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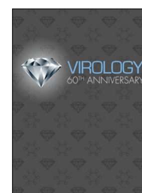

Review

Adenovirus membrane penetration: Tickling the tail of a sleeping dragon

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ABSTRACT

As is the case for nearly every viral pathogen, non-enveloped viruses (NEV) must maintain their integrity under potentially harsh environmental conditions while retaining the ability to undergo rapid disassembly at the right time and right place inside host cells. NEVs generally exist in this metastable state until they encounter key cellular stimuli such as membrane receptors, decreased intracellular pH, digestion by cellular proteases, or a combination of these factors. These stimuli trigger conformational changes in the viral capsid that exposes a sequestered membrane-perturbing protein. This protein subsequently modifies the cell membrane in such a way as to allow passage of the virion and accompanying nucleic acid payload into the cell cytoplasm. Different NEVs employ variations of this general pathway for cell entry (Moyer and Nemerow, 2011, *Curr. Opin. Virol.*, 1, 44–49), however this review will focus on significant new knowledge obtained on cell entry by human adenovirus (HAdV).
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Introduction

There are more than 60 different types of human adenovirus belonging to 6 distinct subgroups (A–G) (King et al., 2012; Liu et al., 2012) and many of these are associated with acute respiratory, gastrointestinal and ocular infections. Although usually self-limiting, these infections can lead to fatal disseminated infections in immunocompromised individuals (Lion, 2014). Conditionally-replicating (i.e., oncolytic) or replication-defective adenoviruses are now well known for their use in gene transfer or vaccine delivery. However, optimal targeting to specific cell types remains an unresolved goal. Thus, an understanding of complex interactions of HAdV with the host is crucial. Over the past several years, detailed knowledge of cell entry by this relatively large (150 MDa) virus has been obtained and some of its most closely held secrets have been revealed. These include the mechanism of

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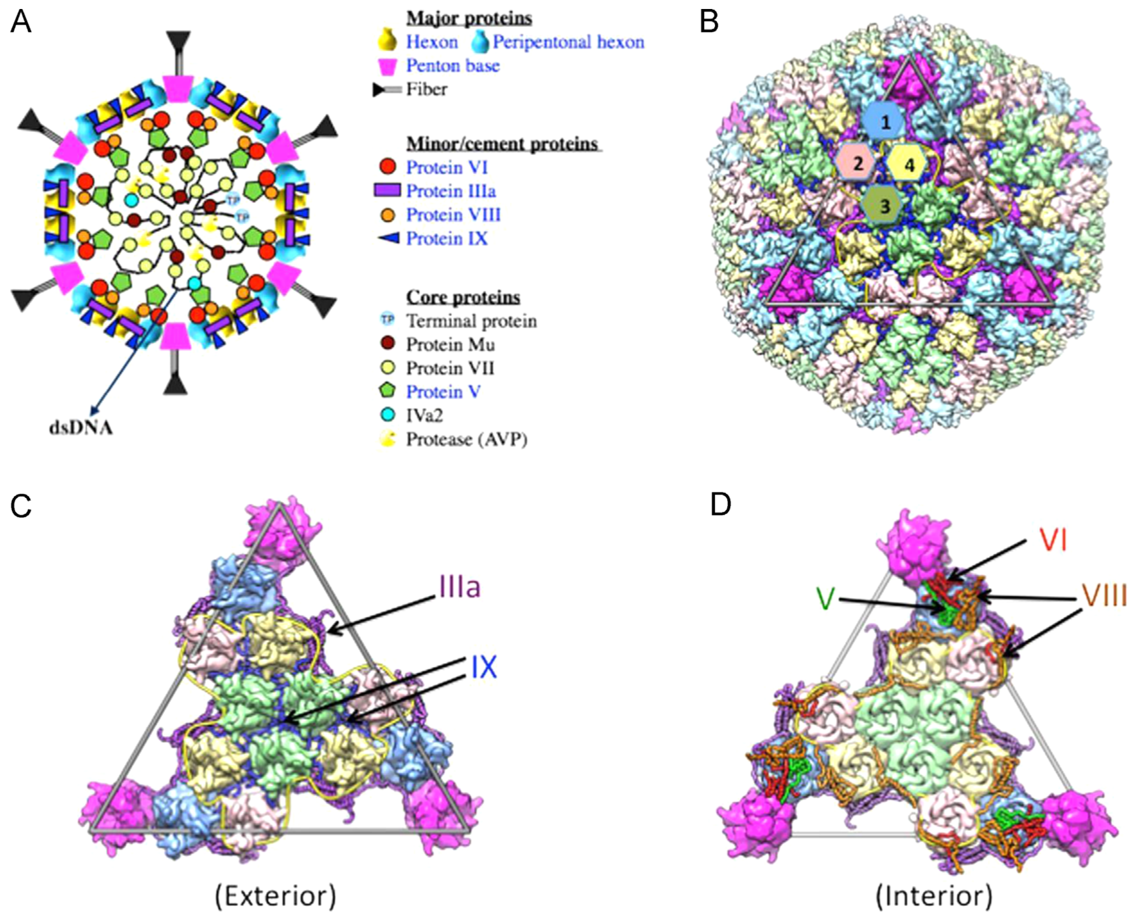

