Published in final edited form as:

Curr Top Microbiol Immunol. 2010 ; 343: 195–224. doi:10.1007/82_2010_16.

Adenovirus

Jason G. Smith,

Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 N. Torrey Pines Road, IMM-19, La Jolla, CA 92037, USA

Christopher M. Wiethoff,

Department of Microbiology and Immunology, Loyola University Medical Center, Maywood, IL 60153, USA

Phoebe L. Stewart, and

Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN 37232, USA

Glen R. Nemerow

Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 N. Torrey Pines Road, IMM-19, La Jolla, CA 92037, USA

Glen R. Nemerow: gnemerow@scripps.edu

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 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 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 numbers of the AdV structural proteins are known from a biochemical analysis (van Oostrum and Burnett 1985) as well as from mass spectrometry (Lehmborg 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 (Dechechi 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 (Kalyuzhnyi 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 HSPG 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*tsI*, 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 ($K_D \approx 50\text{--}80\text{ nM}$) of the AdV penton base protein with the vitronectin-binding integrins $\alpha\beta 3$, $\alpha\beta 5$, and $\alpha\beta 1$ 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 α_v 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 phosphatidylinositol 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 $\alpha_v\beta_5$ can also facilitate AdV entry events occurring postinternalization. Previous studies showed that expression of integrin $\alpha_v\beta_5$ 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 β_5 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 β_5 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 β_5 -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 $\alpha_v\beta_5$ but not $\alpha_v\beta_3$ 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 pentonless 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 pleiotropic 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 endosomolytic 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 endosomal analysis.

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 permeabilization of endosomal membranes (Greber et al. 1996; Wickham et al. 1993). So, these results cannot directly implicate the penton base in the permeabilization 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 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 spectropolarimetry (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 $\mu 1$ protein, which is responsible for reovirus penetration of endosomal membranes, is located directly underneath the major capsid protein $\sigma 3$. Upon digestion of $\sigma 3$ by intestinal or endosomal proteases, $\mu 1$ becomes exposed, and a myristoylated N-terminal $\mu 1$ 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 μ M 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 μ M chloroquine, 10 mM ammonium chloride, 5 mM methylamine, 100 μ M *N*-hexylamine, and 1 μ M 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 endosomal lysis, 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 μ M chloroquine, 50 mM ammonium chloride, and 50 μ M 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 μ M 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 μ M 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 μ M 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 μ M amantadine, 2.5 and 5 μ M 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 μ M bafilomycin A1, 100 μ M 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 μ M chloroquine, 5 μ M 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.

Acknowledgments

This work is manuscript No. 19961 at The Scripps Research Institute.

References

- Agosto MA, Ivanovic T, Nibert ML. Mammalian reovirus, a nonfusogenic nonenveloped virus, forms size-selective pores in a model membrane. *Proc Natl Acad Sci USA*. 2006; 103:16496–16501. [PubMed: 17053074]
- Albinsson B, Kidd AH. Adenovirus type 41 lacks an RGD $\alpha(v)$ -integrin binding motif on the penton base and undergoes delayed uptake in A549 cells. *Virus Res*. 1999; 64:125–136. [PubMed: 10518709]
- Alemayehu R, Curiel DT. CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene Ther*. 2001; 8:1347–1353. [PubMed: 11571572]
- Amstutz B, Gastaldelli M, Kalin S, Imelli N, Boucke K, Wandeler E, Mercer J, Hemmi S, Greber UF. Subversion of CtBP1-controlled macropinocytosis by human adenovirus serotype 3. *EMBO J*. 2008; 27:956–969. [PubMed: 18323776]
- Arnberg N, Kidd AH, Edlund K, Nilsson J, Pring-Akerblom P, Wadell G. Adenovirus type 37 binds to cell surface sialic acid through a charge-dependent interaction. *Virology*. 2002a; 302:33–43. [PubMed: 12429514]
- Arnberg N, Pring-Akerblom P, Wadell G. Adenovirus type 37 uses sialic acid as a cellular receptor on Chang C cells. *J Virol*. 2002b; 76:8834–8841. [PubMed: 12163603]
- Bai M, Harfe B, Freimuth P. Mutations that alter an Arg-Gly-Asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. *J Virol*. 1993; 67:5198–5205. [PubMed: 8350395]
- Bailey CJ, Crystal RG, Leopold PL. Association of adenovirus with the microtubule organizing center. *J Virol*. 2003; 77:13275–13287. [PubMed: 14645584]
- Belnap DM, Filman DJ, Trus BL, Cheng N, Booy FP, Conway JF, Curry S, Hiremath CN, Tsang SK, Steven AC, Hogle JM. Molecular tectonic model of virus structural transitions: the putative cell entry states of poliovirus. *J Virol*. 2000; 74:1342–1354. [PubMed: 10627545]
- Benson SD, Bamford JK, Bamford DH, Burnett RM. Viral evolution revealed by bacteriophage PRD1 and human adenovirus coat protein structures. *Cell*. 1999; 98:825–833. [PubMed: 10499799]
- Bergelson JM, Cunningham JA, Droguett G, Kurt-Jones EA, Krithivas A, Hong JS, Horwitz MS, Crowell RL, Finberg RW. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*. 1997; 275:1320–1323. [PubMed: 9036860]
- Bergelson JM, Krithivas A, Celi L, Droguett G, Horwitz MS, Wickham T, Crowell RL, Finberg RW. The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J Virol*. 1998; 72:415–419. [PubMed: 9420240]
- Bewley MC, Springer K, Zhang YB, Freimuth P, Flanagan JM. Structural analysis of the mechanism of adenovirus binding to its human cellular receptor, CAR. *Science*. 1999; 286:1579–1583. [PubMed: 10567268]
- Blumenthal R, Seth P, Willingham MC, Pastan I. pH-dependent lysis of liposomes by adenovirus. *Biochemistry*. 1986; 25:2231–2237. [PubMed: 3754765]
- Boulanger PA, Hennache B. Adenovirus uncoating: an additional evidence for the involvement of cell surface in capsid labilization. *FEBS Lett*. 1973; 35:15–18. [PubMed: 4751992]
- Boulanger PA, Breynaert MD, Biserte G. Lysosomes and the problem of adenovirus uncoating. *Exp Mol Pathol*. 1970; 12:235–242. [PubMed: 5434757]
- Brown DT, Burlingham BT. Penetration of host cell membranes by adenovirus 2. *J Virol*. 1973; 12:386–396. [PubMed: 4127052]
- Buck CB, Day PM, Thompson CD, Lubkowski J, Lu W, Lowy DR, Schiller JT. Human α -defensins block papillomavirus infection. *Proc Natl Acad Sci USA*. 2006; 103:1516–1521. [PubMed: 16432216]
- Burmeister WP, Guilligay D, Cusack S, Wadell G, Arnberg N. Crystal structure of species D adenovirus fiber knobs and their sialic acid binding sites. *J Virol*. 2004; 78:7727–7736. [PubMed: 15220447]
- Caravokyri C, Leppard KN. Constitutive episomal expression of polypeptide IX (pIX) in a 293-based cell line complements the deficiency of pIX mutant adenovirus type 5. *J Virol*. 1995; 69:6627–6633. [PubMed: 7474071]

