Mechanisms of Adenovirus Membrane Permeabilization

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ABSTRACT

For a successful infection to occur, a virus must first penetrate host cell membranes to access intracellular sites of viral replication. Currently the mechanism through which adenovirus, a non-enveloped, dsDNA virus, disrupts the endosomal membrane during cell entry is not well characterized.

Recent studies suggest that adenovirus protein VI, which is released from the interior of the capsid during cell entry, has all of the in vitro membrane lytic activity of the virion. We found that protein VI binds membranes via an amino-terminal 80 residue α-helical domain. Critical to this interaction are conserved hydrophobic and basic lysine residues within this domain. Membrane disruption can occur by two different mechanisms: transmembrane pore formation or membrane fragmentation. Our studies indicate that protein VI fragments membranes by inducing membrane curvature stress.

The observed mechanism for protein VI membrane disruption in vitro correlates with events during adenovirus endosomal escape. Recombinant viruses with mutations in protein VI α-helical domain that either decrease hydrophobicity or reverse the positive charge of lysine residues have a defect in endosomal escape. These data suggest that electrostatic and hydrophobic interactions between protein VI and the endosomal membrane are important for adenovirus endosomal escape.
Furthermore, the gross membrane reorganization by protein VI observed \textit{in vitro} is consistent with our observations that adenovirus endosomal membrane rupture allows the interaction of a 30kD cytosolic protein, galectin-3 with exo-domains of endosomal transmembrane proteins. Galectin-3 recruitment to adenovirus-disrupted endosomal membranes can therefore serve as a marker for adenovirus membrane penetration. Using this marker, we have conclusively demonstrated that protein VI release from the capsid interior precedes endosomal membrane rupture. Thus, use of galectin-3 to detect adenovirus rupture of endosomal membranes by microscopy will likely contribute greatly to our further understanding of intracellular viral trafficking during cell entry.

The work presented in this dissertation provides significant insight into the mechanism used by adenovirus to penetrate cell membranes during entry. Furthermore, since endosomal disruption is necessary for this virus to activate the innate immune response, these studies also have important implications for understanding how the immune system recognizes adenovirus infection.
CHAPTER I

INTRODUCTION

COMPARISON OF ENVELOPED AND NONENVELOPED VIRUS ENTRY

A virus is a microscopic infectious agent that can only replicate within a host cell. Therefore, to establish a productive infection, viruses have to deliver their genetic material into a cell. To do so these agents must first bind a target cell and permeabilize its membrane. Viruses have developed unique strategies of disrupting cellular membranes depending on the virus structure (Tsai 2007). One important structural feature is the presence of a lipid bilayer surrounding the virus. Enveloped viruses are viruses that have this membrane layer, while viruses that lack this characteristic are classified as nonenveloped viruses.

The presence or absence of the lipid bilayer dictates how a virus penetrates cellular membranes. Enveloped viruses induce the fusion of the viral envelope with the cellular membrane, while nonenveloped viruses must lyse the limiting cell membrane (Poranen, Daugelavicius et al. 2002; Smith and Helenius 2004). Although the mechanism of membrane penetrations (fusion vs membrane lysis) is different, the series of events that lead to this process is similar between the two virus groups. One such similarity is that the fusion peptide or the membrane disrupting factor is buried within the virion, and is only exposed at the site of membrane penetration.
Enveloped virions are decorated with glycoproteins protruding from the viral bilayer, and they can function in cell binding as well as membrane fusion (Takimoto, Taylor et al. 2002). Membrane fusion is mediated by a fusion peptide often buried within these glycoproteins. The peptide is exposed only when a conformational change occurs in the glycoprotein (Harrison 2008). The triggering event that mediates this process dictates the site of membrane penetration. For example, HIV-1 fusion occurs at the cell surface and is triggered by the sequential interaction of the viral glycoprotein with a cellular receptor and a co-receptor (Harrison 2005). Other enveloped viruses such as influenza are first taken up by endocytosis, and the low-pH in the endosome triggers a conformational change in the hemagglutinin (HA) protein that exposes the fusion peptide (Bullough, Hughson et al. 1994). This peptide then induces fusion of the viral membrane with the endosomal membrane (Han, Bushweller et al. 2001; Borrego-Diaz, Peeples et al. 2003).

Similar to the enveloped virus glycoproteins, nonenveloped viruses have outer-layer capsid proteins that can serve in membrane binding and membrane penetration. Membrane penetration is mediated either by a membrane lytic factor that is released from the capsid, or by exposure of a hydrophobic moiety in the capsid proteins (Tsai 2007). These factors are buried within the capsid, and are exposed at the site of membrane penetration by triggering events such as receptor interaction, a decrease in pH, or cellular proteases (Fricks and Hogle 1990; Ebert, Deussing et al. 2002; Wiethoff, Wodrich et al. 2005). Enveloped virus membrane fusion has been studied in great detail, and as a result the different steps in this process are well characterized (Poranen, Daugelavicius et al. 2002; Harrison 2008). However, the molecular mechanism of membrane penetration by nonenveloped viruses is still poorly understood.
NONENVELOPED VIRUS ENTRY

Nonenveloped virus entry can be divided into four steps: traffic to the site of membrane penetration, exposure of a membrane lytic factor, membrane disruption, and virion translocation across this membrane. The cellular conditions that trigger the membrane lytic protein to be exposed, as well as the identity of many of these proteins are now known. However the molecular mechanism of membrane disruption is not well characterized. The work described in this dissertation has shed some light on the process used by a nonenveloped virus to disrupt membranes.