Fig. 1. Structure and organization of human adenovirus. (A) A schematic illustration of the organization of capsid and core proteins in human adenovirus. The locations of various proteins are represented by different-colored symbols and the corresponding names are shown (*Right*). The indicated locations of the core proteins are approximate. Shown in blue-colored lettering are the proteins whose structures have been identified in this study. (B) Overall organization of hexon and penton base subunits exhibiting pseudo-T=25 icosahedral symmetry. Structurally unique hexons (1–4) are color-coded in light blue, pink, green, and khaki, respectively. Penton vertices are shown in magenta. Outer cement proteins IIIa and IX are shown in purple and blue, respectively. Fiber molecules associated with the penton base are disordered. The outline of the triangular icosahedral facet is shown as a gray triangle, whereas the border of the GON hexons is indicated by yellow-colored rope. (C) An exterior view of the triangular icosahedral facet that comprises 12 hexons along with penton base vertices shown in magenta. Color representations are the same as in *B*. (*D*) An interior view of the facet in C, with three minor proteins, V (green), VI (red), and VIII (orange). It is noteworthy that a copy of V, VI, and VIII forms a ternary complex beneath the vertices, whereas VIII (orange) molecules are arranged as staples along the border (yellow-colored rope) of the GON hexons. Reddy V S, and Nemerow G R. PNAS 2014;111:11715–11720.

virus internalization, the location and structure of the virus membrane lytic protein inside the virus capsid, as well as the host immune and cellular responses triggered by HAdV cell entry.

Receptor-mediated virus attachment and internalization

Due to its size and complexity, HAdV presents significant challenges for studying its mode of cell entry. This naked virus is ~90 nm in diameter and contains a 36-KB dsDNA genome encoding 13 distinct structural proteins including a cysteine protease whose activity is required for capsid maturation (Fig. 1). Each of the 12 vertices of the virus possess two major outer capsid proteins known as the fiber (van Raaij et al., 1999) and penton base (Zubieta et al., 2005; Wickham et al., 1993) that serve as attachment and internalization receptor binding proteins, respectively. Attachment of most HAdV types is mediated by a cell receptor known as the Coxsackie and Adenovirus Receptor (CAR), a member of the Ig superfamily (Bergelson et al., 1997). CAR plays a role in maintaining the integrity of tight junctions in polarized epithelial cells and is normally sequestered on the basolateral surface of these cells (Walters et al., 2002). This situation has hindered the use of Ad gene delivery for treatment of human airway diseases (Zabner et al., 1997; Walters et al., 1999). However, a single isoform of HCAR, designated CAR^{Ex8}, appears to traffic to the apical surface of airway epithelial cells and allows a modest level of HAdV infection from this location (Excoffon et al., 2010). Interestingly, on certain cell types such as motor neurons, CAR can serve as both an attachment receptor as well as an internalization to promote virus uptake (Salinas et al., 2009; Salinas et al., 2014).

As noted above, not all species of HAdV use CAR as their primary receptor. For example, the fiber proteins of certain subgroup B adenoviruses including types 3, 7, 11 and 14 use desmoglein-2 (DSG-2) as a high affinity attachment receptor (Wang et al., 2011). DSG-2 ligation can regulate access to epithelial cell junctions and thus the use of type B Ad vectors that recognize this receptor may prove useful for targeting oncolytic HAdV to tumor cells. The fiber proteins of other subgroup B viruses including Ad types 35 and 16, have been shown to use CD46, a complement regulatory protein family member, for cell attachment (Gaggar et al., 2003). Virus types such as Ad19 and Ad37 associated with epidemic keratoconjunctivitis, a serious ocular disease, bind to α2,3-linked sialic acid (Arnberg et al., 2000; Nilsson et al., 2011) or CD46 (Wu et al., 2004). These cell receptors are widely distributed on various cell types in vivo, and thus their presence on certain tissues does not readily explain adenovirus tropism. Nonetheless, significant progress has been made in elucidating the structural basis of Ad fiber interactions with CAR

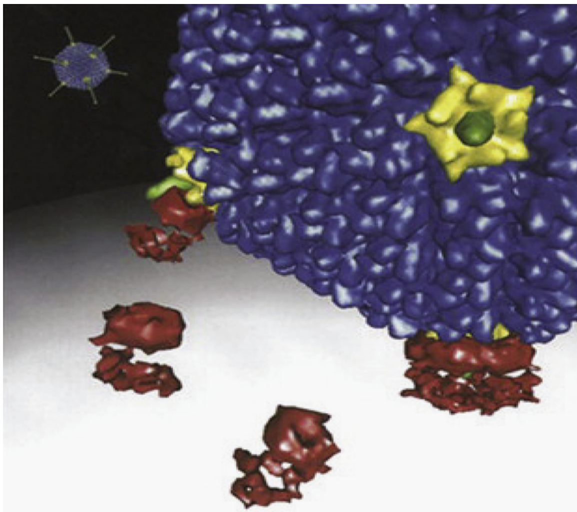

Fig. 2. CryoEM structure of Ad12 complexed with a soluble recombinant form of integrin αvβ5. The structure is shown artistically as if one virion were interacting with a host cell in the foreground. A second Ad virion with modeled full-length fibers is shown in the distance. The cryoEM structure revealed a compact ring of integrin density over each penton base (yellow) with the RGD-loop (surface protrusions) spacing of the penton base promoting interaction between integrin heterodimers (red) (bottom). Segmented density regions corresponding roughly to individual integrin molecules are shown in solution on the lower left. Copyright American Society for Microbiology, Journal of Virology, Volume 73, Number 8, p. 6759–6768, cover image, 1999. G.R. Nemerow, L. Pache, V. Reddy, P.L. Stewart. Virology, Volume 384, Issue 2, 2009, 380–388.