- Carey B, Staudt MK, Bonaminio D, van der Loo JC, Trapnell BC. PU.1 redirects adenovirus to lysosomes in alveolar macrophages, uncoupling internalization from infection. *J Immunol.* 2007; 178:2440–2447. [PubMed: 17277151]
- Carrasco L. Entry of animal viruses and macromolecules into cells. *FEBS Lett.* 1994; 350:151–154. [PubMed: 7915239]
- Cattaneo R. Four viruses, two bacteria, and one receptor: membrane cofactor protein (CD46) as pathogens' magnet. *J Virol.* 2004; 78:4385–4388. [PubMed: 15078919]
- Chardonnet Y, Dales S. Early events in the interaction of adenoviruses with HeLa cells. I. Penetration of type 5 and intracellular release of the DNA genome. *Virology.* 1970; 40:462–477. [PubMed: 5437781]
- Chatterjee PK, Vayda ME, Flint SJ. Interactions among the three adenovirus core proteins. *J Virol.* 1985; 55:379–386. [PubMed: 4020954]
- Chillon M, Kremer EJ. Trafficking and propagation of canine adenovirus vectors lacking a known integrin-interacting motif. *Hum Gene Ther.* 2001; 12:1815–1823. [PubMed: 11560774]
- Chiu CY, Mathias P, Nemerow GR, Stewart PL. Structure of adenovirus complexed with its internalization receptor, alphavbeta5 integrin. *J Virol.* 1999; 73:6759–6768. [PubMed: 10400774]
- Chiu CY, Wu E, Brown SL, Von Seggern DJ, Nemerow GR, Stewart PL. Structural analysis of a fiber-pseudotyped adenovirus with ocular tropism suggests differential modes of cell receptor interactions. *J Virol.* 2001; 75:5375–5380. [PubMed: 11333920]
- Chroboczek J, Ruigrok RW, Cusack S. Adenovirus fiber. *Curr Top Microbiol Immunol.* 1995; 199 (Pt 1):163–200. [PubMed: 7555054]
- Cohen CJ, Gaetz J, Ohman T, Bergelson JM. Multiple regions within the coxsackievirus and adenovirus receptor cytoplasmic domain are required for basolateral sorting. *J Biol Chem.* 2001a; 276:25392–25398. [PubMed: 11316797]
- Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proc Natl Acad Sci USA.* 2001b; 98:15191–15196. [PubMed: 11734628]
- Cotten M, Weber JM. The adenovirus protease is required for virus entry into host cells. *Virology.* 1995; 213(2):494–502. [PubMed: 7491774]
- Cotten M, Wagner E, Zatloukal K, Phillips S, Curiel DT, Birnstiel ML. High-efficiency receptor-mediated delivery of small and large (48 kilobase gene constructs using the endo-some-disruption activity of defective or chemically inactivated adenovirus particles. *Proc Natl Acad Sci USA.* 1992; 89:6094–6098. [PubMed: 1352882]
- Dales S, Chardonnet Y. Early events in the interaction of adenoviruses with HeLa cells. IV. Association with microtubules and the nuclear pore complex during vectorial movement of the inoculum. *Virology.* 1973; 56:465–483. [PubMed: 4271287]
- Daniels R, Rusan NM, Wadsworth P, Hebert DN. SV40 VP2 and VP3 insertion into ER membranes is controlled by the capsid protein VP1: implications for DNA translocation out of the ER. *Mol Cell.* 2006; 24:955–966. [PubMed: 17189196]
- Dechecchi MC, Tamanini A, Bonizzato A, Cabrini G. Heparan sulfate glycosaminoglycans are involved in adenovirus type 5 and 2-host cell interactions. *Virology.* 2000; 268:382–390. [PubMed: 10704346]
- Ding J, McGrath WJ, Sweet RM, Mangel WF. Crystal structure of the human adenovirus proteinase with its 11 amino acid cofactor. *Embo J.* 1996; 15:1778–1783. [PubMed: 8617222]
- Einfeld DA, Schroeder R, Roelvink PW, Lizonova A, King CR, Kovesdi I, Wickham TJ. Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. *J Virol.* 2001; 75:11284–11291. [PubMed: 11689608]
- Everitt E, Philipson L. Structural proteins of adenoviruses. XI. Purification of three low molecular weight virion proteins of adenovirus type 2 and their synthesis during productive infection. *Virology.* 1974; 62:253–269. [PubMed: 4213750]
- Everitt E, Persson MJ, Wohlfart C. pH-dependent exposure of endoproteolytic cleavage sites of the adenovirus 2 hexon protein. *FEMS Microbiol Lett.* 1988; 49:229–233.
- Everitt E, de Luca A, Blixt Y. Antibody-mediated uncoating of adenovirus *in vitro*. *FEMS Microbiol Lett.* 1992; 77:21–27. [PubMed: 1459411]

