdV pathogenesis. In contrast, we

lack sufficient information on the intracellular events that trigger AdV capsid disassembly or of the precise regions of the virus that undergo conformational changes as the "weak link" in uncoating. Important clues to these processes have been obtained via cryoEM structural and cell biological studies, but it is clear that additional structural analyses of AdV particles at high resolution (i.e., 3 4 Å) may provide complementary information. Finally, as AdV elicits one of the more potent antiviral responses, further knowledge of viral entry mechanisms may also shed light on crucial interactions with components of the innate immune system and could help shape the design of gene delivery vectors. As a member of the community of nonenveloped viruses, AdV may also help formulate the general rules for membrane penetration by these naked microbes.

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