(Seiradake et al., 2006; Bewley et al., 1999), CD46 (Persson et al., 2007; Pache et al., 2008; Cupelli et al., 2010), and sialic acid (Burmeister et al., 2004). In addition to specific amino acid sequences present in the fiber knob domain that mediate receptor interaction, the length and flexibility of the fiber shaft domain also play roles in receptor usage (Wu et al., 2004). These structural, biochemical, and functional studies continue to foster the development of chimeric/recombinant HAdV vectors with unique cell receptor specifities (Schagen et al., 2006).

The vitronectin-binding integrins αvβ3 and αvβ5 promote HAdV2 internalization but not cell attachment (Wickham et al., 1993). αv integrins bind to an extended and flexible loop on the virus penton base that contains an arginine, glycine, aspartic acid (RGD) sequence (Stewart et al., 1997) (Fig. 2) and facilitate virus uptake into clathrin-coated early endosomes (Wang et al., 1998). Virus uptake appears to be stimulated by cell signaling events that involve activation of PI3K and rearrangement of the actin cystoskeleton (Li et al., 1998). It is also possible that integrin-mediated signaling events enhance adenovirus cell entry via macropinocytosis (Amstutz et al., 2008). More recent fluorescence microscopy studies showed that the different physical motions of CAR and integrins in the cell plasma membrane following virus interactions, may initiate disassembly of the virus particle even before it reaches the early endosome (Burckhardt et al., 2011). In keeping with this possibility, cryoelectron microscopy structural studies of a complex of integrin αvβ5 bound to HAdV revealed a subtle conformational change in the penton base (i.e. clockwise twist) that could represent the initial changes in the viral vertex region associated with the capsid disassembly process (Lindert et al., 2009).

Despite the accumulating knowledge of HAdV cell receptors in cell culture systems, the host factors that regulate HAdV cell tropism and immune responses in vivo are considerably more complex. This was brought into focus by the tragic death of a patient in a gene therapy clinical trial for liver disease using systemic delivery of an adenoviral vector (Raper et al., 2003). It was subsequently discovered that HAdV5-mediated gene delivery to the liver occurs without the use of CAR or integrins (Smith, 2003). Instead, coagulation factor X (FX) was shown to mediate Ad5 vector binding to hepatocytes (Waddington et al., 2008; Kalyuzhniy et al., 2008). In this situation, the γ-carboxy glutamic acid domain of FX binds to the hexon protein of Ad5 and this allows association of the C-terminal serine protease domain of FX with cell surface heparin-sulfated molecules (Waddington et al., 2008). More recent studies have also suggested that the binding of FX to the HAdV5 capsid may restrict coating of the capsid by IgM and complement components, thereby enhancing hepatocyte interactions (Xu et al., 2013). Taken together, these in vivo studies illustrate the diverse modes of HAdV–tissue interactions and serve as a reminder of the safety concerns of adenoviral vectors.

Membrane disruption (historical perspective)

After attachment to cells, early electron microscopy studies showed that adenovirus is endocytosed and that a largely intact capsid gains access to the cytoplasm (Morgan et al., 1969). Adenovirus was initially presumed to cause global disruption of cell membranes in a manner distinct from other nonenveloped viruses (e.g. picornaviruses). This observation was based on the capacity of virions to permeabilize cell membranes to small ions like chromium and choline (Seth et al., 1984; Seth et al., 1985), as well as to larger molecules such as alpha-sarcin (16 kDa) (Wiethoff et al., 2005) and fluorescent dextrans (70 kDa FITC-dextran) (Prchla et al., 1995) and even a 25 nm diameter mutant parvovirus defective in endosomal escape (Farr et al., 2005). From these studies it was concluded that adenovirus induced large-scale fragmentation of cell membranes rather than formation of discrete pores. Early studies did not reveal the specific site of membrane disruption during cell entry. However, negative stain-electron microscopy and immunofluorescence microscopic analyses as well as the use of chemical inhibitors of endosomal acidification supported the idea that endosomal membranes were the sites of membrane damage (Morgan et al., 1969; FitzGerald et al., 1983; Greber et al., 1993). In one study, flow cytometric analyses showed a decrease in the number of endosomes obtained from cells after adenovirus cell entry, leading the authors to suggest that adenovirus ruptured endosomes in order to gain access to the cytoplasm (Brabec et al., 2005).

Initial studies of adenovirus permeablization of cell membranes showed that virus-mediated release of small ions was augmented at lower pH. This was in agreement with previous observations that adenovirus escape from endosomes into the cytoplasm could be inhibited by pharmacological inhibition of endosomal acidification (Seth et al., 1984). Thus, early on it was proposed that adenovirus membrane penetration was a pH dependent process. Later studies however suggested that pharmacological inhibition of endosomal acidification did not affect adenovirus cell entry or that low pH may impact an event upstream of endosomal escape such as endocytosis or endosomal trafficking (Rodríguez and Everitt, 1996; Pérez and Carrasco, 1994). Such events could include the partial disassembly of the adenovirus capsid, a step necessary for endosomal escape. However, more recent studies have concluded that, at least in the case of subgroup C adenovirus, capsid disassembly may be initially induced at the cell surface in a manner dependent on the dual engagement of mobile Coxsackievirus and Adenovirus Receptor and the less mobile α_V integrins (Burckhardt et al., 2011).