- Fabry CM, Rosa-Calatrava M, Conway JF, Zubieta C, Cusack S, Ruigrok RW, Schoehn G. A quasi-atomic model of human adenovirus type 5 capsid. *Embo J*. 2005; 24:1645–1654. [PubMed: 15861131]
- Fabry CM, Rosa-Calatrava M, Moriscot C, Ruigrok RW, Boulanger P, Schoehn G. The C-terminal domains of adenovirus serotype 5 protein IX assemble into an antiparallel structure on the facets of the capsid. *J Virol*. 2009; 83:1135–1139. [PubMed: 19004948]
- Farr GA, Zhang LG, Tattersall P. Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. *Proc Natl Acad Sci USA*. 2005; 102:17148–17153. [PubMed: 16284249]
- FitzGerald DJ, Padmanabhan R, Pastan I, Willingham MC. Adenovirus-induced release of epidermal growth factor and pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. *Cell*. 1983; 32:607–617. [PubMed: 6130853]
- Furcinitti PS, van Oostrum J, Burnett RM. Adenovirus polypeptide IX revealed as capsid cement by difference images from electron microscopy and crystallography. *EMBO J*. 1989; 8:3563–3570. [PubMed: 2583109]
- Gaggar A, Shayakhmetov DM, Lieber A. CD46 is a cellular receptor for group B adeno-viruses. *Nat Med*. 2003; 9:1408–1412. [PubMed: 14566335]
- Gastaldelli M, Imelli N, Boucke K, Amstutz B, Meier O, Greber UF. Infectious adenovirus type 2 transport through early but not late endosomes. *Traffic*. 2008; 9(12):2265–2278. [PubMed: 18980614]
- Goldman MJ, Wilson JM. Expression of alpha v beta 5 integrin is necessary for efficient adenovirus-mediated gene transfer in the human airway. *J Virol*. 1995; 69:5951–5958. [PubMed: 7545239]
- Goldman M, Su Q, Wilson JM. Gradient of RGD-dependent entry of adenoviral vector in nasal and intrapulmonary epithelia: implications for gene therapy of cystic fibrosis. *Gene Ther*. 1996; 3:811–818. [PubMed: 8875230]
- Greber UF. Virus assembly and disassembly: the adenovirus cysteine protease as a trigger factor. *Rev Med Virol*. 1998; 8:213–222. [PubMed: 10398510]
- Greber UF, Willetts M, Webster P, Helenius A. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell*. 1993; 75:477–486. [PubMed: 8221887]
- Greber UF, Webster P, Weber J, Helenius A. The role of the adenovirus protease on virus entry into cells. *Embo J*. 1996; 15:1766–1777. [PubMed: 8617221]
- Gustin KE, Imperiale MJ. Encapsidation of viral DNA requires the adenovirus L1 52/55-kilodalton protein. *J Virol*. 1998; 72:7860–7870. [PubMed: 9733823]
- Guy J, Drabek D, Antoniou M. Delivery of DNA into mammalian cells by receptor-mediated endocytosis and gene therapy. *Mol Biotechnol*. 1995; 3:237–248. [PubMed: 7552693]
- Horne WS, Wiethoff CM, Cui C, Wilcoxon KM, Amorin M, Ghadiri MR, Nemerow GR. Antiviral cyclic D, L-alpha-peptides: targeting a general biochemical pathway in virus infections. *Bioorg Med Chem*. 2005; 13:5145–5153. [PubMed: 15993611]
- Ivanovic T, Agosto MA, Zhang L, Chandran K, Harrison SC, Nibert ML. Peptides released from reovirus outer capsid form membrane pores that recruit virus particles. *Embo J*. 2008; 27:1289–1298. [PubMed: 18369316]
- Janshoff A, Bong DT, Steinem C, Johnson JE, Ghadiri MR. An animal virus-derived peptide switches membrane morphology: possible relevance to nodaviral transfection processes. *Biochemistry*. 1999; 38:5328–5336. [PubMed: 10220319]
- Kalyuzhnyi O, Di Paolo NC, Silvestry M, Hofherr SE, Barry MA, Stewart PL, Shayakhmetov DM. Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes *in vivo*. *Proc Natl Acad Sci USA*. 2008; 105:5483–5488. [PubMed: 18391209]
- Laver WG, Wrigley NG, Pereira HG. Removal of pentons from particles of adenovirus type 2. *Virology*. 1969; 39:599–604. [PubMed: 4982541]
- Law LK, Davidson BL. What does it take to bind CAR? *Mol Ther*. 2005; 12:599–609. [PubMed: 16109509]
- Lawrence WC, Ginsberg HS. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. *J Virol*. 1967; 1:851–867. [PubMed: 5621482]