Traffic to site of membrane penetration

The first step in successfully permeabilizing cellular membranes is virus trafficking to the appropriate site of membrane penetration. For some viruses these sites are cellular organelles such as the endosome or the ER. There are two main pathways a virus can use to reach these sites: clathrin or caveolae mediated endocytosis (Marsh and Helenius 2006). These two pathways differ in the mechanism used to initiate endocytosis as well as the factors involved. Clathrin dependent endocytosis is mediated by the protein clathrin and occurs in clathrin coated pits on the plasma membrane (Reider and Wendland; Meier and Greber 2004). On the other hand, caveolae mediated endocytosis occurs in membrane microdomains rich in cholesterol, glycosphingolipids and the cholesterol binding protein caveolin (Li, Song et al. 1996). The best studied viruses that use caveolae dependent endocytosis belong to the polyomavirus family and include SV40, mouse polyomavirus, and the human pathogens BK and JC viruses (Norkin, Anderson et al. 2002). Similar to many viruses that use this pathway, polyomaviruses use different gangliosides as receptors. Receptor binding triggers caveolae mediated
endocytosis and subsequent transport to the ER, where these viruses are thought to cross the ER membrane to the cytoplasm (Qian and Tsai; Gilbert and Benjamin 2004). On the other hand, canine parvovirus binds the transferrin receptor, and is internalized via clathrin mediated endocytosis. This virus is then transported to the recycling endosome, however the site of membrane penetration is not clear (Suikkanen, Antila et al. 2003).

**Release of the membrane lytic factor**

Upon reaching the membrane penetration site, the viral capsid protein of nonenveloped viruses must undergo a series of structural rearrangements to lyse the limiting membrane. These rearrangements result in release of a membrane lytic factor or the exposure of a hydrophobic moiety that mediates membrane lysis. The cellular factors that trigger these conformational changes differ depending on the site of membrane penetrations. For instance, poliovirus interaction with its receptor PVR on the target cell initiates the membrane penetration process. Receptor binding induces a conformational change in the virion that exposes the viral protein 4 (VP4) and the N terminus of the virus protein 1 (VP1) (Fricks and Hogle 1990). The myristilated VP4 and N terminus of VP1 insert into the membrane to form a transmembrane pore (Tosteson and Chow 1997).

Other viruses use cellular proteases to activate the virus into the membrane disrupting intermediate. During reovirus infection the outermost capsid protein σ3 is digested by the endolysosomal proteases cathepsin L and cathepsin B exposing the capsid protein μ1 (Nibert and Fields 1992; Danthi, Guglielmi et al. 2010). An autocatalytic event then occurs resulting in the release of a myristolated N terminal fragment of μ1, μ1N, which has lytic activity (Zhang, Chandran et al. 2006). Other cellular proteases such as
furin and trypsin have been implicated in activating viruses such as papillomavirus and rotavirus respectively (Estes, Graham et al. 1981; Richards, Lowy et al. 2006).

Similar to their enveloped counterpart, where low pH induces membrane fusion, a drop in pH also triggers nonenveloped virus membrane penetration. The parvovirus minute virus of mice (MVM) deploys the lipolytic enzyme phospholipase A2 (PLA2) to lyse membranes (Farr, Zhang et al. 2005). This enzyme is located at the N terminus of the coat protein VP1 and studies suggest that a decrease in pH is important for PLA2 activity (Suikkanen, Antila et al. 2003; Mani, Baltzer et al. 2006).