Studies employing a temperature sensitive mutant of human adenovirus 2, *H2ts1 (ts1)*, suggested that structural lability (commonly referred to as metastable) of the capsid is required for cell membrane penetration. The *ts1* capsids produced at the nonpermissive temperature are known to be hyperstable and incapable of releasing vertex proteins upon heating or when incubated at low pH, in contrast to what is observed for capsids formed at the

permissive temperature (Hannan et al., 1983). Structural studies have demonstrated that this enhanced stability of the *ts1* capsid is related to a more intimate association between the capsid proteins near the vertex and the DNA core (Silvestry et al., 2009). Electron microscopic studies demonstrated that, when grown at the non-permissive temperature, the *ts1* virion failed to escape cell endosomes (Miles et al., 1980; Greber et al., 1996). Further studies provided biochemical evidence that *ts1* could not permeabilize cell membranes to small ions or larger biomolecules (Wiethoff et al., 2005; Cotten and Weber, 1995). The defect in cell membrane permeablization by the *ts1* mutant was confirmed by studies demonstrating the failure of this mutant virus to permeabilize model liposomal membranes to small molecular weight fluorophores in the absence of cellular receptors or other factors (Wiethoff et al., 2005).

Discovery of protein VI membrane lytic activity

While considerable effort has gone into the study of how adenovirus disrupts cellular membranes to gain access to the cytoplasm and nucleus, prior to 2005 there has been little conclusive evidence that one or more of the 13 structural proteins of the virion mediate membrane rupture. Some of the first studies aimed at identifying the membrane lytic protein of the adenovirus capsid used antibodies against the penton base and hexon to assess their role in cellular membrane permeablization. When the virus was added to cells loaded with radiolabeled choline in the presence of anti-penton base antibodies, release of radioactivity into the cell supernatant was greatly reduced suggesting that the penton base was involved in membrane rupture (Seth, 1994). Other studies demonstrated that when viruses were mixed with liposomes, these lipid membranes were bound at the vertex of the virions, suggesting that the penton could be involved in membrane lysis.

Attempts to demonstrate that recombinant penton base was capable of permeabilizing artificial liposomal membranes were unsuccessful, as we were unable to observe any membrane lytic activity compared to purified virions that efficiently disrupted membranes (Wiethoff and Nemerow, unpublished observations and (Wiethoff et al., 2005)). However, when mixed with liposomes, we observed membrane permeabilization only when the virus was first incubated at low pH, suggesting that a pH dependent conformational change in the capsid was required for membrane rupture. Heating the virus above 42° to release the vertex proteins, also caused the virus to partially uncoat. Thus, virus-induced liposome disruption occurred independent of low pH when the capsid was first exposed to elevated temperatures. Therefore, the presumed pH dependence of adenovirus rupture of cell membranes seemed to correlate with capsid uncoating rather than with the actual process of membrane lytic activity.

Further, when the capsid was disrupted by incubation at elevated temperature, the released proteins could be separated from the partially disassembled virion by density gradient centrifugation (Wiethoff et al., 2005). Membrane lytic activity was present in the fraction of the soluble proteins rather than in the partially disassembled capsid. Biochemically we observed that these proteins were composed mostly of hexon, penton base, fiber, IIIa and protein VI (Wiethoff et al., 2005). Further studies showed that protein IX and protein VIII were incompletely released from the capsid as well (C.M. Wiethoff, unpublished observation). in vitro membrane lytic activity was not observed with the purified hexon, recombinant penton base, or the fiber knob. Therefore, we examined the primary sequences of other capsid proteins such as protein IIIa and VI. A highly-conserved amphipathic helix domain was found to be present in protein VI at residues 34–54, immediately following the cleavage site processed by the virally encoded 23 K cysteine protease.

To determine whether protein VI actually possessed the membrane lytic activity, we immunodepleted protein VI or penton base from the mixture of heat-released proteins and found that only protein VI immunodepletion reduced the membrane lytic activity of from this mixture of released vertex proteins. Recombinant protein VI possessed the same pH-independent membrane lytic activity as heat disrupted adenovirus virions and the N-terminal amphiphathic helix in protein VI was required for this membrane lytic activity. Subsequent studies demonstrated that antibodies to protein VI abrogated adenovirus-mediated membrane permeabilization as well as the delivery of the adenovirus genome to the nucleus (Maier et al., 2010). Genetic manipulation of the amphipathic alpha helix in the context of adenovirus provided further evidence that protein VI was involved in adenovirus membrane penetration. A mutant virus with single point mutation in protein VI, L40Q, is defective in endosomal escape and the corresponding recombinant L40Q mutant protein has reduced membrane binding and liposome-disrupting activity in vitro (Moyer et al., 2011).