- Legrand V, Spehner D, Schlesinger Y, Settelen N, Pavirani A, Mehtali M. Fiberless recombinant adenoviruses: virus maturation and infectivity in the absence of fiber. *J Virol.* 1999; 73:907–919. [PubMed: 9882291]
- Lehmberg E, Traina JA, Chakel JA, Chang RJ, Parkman M, McCaman MT, Murakami PK, Lahidji V, Nelson JW, Hancock WS, Nestaas E, Pungor E Jr. Reversed-phase high-performance liquid chromatographic assay for the adenovirus type 5 proteome. *J Chromatogr B Biomed Sci Appl.* 1999; 732:411–423. [PubMed: 10517364]
- Leopold PL, Kreitzer G, Miyazawa N, Rempel S, Pfister KK, Rodriguez-Boulan E, Crystal RG. Dynein- and microtubule-mediated translocation of adenovirus serotype 5 occurs after endosomal lysis. *Hum Gene Ther.* 2000; 11:151–165. [PubMed: 10646647]
- Li E, Stupack D, Bokoch GM, Nemerow GR. Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J Virol.* 1998a; 72:8806–8812. [PubMed: 9765425]
- Li E, Stupack D, Klemke R, Cheresch DA, Nemerow GR. Adenovirus endocytosis via alpha (v) integrins requires phosphoinositide-3-OH kinase. *J Virol.* 1998b; 72:2055–2061. [PubMed: 9499060]
- Li E, Stupack DG, Brown SL, Klemke R, Schlaepfer DD, Nemerow GR. Association of p130CAS with phosphatidylinositol-3-OH kinase mediates adenovirus cell entry. *J Biol Chem.* 2000; 275:14729–14735. [PubMed: 10799562]
- Li E, Brown SL, Stupack DG, Puente XS, Cheresch DA, Nemerow GR. Integrin alpha(v) beta1 is an adenovirus coreceptor. *J Virol.* 2001; 75:5405–5409. [PubMed: 11333925]
- Lisewski U, Shi Y, Wrackmeyer U, Fischer R, Chen C, Schirdewan A, Juttner R, Rathjen F, Poller W, Radke MH, Gotthardt M. The tight junction protein CAR regulates cardiac conduction and cell-cell communication. *J Exp Med.* 2008; 205:2369–2379. [PubMed: 18794341]
- Lonberg-Holm K, Philipson L. Early events of virus-cell interaction in an adenovirus system. *J Virol.* 1969; 4:323–338. [PubMed: 4898584]
- Lortat-Jacob H, Chouin E, Cusack S, van Raaij MJ. Kinetic analysis of adenovirus fiber binding to its receptor reveals an avidity mechanism for trimeric receptor-ligand interactions. *J Biol Chem.* 2001; 276:9009–9015. [PubMed: 11124261]
- Lukashok SA, Horwitz MS. New perspectives in adenoviruses. *Curr Clin Top Infect Dis.* 1998; 18:286–305. [PubMed: 9779361]
- Magnuson B, Rainey EK, Benjamin T, Baryshev M, Mkrtchian S, Tsai B. ERp29 triggers a conformational change in polyomavirus to stimulate membrane binding. *Mol Cell.* 2005; 20:289–300. [PubMed: 16246730]
- Mani B, Baltzer C, Valle N, Almendral JM, Kempf C, Ros C. Low pH-dependent endosomal processing of the incoming parvovirus minute virus of mice virion leads to externalization of the VP1 N-terminal sequence (N-VP1), N-VP2 cleavage, and uncoating of the full-length genome. *J Virol.* 2006; 80:1015–1024. [PubMed: 16379002]
- Marsh MP, Campos SK, Baker ML, Chen CY, Chiu W, Barry MA. Cryoelectron microscopy of protein IX-modified adenoviruses suggests a new position for the C terminus of protein IX. *J Virol.* 2006; 80:11881–11886. [PubMed: 16987967]
- Martin-Fernandez M, Longshaw SV, Kirby I, Santis G, Tobin MJ, Clarke DT, Jones GR. Adenovirus type-5 entry and disassembly followed in living cells by FRET, fluorescence anisotropy, and FLIM. *Biophys J.* 2004; 87:1316–1327. [PubMed: 15298934]
- Mathias P, Wickham T, Moore M, Nemerow G. Multiple adenovirus serotypes use alpha v integrins for infection. *J Virol.* 1994; 68:6811–6814. [PubMed: 8084019]
- McGrath WJ, Abola AP, Toledo DL, Brown MT, Mangel WF. Characterization of human adenovirus proteinase activity in disrupted virus particles. *Virology.* 1996; 217:131–138. [PubMed: 8599197]
- McGrath WJ, Ding J, Didwania A, Sweet RM, Mangel WF. Crystallographic structure at 1.6-Å resolution of the human adenovirus proteinase in a covalent complex with its 11-aminoacid peptide cofactor: insights on a new fold. *Biochim Biophys Acta.* 2003; 1648:1–11. [PubMed: 12758141]

- Meier O, Boucke K, Hammer SV, Keller S, Stidwill RP, Hemmi S, Greber UF. Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J Cell Biol.* 2002; 158:1119–1131. [PubMed: 12221069]
- Meunier-Durmort C, Picart R, Ragot T, Perricaudet M, Hainque B, Forest C. Mechanism of adenovirus improvement of cationic liposome-mediated gene transfer. *Biochim Biophys Acta.* 1997; 1330:8–16. [PubMed: 9375808]
- Miles BD, Luftig RB, Weatherbee JA, Weihing RR, Weber J. Quantitation of the interaction between adenovirus types 2 and 5 and microtubules inside infected cells. *Virology.* 1980; 105:265–269. [PubMed: 7414953]
- Miyazawa N, Crystal RG, Leopold PL. Adenovirus serotype 7 retention in a late endosomal compartment prior to cytosol escape is modulated by fiber protein. *J Virol.* 2001; 75:1387–1400. [PubMed: 11152512]
- Morgan C, Rosenkranz HS, Mednis B. Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating of adenovirus. *J Virol.* 1969; 4:777–796. [PubMed: 5360527]
- Nakano MY, Greber UF. Quantitative microscopy of fluorescent adenovirus entry. *J Struct Biol.* 2000; 129:57–68. [PubMed: 10675297]
- Nakano MY, Boucke K, Suomalainen M, Stidwill RP, Greber UF. The first step of adenovirus type 2 disassembly occurs at the cell surface, independently of endocytosis and escape to the cytosol. *J Virol.* 2000; 74:7085–7095. [PubMed: 10888649]
- National Institutes of Health Recombinant DNA Advisory Committee. Assessment of adenoviral vector safety and toxicity: report of the National Institutes of Health Recombinant DNA Advisory Committee. *Hum Gene Ther.* 2002; 13:3–13. [PubMed: 11779411]
- Nicklin SA, Wu E, Nemerow GR, Baker AH. The influence of adenovirus fiber structure and function on vector development for gene therapy. *Mol Ther.* 2005; 12:384–393. [PubMed: 15993650]
- Otero MJ, Carrasco L. Proteins are coinernalized with virion particles during early infection. *Virology.* 1987; 160:75–80. [PubMed: 2820142]
- Pache L, Venkataraman S, Nemerow GR, Reddy VS. Conservation of fiber structure and CD46 usage by subgroup B2 adenoviruses. *Virology.* 2008; 375:573–579. [PubMed: 18336857]
- Perez L, Carrasco L. Involvement of the vacuolar H(+)-ATPase in animal virus entry. *J Gen Virol.* 1994; 75(Pt 10):2595–2606. [PubMed: 7931146]
- Persson BD, Reiter DM, Marttila M, Mei YF, Casasnovas JM, Arnberg N, Stehle T. Adenovirus type 11 binding alters the conformation of its receptor CD46. *Nat Struct Mol Biol.* 2007; 14:164–166. [PubMed: 17220899]
- Philipson L. Attachment and eclipse of adenovirus. *J Virol.* 1967; 1:868–875. [PubMed: 5621483]
- Philipson L, Lonberg-Holm K, Pettersson U. Virus-receptor interaction in an adenovirus system. *J Virol.* 1968; 2:1064–1075. [PubMed: 5723709]
- Philpott NJ, Nociari M, Elkon KB, Falck-Pedersen E. Adenovirus-induced maturation of dendritic cells through a PI3 kinase-mediated TNF- α induction pathway. *Proc Natl Acad Sci USA.* 2004; 101:6200–6205. [PubMed: 15071185]
- Prage L, Pettersson U, Philipson L. Internal basic proteins in adenovirus. *Virology.* 1968; 36:508–511. [PubMed: 4972585]
- Prage L, Pettersson U, Hoglund S, Lonberg-Holm K, Philipson L. Structural proteins of adenoviruses. IV. Sequential degradation of the adenovirus type 2 virion. *Virology.* 1970; 42:341–358. [PubMed: 4099069]
- Rajala MS, Rajala RV, Astley RA, Butt AL, Chodosh J. Corneal cell survival in adenovirus type 19 infection requires phosphoinositide 3-kinase/Akt activation. *J Virol.* 2005; 79:12332–12341. [PubMed: 16160160]
- Raman S, Hsu TH, Ashley SL, Spindler KR. Integrin and heparan sulfate usage as receptors for mouse adenovirus type 1. *J Virol.* 2009; 83:2831–2838. [PubMed: 19176624]
- Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab.* 2003; 80:148–158. [PubMed: 14567964]