Biochemistry of membrane disruption by VI

While initial studies determined that an N-terminal helix from residues 34–53 was required for protein VI membrane lytic activity, the mechanism by which protein VI ruptured membranes remained incompletely characterized. All evidence in vitro and in tissue culture suggested that membrane damage by adenovirus is catastrophic, allowing for the passage of a ~90 nm virion through the membrane. Given the biochemical copy number of protein VI, it is unlikely that a stable pore of that size would be possible. A better understanding of protein VI membrane lytic activity required further study.

Investigations using recombinant proteins demonstrated that this helix was a critical determinant for binding to model liposomal membranes (Maier et al., 2010). When this N-terminal amphipathic α-helix is replaced by a 6xHis tag the membrane binding capacity is reduced 40-fold and membrane lytic activity is reduced by ~100-fold (Maier et al., 2010). However, if membrane affinity of this 6xHis-tag-replaced protein VI is artificially enhanced by using liposomes containing NTA-Ni2+ headgroups for binding to the 6xHis tag, then the membrane lytic activity of the protein can be restored to normal levels (Maier and Wiethoff, 2010). Further genetic evidence that this N-terminal amphipathic α-helix is required for membrane binding and rupture was obtained when L40 was mutated to glutamine, L40Q (Moyer et al., 2011). This mutation led to a 10-fold reduction in recombinant protein VI membrane affinity as well as a 10-fold reduction in in vitro membrane lytic activity. A recombinant E1, E3-deleted human adenovirus 5 encoding the L40Q mutation in protein VI was also found to be roughly 10-fold less infectious than virus encoding the wild type protein. The decreased infectivity of the L40Q mutant virus may also be related to an additional lability of the capsid that causes it to release the receptor binding penton prior to virus attachment to cells (Martinez et al., 2014).

The mechanism of protein VI membrane rupture remained uncertain even after the protein was established as the membrane lytic factor in the adenovirus capsid. To understand how protein VI ruptured membranes, fluorescence microscopy was used to visualize topological changes in giant lipid vesicles upon protein VI binding. Within seconds after addition of protein VI, these lipid vesicles are completely fragmented. In many cases, these fragments are rearranged into elongated tubules (Maier and Wiethoff, 2010), although the limits of light microscopy spatial resolution has precluded further insights into the structure of these tubules. Nonetheless, these observations fit with a model in which protein VI binds membranes and, much like a

detergent, fragments the lipid bilayer into smaller, highly curved structures. This is akin to how certain antimicrobial peptides might rupture membranes (Domanov and Kinnunen, 2006) and involves the induction of either severe positive or negative membrane curvature. In the case of protein VI, incorporating small amounts of lysophosphatidylcholine, a molecule with a propensity to adopt positively curved lipidic structures, enhanced protein VI membrane lysis. In contrast, incorporating phosphatidylethanolamine, a molecule with a propensity to adopt negatively curved lipidic structures, attenuated protein VI membrane lysis (Maier et al., 2010). Thus, protein VI appears to fragment membranes by binding to them and subjecting them to severe positive curvature-inducing forces.

To understand how protein VI might be inducing positive membrane curvature in lipid bilayers, studies were performed to assess the topology of protein VI on lipid bilayers. A highly conserved 80 residue domain at the N-terminus of protein VI is ~85% α-helical as measured by CD spectrapolarimetry. Secondary structure predictions suggest that this domain contains 4 α-helices (Wiethoff et al., 2005). We used bromine atoms appended to phospholipids to cause tryptophan fluorescence quenching of three tryptophan residues in the 80-residue VI amphipathic helix. The extent of quenching depended on the depths or the tryptophan residues within the lipid bilayer. The data obtained in these analyses suggested that protein VI penetrates lipid bilayers superficially, near the lipid headgroups (Maier et al., 2010). This type of membrane topology is often observed for helical peptides that induce positive membrane curvature (Epand and Epand, 2000). By pushing apart the lipid headgroups to accommodate the protein, one leaflet encounters substantial positive membrane curvature stress.

A structure for a portion of this 80-residue α-helical domain of protein VI was recently revealed in X-ray crystallographic studies (Reddy and Nemerow, 2014). Interestingly, this domain in the virus does not appear to possess the same degree of α-helicity as that contained in the recombinant purified protein. It seems likely that protein VI, following its release from the virus, must undergo a significant structural rearrangement upon membrane binding. Further studies of the structure of protein VI on membranes and in the capsid are needed to shed light on this possibility.

Structure and localization of protein VI in the HAdV capsid

The continued advancements in cryoEM, X-ray diffraction, atomic force microscopy, and mass spectrometry techniques over the past two decades have significantly increased our knowledge of adenovirus capsid structure as well as provided clues about the processes of assembly and disassembly. Historically, the first animal virus protein to be crystallized (Franklin et al., 1971) and then structurally characterized (Roberts et al., 1986) was the hexon, the major outer capsid protein of adenovirus. These early studies paved the way for subsequent cryoEM studies of adenovirus at 6 Å (Saban et al., 2006) and 10 Å resolution (Fabry et al., 2005). These cryoEM structural studies of HAdV provided further structural knowledge of this 90 nm (dia.) icosahedral virus including the location of specific cement (minor) proteins IIIa and IX. Unfortunately, the cryoEM density maps were not of sufficient resolution to definitively assign the location and interactions of all of the capsid proteins including protein VI. This situation improved after 3.5 Å resolution structures of HAdV were obtained by X-ray diffraction (Reddy et al., 2010) and enhanced cryoEM techniques (Liu et al., 2010). Complete agreement on the location and structures of some of the cement proteins was not forthcoming however. Recently, the location of protein VI in the virus capsid and structure was determined by using advanced X-ray diffraction techniques (Reddy and Nemerow, 2014) (Fig. 3). A significant portion of protein VI, residues 6–157, was revealed underneath the vertex region closely associated with the peripentonal hexons. Interestingly, the first 33 amino acids of preprotein VI that are cleaved by the viral cysteine protease, were seen buried inside the cavity of the hexon trimer. This observation obtained by X-ray diffraction was corroborated by mass spectrometry analyses that revealed a pH dependent association of the amino terminal 33 residues of preprotein VI (pVIn) with the peripentonal hexons (Snijder et al., 2014). In addition, hydrogen-deuterium exchanges analyses indicated that up to 3 molecules of pVIn bind on the inner surface of the hexon trimer (Snijder et al., 2014). Together these studies suggested that the pVIn domain helps anchor the entire