- Rexroad J, Evans RK, Middaugh CR. Effect of pH and ionic strength on the physical stability of adenovirus type 5. *J Pharm sci.* 2006; 95:237–247. [PubMed: 16372304]
- Rodriguez E, Everitt E. Adenovirus uncoating and nuclear establishment are not affected by weak base amines. *J Virol.* 1996; 70:3470–3477. [PubMed: 8648679]
- Roelvink PW, Lizonova A, Lee JG, Li Y, Bergelson JM, Finberg RW, Brough DE, Kovesdi I, Wickham TJ. The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J Virol.* 1998; 72:7909–7915. [PubMed: 9733828]
- Roelvink PW, Mi Lee G, Einfeld DA, Kovesdi I, Wickham TJ. Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science.* 1999; 286:1568–1571. [PubMed: 10567265]
- Roy S, Clawson DS, Calcedo R, Lebherz C, Sanmiguel J, Wu D, Wilson JM. Use of chimeric adenoviral vectors to assess capsid neutralization determinants. *Virology.* 2005; 333:207–214. [PubMed: 15721355]
- Russell WC, Valentine RC, Pereira HG. The effect of heat on the anatomy of the adenovirus. *J Gen Virol.* 1967; 1:509–522. [PubMed: 6081701]
- Rux JJ, Kuser PR, Burnett RM. Structural and phylogenetic analysis of adenovirus hexons by use of high-resolution x-ray crystallographic, molecular modeling, and sequence-based methods. *J Virol.* 2003; 77:9553–9566. [PubMed: 12915569]
- Saban SD, Nepomuceno RR, Gritton LD, Nemerow GR, Stewart PL. CryoEM structure at 9 Å resolution of an adenovirus vector targeted to hematopoietic cells. *J Mol Biol.* 2005; 349:526–537. [PubMed: 15890367]
- Saban SD, Silvestry M, Nemerow GR, Stewart PL. Visualization of alpha-helices in a 6-angstrom resolution cryoelectron microscopy structure of adenovirus allows refinement of capsid protein assignments. *J Virol.* 2006; 80:12049–12059. [PubMed: 17005667]
- San Martin C, Glasgow JN, Borovjagin A, Beatty MS, Kashentseva EA, Curiel DT, Marabini R, Dmitriev IP. Localization of the N-terminus of minor coat protein IIIa in the adenovirus capsid. *J Mol Biol.* 2008; 383:923–934. [PubMed: 18786542]
- Segerman A, Atkinson JP, Marttila M, Dennerquist V, Wadell G, Arnberg N. Adenovirus type 11 uses CD46 as a cellular receptor. *J Virol.* 2003; 77:9183–9191. [PubMed: 12915534]
- Seki T, Dmitriev I, Kashentseva E, Takayama K, Rots M, Suzuki K, Curiel DT. Artificial extension of the adenovirus fiber shaft inhibits infectivity in coxsackievirus and adenovirus receptor-positive cell lines. *J Virol.* 2002; 76:1100–1108. [PubMed: 11773386]
- Seth P. Adenovirus-dependent release of choline from plasma membrane vesicles at an acidic pH is mediated by the penton base protein. *J Virol.* 1994; 68:1204–1206. [PubMed: 8289352]
- Seth P, Fitzgerald D, Ginsberg H, Willingham M, Pastan I. Evidence that the penton base of adenovirus is involved in potentiation of toxicity of *Pseudomonas* exotoxin conjugated to epidermal growth factor. *Mol Cell Biol.* 1984a; 4:1528–1533. [PubMed: 6333584]
- Seth P, Fitzgerald DJ, Willingham MC, Pastan I. Role of a low-pH environment in adenovirus enhancement of the toxicity of a *Pseudomonas* exotoxin-epidermal growth factor conjugate. *J Virol.* 1984b; 51:650–655. [PubMed: 6332200]
- Seth P, Willingham MC, Pastan I. Adenovirus-dependent release of ⁵¹Cr from KB cells at an acidic pH. *J Biol Chem.* 1984c; 259:14350–14353. [PubMed: 6334083]
- Seth P, Pastan I, Willingham MC. Adenovirus-dependent increase in cell membrane permeability. *J Biol Chem.* 1985a; 260:9598–9602. [PubMed: 4019489]
- Seth P, Willingham MC, Pastan I. Binding of adenovirus and its external proteins to Triton X-114. Dependence on pH. *J Biol Chem.* 1985b; 260:14431–14434. [PubMed: 4055783]
- Shayakhmetov DM, Lieber A. Dependence of adenovirus infectivity on length of the fiber shaft domain. *J Virol.* 2000; 74:10274–10286. [PubMed: 11044071]
- Shayakhmetov DM, Li ZY, Ternovoi V, Gaggar A, Gharwan H, Lieber A. The interaction between the fiber knob domain and the cellular attachment receptor determines the intracellular trafficking route of adenoviruses. *J Virol.* 2003; 77:3712–3723. [PubMed: 12610146]
- Shayakhmetov DM, Gaggar A, Ni S, Li ZY, Lieber A. Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J Virol.* 2005; 79:7478–7491. [PubMed: 15919903]

- Sirena D, Lilienfeld B, Eisenhut M, Kalin S, Boucke K, Beerli RR, Vogt L, Ruedl C, Bachmann MF, Greber UF, Hemmi S. The human membrane cofactor CD46 is a receptor for species B adenovirus serotype 3. *J Virol.* 2004; 78:4454–4462. [PubMed: 15078926]
- Smith JG, Nemerow GR. Mechanism of adenovirus neutralization by human alpha-defensins. *Cell Host Microbe.* 2008; 3:11–19. [PubMed: 18191790]
- Smith T, Idamakanti N, Kylefjord H, Rollence M, King L, Kaloss M, Kaleko M, Stevenson SC. In vivo hepatic adenoviral gene delivery occurs independently of the coxsackievirus-adenovirus receptor. *Mol Ther.* 2002; 5:770–779. [PubMed: 12027562]
- Smith TA, Idamakanti N, Marshall-Neff J, Rollence ML, Wright P, Kaloss M, King L, Mech C, Dinges L, Iverson WO, Sherer AD, Markovits JE, Lyons RM, Kaleko M, Stevenson SC. Receptor interactions involved in adenoviral-mediated gene delivery after systemic administration in non-human primates. *Hum Gene Ther.* 2003; 14:1595–1604. [PubMed: 14633402]
- Smith JG, Cassany A, Gerace L, Ralston R, Nemerow GR. Neutralizing antibody blocks adenovirus infection by arresting microtubule-dependent cytoplasmic transport. *J Virol.* 2008; 82:6492–6500. [PubMed: 18448546]
- Stewart PL, Nemerow GR. Cell integrins: commonly used receptors for diverse viral pathogens. *Trends Microbiol.* 2007; 15:500–507. [PubMed: 17988871]
- Stewart PL, Fuller SD, Burnett RM. Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. *Embo J.* 1993; 12:2589–2599. [PubMed: 8334984]
- Stewart PL, Chiu CY, Huang S, Muir T, Zhao Y, Chait B, Mathias P, Nemerow GR. Cryo-EM visualization of an exposed RGD epitope on adenovirus that escapes antibody neutralization. *Embo J.* 1997; 16:1189–1198. [PubMed: 9135136]
- Stonebraker JR, Wagner D, Lefensty RW, Burns K, Gendler SJ, Bergelson JM, Boucher RC, O'Neal WK, Pickles RJ. Glycocalyx restricts adenoviral vector access to apical receptors expressed on respiratory epithelium *in vitro* and *in vivo*: role for tethered mucins as barriers to luminal infection. *J Virol.* 2004; 78:13755–13768. [PubMed: 15564484]
- Strunze S, Trotman LC, Boucke K, Greber UF. Nuclear targeting of adenovirus type 2 requires CRM1-mediated nuclear export. *Mol Biol Cell.* 2005; 16:2999–3009. [PubMed: 15814838]
- Sumida SM, Truitt DM, Lemckert AA, Vogels R, Custers JH, Addo MM, Lockman S, Peter T, Peyrel FW, Kishko MG, Jackson SS, Gorgone DA, Lifton MA, Essex M, Walker BD, Goudsmit J, Havenga MJ, Barouch DH. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol.* 2005; 174:7179–7185. [PubMed: 15905562]
- Suomalainen M, Nakano MY, Keller S, Boucke K, Stidwill RP, Greber UF. Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus. *J Cell Biol.* 1999; 144:657–672. [PubMed: 10037788]
- Sussenbach JS. Early events in the infection process of adenovirus type 5 in HeLa cells. *Virology.* 1967; 33:567–574. [PubMed: 18614078]
- Svensson U. Role of vesicles during adenovirus 2 internalization into HeLa cells. *J Virol.* 1985; 55:442–449. [PubMed: 2862291]
- Svensson U, Persson R. Entry of adenovirus 2 into HeLa cells. *J Virol.* 1984; 51:687–694. [PubMed: 6471167]
- Tomko RP, Xu R, Philipson L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci USA.* 1997; 94:3352–3356. [PubMed: 9096397]
- Toogood CI, Crompton J, Hay RT. Antipeptide antisera define neutralizing epitopes on the adenovirus hexon. *J Gen Virol.* 1992; 73(Pt 6):1429–1435. [PubMed: 1376769]
- van Oostrum J, Burnett RM. Molecular composition of the adenovirus type 2 virion. *J Virol.* 1985; 56:439–448. [PubMed: 4057357]
- van Raaij MJ, Mitraki A, Lavigne G, Cusack S. A triple beta-spiral in the adenovirus fibre shaft reveals a new structural motif for a fibrous protein. *Nature.* 1999; 401:935–938. [PubMed: 10553913]