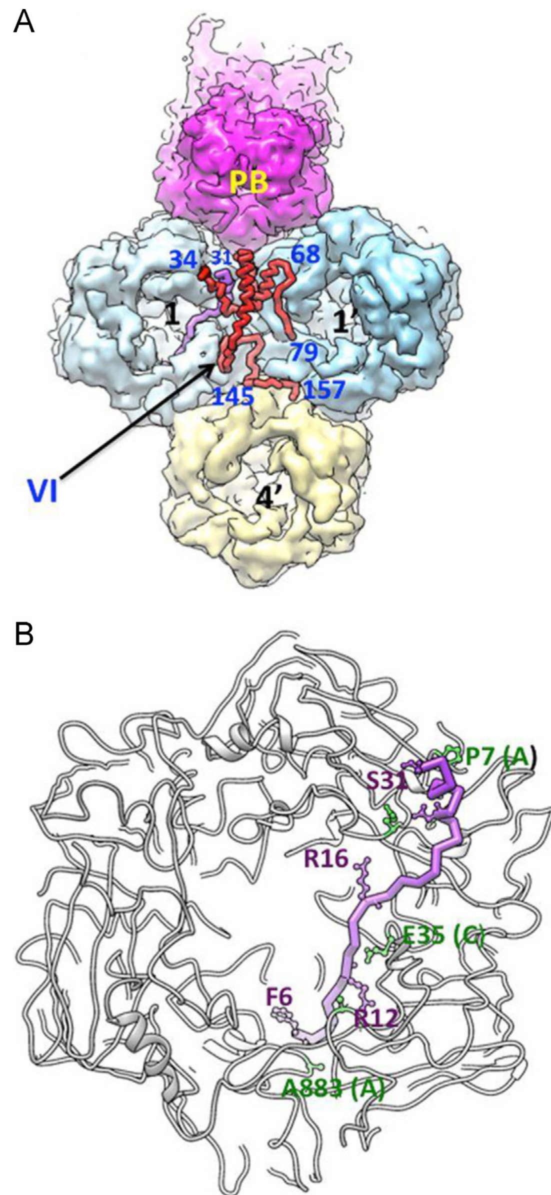

Fig. 3. Interactions between protein VI and the PPHs. (A) A hybrid (surface and tube) representation illustrating associations mediated by one copy of VI (red tube) gluing the adjacent PPHs (1,1′) and connecting them to hexon-4′ arising from the neighboring GON tile. The cleaved propeptide of VI (purple tube) remains associated with PPH-1 inside the hexon cavity. Certain residues of VI are identified with blue-colored labels. The penton base (PB) is shown in magenta. (B) A close-up view of the propeptide (purple tube) interactions with PPH-1, shown as a gray ribbon. A few residues that are involved in propeptide (purple) and hexon (green) interactions are labeled. Names of hexon subunits (A and C) are shown in parentheses. Reddy V S, and Nemerow G R. PNAS 2014;111:11715–11720.

protein VI molecule inside the virus capsid. Cleavage of the amino terminus of pVI (pVIn) during capsid maturation probably facilitates release of the membrane lytic domain during virus infection. The remaining amino acid residues that represent the mature/cleaved form of protein VI (a.a. 34–157) including the membrane lytic domain (a.a. 34–54) are situated between neighboring peripentonal hexons and help stabilize hexons on the inside of the virus capsid (Fig. 3). Protein VI is also in close contact with two other capsid proteins, V and VIII that together form a ternary complex (VI–V–VIII) underneath the vertex. The protein VI fold appears to be distinct and does not have any apparent structural homologs. As mentioned earlier, residues 34–54 of VI that have been predicted to form an amphipathic alpha helix (Wiethoff et al., 2005), do not form a helical structure in the virus, however it is possible that they adopt a helical conformation upon association with host cell membranes.

A puzzling aspect of protein VI relates to its copy number in the virus particle. Recent mass spectrometry analyses of HAdV using in depth quantitative proteomics demonstrated that virions contain ~360 copies of protein VI (Benevento et al., 2014). However the X-ray structure of HAdV revealed only a maximum of 180 copies of protein VI. One possibility that might explain this discrepancy is that an additional 180 copies of protein VI are located in the inner core of the virus and associated with the dsDNA genome. This region of the virus is not icosahedarally ordered and thus it has not been possible to analyze its structure. However, it is known that protein VI and the C-terminus of this protein in particular, acts as a molecular sled to facilitate the translocation of the viral cysteine protease along DNA during virus capsid maturation (Blainey et al., 2013; Baniecki et al., 2013).

The key function of protein VI is membrane disruption, a process that requires virus disassembly in the early endosome to expose this molecule (Nguyen et al., 2010). Recent atomic force microscopy (AFM) studies have provided new information on the biophysical properties of the viral capsid and how they relate to capsid disassembly (Snijder et al., 2013). Using AFM in the nanoindentation (also defined as virus-poking) mode, the vertex region of the virus containing the penton base and fiber was found to be the least stable region of the capsid (Ortega-Esteban et al., 2013). Moreover, AFM nanoindentation studies showed that the binding of integrin αvβ5 to the penton base further softens the capsid vertex region whereas the binding of a defensin HD5 has precisely the opposite effect and strengthens the vertex. These studies are consistent with previous cryoEM structural analyses showing that αv integrin causes an untwisting of the penton base (Lindert et al., 2009) whereas binding of defensing HD5 prevents vertex removal and release of protein VI (Nguyen et al., 2010; Smith et al., 2010).

These structural, biochemical and biophysical analyses have shed further light on the molecular basis of virus assembly and disassembly. However, further investigation is required to understand precisely how pVI is released from the capsid and how it undergoes conformational changes during membrane association.

Cell signaling and trafficking events initiated by VI and endosome disruption

Structural, biochemical and cell biological studies have provided important clues to the mechanisms involved in adenovirus rupture and penetration of cellular membranes. However, little is known of the host response to this assault, particularly on one of the defining features of the cell itself, cellular membranes. By all accounts, adenovirus induces large membrane defects capable of transmitting not only a ~90 nm diameter viral particle, but many soluble factor present near the membrane defect as well. Since membrane integrity is a crucial aspect of normal cell physiology, it is very likely that membrane damage is capable of being sensed by the host. In other systems, pathogen-associated membrane damage is met by a host stress response that triggers membrane repair pathways (Tam et al., 2010; Gurcel et al., 2006). But it has also been shown to trigger pathways leading to both innate and adaptive immune activation (Thurston et al., 2012; Hara et al., 2007). Thus adenovirus cell entry must somehow counterbalance the responses mounted by the host upon virus induced membrane damage.

Studies of the *ts1* mutant provided some of the first evidence that membrane damage during adenovirus cell entry triggers a stress response. This mutant virus is incapable of uncoating and releasing protein VI to rupture cell membranes. Studies examining the proinflammatory response to adenovirus in mice demonstrated that while replication defective HAdV-C5 induces a very rapid activation of the mitogen activated kinases, P38 and ERK, these kinases were not activated early during infection with *ts1* (Smith et al., 2011). Additional in vivo studies found that JNK1/2 activation in mice is dependent on capsid uncoating (Fejer et al., 2008). These signaling pathways could be activated by sensing of the viral DNA genome (Nociari et al., 2009) or early expression of small virally associated, noncoding RNAs (Yamaguchi et al., 2010). However, other studies have suggested that the viral capsid, acting through αvβ3 integrins, is responsible for some of these responses (Di Paolo et al., 2009; Chintakuntlawar et al., 2010; Higginbotham et al., 2002).

More recently, adenovirus-induced membrane damage occurring during entry of macrophages and dendritic cells was found to activate an innate proflammatory signaling pathway leading to augmented expression of proinflammtory cytokines and activation of the NLRP3 inflammasome (Barlan et al., 2011; Muruve et al., 2008). This pathway is related to the release of lysosomal cathepsins after adenovirus induced membrane damage (Barlan et al., 2011). Cathepsin release is observed within minutes of membrane damage and cathepsin-dependent events in the cytoplasm initiate a mitochondrial stress pathway leading to increased oxidative stress, all detectable within minutes after infection (McGuire et al., 2011). This response is strongly correlated with the timing of adenovirus membrane penetration (Greber et al., 1993) and does not occur during infection with the *ts1* mutant (Barlan et al., 2011). Interestingly, another nonenveloped virus thought to permeabilize membranes during cell entry, reovirus, did not release lysosomal cathepsins into the cytoplasm or induce oxidative stress signaling in cells, suggesting the mechanism of adenoviruses cell membrane penetration is much more catastrophic and thus capable of being sensed by the host (Barlan et al., 2011).

Evidence that adenoviruses may have evolved strategies to evade host detection of rupture of specific membrane compartments is apparent from the fact that certain virus serotypes appear to traffic less efficiently to lysosomes prior to escape into the cytoplasm than others. For example, subgroup A, B and D human adenoviruses have been shown to traffic more readily to lysosomes than subgroup C viruses (Shayakhmetov et al., 2003; Teigler et al., 2014; Miyazawa et al., 1999). This trafficking has been linked to the receptor engaged by the fiber knob (Shayakhmetov et al., 2003; Miyazawa et al., 1999). Barlan et al., found that in macrophages, an HAdV-C5 vector in which the fiber was swapped with that of HAdV-B16 traffics more strongly to lysosomes, resulting in greater release of lysosomal cathepsins than HAdV-C5 resulting in greater inflammasome activation (Barlan et al., 2011). Thus, by avoiding trafficking to lysosomes prior to membrane rupture, subgroup C viruses may have evolved a strategy to avoid triggering innate signaling pathways designed to sense membrane damage.