- Varga MJ, Weibull C, Everitt E. Infectious entry pathway of adenovirus type 2. *J Virol*. 1991; 65:6061–6070. [PubMed: 1920625]
- Varghese R, Mikyas Y, Stewart PL, Ralston R. Postentry neutralization of adenovirus type 5 by an anti-hexon antibody. *J Virol*. 2004; 78:12320–12332. [PubMed: 15507619]
- Von Seggern DJ, Chiu CY, Fleck SK, Stewart PL, Nemerow GR. A helper-independent adenovirus vector with E1, E3, and fiber deleted: structure and infectivity of fiberless particles. *J Virol*. 1999; 73:1601–1608. [PubMed: 9882366]
- Waddington SN, McVey JH, Bhella D, Parker AL, Barker K, Atoda H, Pink R, Buckley SM, Greig JA, Denby L, Custers J, Morita T, Francischetti IM, Monteiro RQ, Barouch DH, van Rooijen N, Napoli C, Havenga MJ, Nicklin SA, Baker AH. Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell*. 2008; 132:397–409. [PubMed: 18267072]
- Walters RW, Grunst T, Bergelson JM, Finberg RW, Welsh MJ, Zabner J. Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. *J Biol Chem*. 1999; 274:10219–10226. [PubMed: 10187807]
- Walters RW, Freimuth P, Moninger TO, Ganske I, Zabner J, Welsh MJ. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. *Cell*. 2002; 110:789–799. [PubMed: 12297051]
- Wang K, Huang S, Kapoor-Munshi A, Nemerow G. Adenovirus internalization and infection require dynamin. *J Virol*. 1998; 72:3455–3458. [PubMed: 9525681]
- Wang K, Guan T, Cheresh DA, Nemerow GR. Regulation of adenovirus membrane penetration by the cytoplasmic tail of integrin beta5. *J Virol*. 2000; 74:2731–2739. [PubMed: 10684289]
- Wang H, Liaw YC, Stone D, Kalyuzhnyi O, Amiraslanov I, Tuve S, Verlinde CL, Shayakhmetov D, Stehle T, Roffler S, Lieber A. Identification of CD46 binding sites within the adenovirus serotype 35 fiber knob. *J Virol*. 2007; 81:12785–12792. [PubMed: 17898059]
- Warren JC, Cassimeris L. The contributions of microtubule stability and dynamic instability to adenovirus nuclear localization efficiency. *Cell Motil Cytoskeleton*. 2007; 64:675–689. [PubMed: 17565754]
- Warren JC, Rutkowski A, Cassimeris L. Infection with replication-deficient adenovirus induces changes in the dynamic instability of host cell microtubules. *Mol Biol Cell*. 2006; 17:3557–3568. [PubMed: 16775012]
- Weber JM. Adenovirus endopeptidase and its role in virus infection. *Curr Top Microbiol Immunol*. 1995; 199(Pt 1):227–235. [PubMed: 7555056]
- Webster A, Russell WC, Kemp GD. Characterization of the adenovirus proteinase: development and use of a specific peptide assay. *J Gen Virol*. 1989; 70(Pt 12):3215–3223. [PubMed: 2691632]
- Webster A, Hay RT, Kemp G. The adenovirus protease is activated by a virus-coded disulphide-linked peptide. *Cell*. 1993; 72:97–104. [PubMed: 8422686]
- Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*. 1993; 73:309–319. [PubMed: 8477447]
- Wickham TJ, Filardo EJ, Cheresh DA, Nemerow GR. Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. *J Cell Biol*. 1994; 127:257–264. [PubMed: 7523420]
- Wiethoff CM, Wodrich H, Gerace L, Nemerow GR. Adenovirus protein VI mediates membrane disruption following capsid disassembly. *J Virol*. 2005; 79:1992–2000. [PubMed: 15681401]
- Wodrich H, Guan T, Cingolani G, Von Seggern D, Nemerow G, Gerace L. Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. *Embo J*. 2003; 22:6245–6255. [PubMed: 14633984]
- Wohlfart C. Neutralization of adenoviruses: kinetics, stoichiometry, and mechanisms. *J Virol*. 1988; 62:2321–2328. [PubMed: 3373570]
- Wohlfart CE, Svensson UK, Everitt E. Interaction between HeLa cells and adenovirus type 2 virions neutralized by different antisera. *J Virol*. 1985; 56:896–903. [PubMed: 4068145]
- Wu E, Nemerow GR. Virus yoga: the role of flexibility in virus host cell recognition. *Trends Microbiol*. 2004; 12:162–169. [PubMed: 15051066]

- Wu E, Pache L, Von Seggern DJ, Mullen TM, Mikyas Y, Stewart PL, Nemerow GR. Flexibility of the adenovirus fiber is required for efficient receptor interaction. *J Virol.* 2003; 77:7225–7235. [PubMed: 12805421]
- Wu E, Trauger SA, Pache L, Mullen TM, von Seggern DJ, Siuzdak G, Nemerow GR. Membrane cofactor protein is a receptor for adenoviruses associated with epidemic keratoconjunctivitis. *J Virol.* 2004; 78:3897–3905. [PubMed: 15047806]
- Xie J, Chiang L, Contreras J, Wu K, Garner JA, Medina-Kauwe L, Hamm-Alvarez SF. Novel fiber-dependent entry mechanism for adenovirus serotype 5 in lacrimal acini. *J Virol.* 2006; 80:11833–11851. [PubMed: 16987972]
- Yoshimura A. Adenovirus-induced leakage of co-endocytosed macromolecules into the cytosol. *Cell Struct Funct.* 1985; 10:391–404. [PubMed: 2417732]
- Zinn KR, Szalai AJ, Stargel A, Krasnykh V, Chaudhuri TR. Bioluminescence imaging reveals a significant role for complement in liver transduction following intravenous delivery of adenovirus. *Gene Ther.* 2004; 11:1482–1486. [PubMed: 15295616]
- Zubieta C, Schoehn G, Chroboczek J, Cusack S. The structure of the human adenovirus 2 penton. *Mol Cell.* 2005; 17:121–135. [PubMed: 15629723]
- Zubieta C, Blanchoin L, Cusack S. Structural and biochemical characterization of a human adenovirus 2/12 penton base chimera. *FEBS J.* 2006; 273:4336–4345. [PubMed: 16939624]

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://viperd.b.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

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

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 and 15% random coil by analysis of the spectrum with the CONTIN software package