Virus trafficking to lysosomes in macrophages has also been correlated with altered expression of integrins (Carey et al., 2007).

In particular, expression of β5 integrin, shown previously to augment the efficiciency of endosomal escape (Wickham et al., 1994). αvβ5 expression is decreased upon activation of the transcription factor, PU.1, making αvβ3 integrin the more abundant integrin and this is correlated with increased viral trafficking to lysosomes (Berclaz et al., 2002). Interestingly, integrin αvβ3 has been linked to greater activation of innate immune signaling in macrophages (Di Paolo et al., 2009). However, it is not known whether αvβ3 integrins usage leads to greater AdV trafficking to lysosomes, or that lysosomal membrane rupture is linked to the inflammatory response to adenovirus observed in mice.

Another recently identified response to pathogen damage of cell membranes involves the sensing of glycoprotein sugars exposed to the cytoplasm upon membrane damage. A subset of cytosolic molecules known as galectins have been recently shown to bind to exodomains when pathogens such as Salmonella (Paz et al., 2010) or Shigella (Dupont et al., 2009) bacteria or adenoviruses (Maier et al., 2012) invade host cells. One of these molecules, galectin-8 has been shown to recruit the autophagy cargo adapter molecule, NDP52 to damaged membranes and target them for macroautophagy (Thurston et al., 2012). This form of xenobiotic autophagy, xenophagy (Boyle and Randow, 2013), has been shown to restrict bacterial replication in cells by targeting them for destruction by lysosomes. Further, autophagy has been shown to be a key mechanism for facilitating antigen presentation to T-cells from infected macrophages or dendritic cells (Schmid et al., 2007).

Maier et al. recently found that galectins are recruited to adenovirus ruptured membranes, much like they are recruited to bacterial membranes (Maier et al., 2012). As with inflammasome activation, another nonenveloped virus, reovirus, did not permeablize cell membranes in a manner that leads to galectin recruitment (Maier et al., 2012). Thus, it appears that some nonenveloped viruses may have evolved strategies to perturb cell membranes without activating these immune detection pathways. Although adenovirus membrane disruption recruits galectins, it is unclear whether galectin-dependent xenophagy restricts adenovirus from efficiently entering cells. In one report, starvation induced macroautophagy was found to augment AdV-C5 delivery and gene expression (Zeng and Carlin, 2013). Thus, it remains to be determined whether adenovirus may have also evolved a strategy to evade, or even usurp, autophagy to efficiently entry cells.

Adenovirus more potently activates innate immune signaling pathways during cell entry, than other nonenveloped viruses (Barlan et al., 2011; Paglino et al., 2014). Perhaps the physical size and nature of the viral genome makes it unavoidable for AdV activation of these pathways. Whatever the case, it appears that AdVs have achieved a delicate balance between rupturing membranes to enter cells and minimizing host cell sensing of this damage or evading the responses to damaged membranes. Richard Feynman once described the criticality experiment, in which nuclear fission is initiated only just until a uncontrolled chain reaction begins, as "tickling the tail of a sleeping dragon". This phrase seems an apt description of adenovirus penetration of cellular membranes.

Unanswered questions and future endeavors

While significant progress has been made in elucidating the complex processes of adenovirus disassembly and membrane penetration, numerous gaps remain in our understanding of the details underlying these events. In particular, clues to the initial steps in capsid uncoating have been gained from highly sophisticated immunofluorescence imaging studies (Burckhardt et al., 2011) as well as biophysical (Snijder et al., 2013; Ortega-Esteban et al., 2013), structural (Saban et al., 2006; Fabry et al., 2005; Reddy et al., 2010; Liu et al., 2010) and functional analyses (Moyer et al., 2011). However, we still lack precise information on the key molecular events in virus disassembly. The proteins located at the vertex region including penton base and fiber likely play central roles in the initial disassembly. How these proteins undergo conformational changes to disengage from the virion is still poorly defined. Further molecular genetic and structural analyses are needed to help solve these mysteries.

With regards to membrane penetration, we now know the key molecule in the virus, protein VI, that mediates this process however the precise details of membrane disruption are not very well understood as is the case for most non-enveloped viruses. However, we now appreciate that the mechanism for adenovirus membrane disruption is likely to be distinct from that used by other non-enveloped viruses (Moyer and Nemerow, 2011). Viruses such as parvovirus and reovirus induce transient lipid modifications or size selective pore formation, respectively. The copy number of protein VI in the virion, 360 per capsid, is now fairly well established (Benevento et al., 2014), however whether all of these are released upon capsid disassembly and whether a smaller subset are adequate for membrane disruption has not been determined. A structure for protein VI in the capsid has been proposed and appears to be largely unstructured (Reddy and Nemerow, 2014). However, the N-terminal 80 residues of mature protein VI appear highly alpha-helical when bound to membranes. Thus, possible conformational changes in protein VI following capsid uncoating and membrane disruption need to be investigated. Moreover, the functional consequences of protease cleavage of protein VI near its amino terminus have yet to be fully elucidated. These and other molecular details regarding virus cell entry await further exploration. Given the role of adenoviruses in human diseases and their uses in vaccine and gene therapy, a failure to fully understand the process of adenovirus cell entry risks awaking a sleeping dragon.

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