**Membrane penetration: pore formation and membrane fragmentation**

Once the virus releases its membrane disrupting protein or virus intermediate, the next step is the actual lysis process. Although the steps that lead to nonenveloped virus membrane disruption are slowly being understood, the mechanism of membrane lysis is poorly characterized. Membrane disruption by antimicrobial peptides on the other hand is well understood. These peptides are small molecular weight proteins that have a broad spectrum antimicrobial activity against bacteria and viruses (Boman 1995). In most cases their mode of action appears to be direct lysis of the pathogenic cell membrane (Shai 1999). This is possible due to the amphiphatic α-helix structure they acquire upon membrane binding (Dathe and Wieprecht 1999). This structure is characterized by the partitioning of the hydrophobic and hydrophilic residues on opposite sides of the helix. There are two distinct mechanisms of membrane disruption by antimicrobial peptides: a detergent-like membrane fragmentation and the formation of transmembrane pores (Shai 2002).
A key difference between these two different mechanisms is the orientation of the peptide on the lipid bilayer. To form a transmembrane pore the amphipathic α-helical peptide must traverse the lipid bilayer. The hydrophobic surface of the helix interacts with the hydrophobic core of the membrane and the hydrophilic surface points inward lining an aqueous pore (Ehrenstein and Lecar 1977). On the other hand, peptides that fragment membranes bind the lipid bilayer with a parallel orientation by electrostatically interacting with negatively charged lipid head groups (Pouny, Rapaport et al. 1992; Gazit, Boman et al. 1995). Surface membrane binding results in the induction of a membrane curvature stress that ultimately fragments the membranes (Brender, Durr et al. 2007). Experimentally discerning between these two different mechanisms of membrane disruption can be made by monitoring the release of different size dyes from model membranes. For example, the mechanism of membrane disruption by the antimicrobial peptide melittin depends on its orientation on the lipid bilayer (Ladokhin and White 2001). In conditions where melittin traverses the lipid bilayer, this peptide forms size selective pores, as determined by its ability to release a 4kD dextran but not a 50kD dextran from model membranes. However, when melittin has a parallel membrane orientation, it fragments the lipid bilayer resulting in the release of both dextrans from liposomes.

These two mechanisms of membrane disruption that have been demonstrated for antimicrobial peptides are also observed with virus lytic peptides. Ivanovic et al has shown that the membrane lytic peptide of reovirus μ1N forms size-selective pores in membranes. This peptide is generated and released from the capsid as a result of autocatalytic event of capsid protein σ1. When added to resealed red blood cell ghosts
that are loaded with fluorescently labeled dextrans of different sizes, \( \mu 1N \) facilitates the release of 3kD and 10kD but not 40kD dextrans. These data suggest that reovirus \( \mu 1N \) forms pores with a 4-9nm diameter (Agosto, Ivanovic et al. 2006; Ivanovic, Agosto et al. 2008). Poliovirus also penetrates membranes by forming a transmembrane pore. Binding its receptor on the target cell triggers a conformational change that exposes the N terminus of the capsid protein VP1 and the myristolated autocleavage peptide VP4 (Fricks and Hogle 1990). Interaction of these proteins with the membranes results in the formation of an ion permeable channel, through which the viral genomic RNA can be translocated to the cytoplasm (Tosteson and Chow 1997).

A different mechanism is used by the parvovirus MVM. As mentioned earlier, the viral capsid protein VP1 contains at its N terminus a PLA2 enzymatic core (Zadori, Szelei et al. 2001; Dorsch, Liebisch et al. 2002). This enzyme is a membrane lytic factor which facilitates MVM endosomal escape (Farr, Zhang et al. 2005). Cellular PLA2 cleaves phospholipid molecules to generated free fatty acid and a lysophospholipid (a lipid with one acyl tail) (Brown, Chambers et al. 2003). This activity has been shown to result in membrane tubule formation with increased membrane curvature (de Figueiredo, Drecktrah et al. 1998). As mentioned earlier, the induction of membrane curvature stress results in membrane fragmentation, although this process has not been demonstrated with MVM.

**Virus translocating across the limiting membrane**

Once the limiting membrane has been permeabilized, the next step is the physical transport of the virus across the limiting membrane. Until now this event has not been directly demonstrated. Membrane penetration has been suggested based on evidence that
viruses, such as adenovirus, can result in the cytoplasmic translocation of cointernalized proteins or DNA (FitzGerald, Padmanabhan et al. 1983; Yoshimura, Rosenfeld et al. 1993; Seth, Rosenfeld et al. 1994) Additionally, electron microscopy studies have shown nonenveloped viruses in the cytoplasm (Imelli, Ruzsics et al. 2009). These results however do not directly demonstrate the actual membrane transport event. The rapid speed by which this process occurs makes it difficult to capture this event in fixed cells. Work presented in this dissertation has identified an assay that can be used to directly monitor membrane disruption by nonenveloped viruses.

GALECTIN 3 AS A MARKER FOR VACUOLE LYSIS

Similar to viruses, bacteria disrupt vacuolar membranes to infect cells. Compared to other steps in the bacterial life cycle, vacuole lysis is not well understood, due to the lack of markers associated with disrupted phagosomes. Paz et al has identified a marker for ruptured phagosomes and this marker is the protein Galectin 3 (Gal-3).

Gal-3 is a member of the galectin family, a conserved family of lectins. These proteins are cytosolic and contain consensus sequences in their carbohydrate binding domain (CRD) which have an affinity for β-galactoside containing glycoconjugates (Hughes 1997). The structure of Gal-3 is unique among the different galectins and it is composed of one CRD at the C terminus and a N-terminal, non-lectin domain consisting of multiple repeats of a peptide sequence rich in proline, glycine, and tyrosine (PGY repeats) (Albrandt, Orida et al. 1987; Cherayil, Weiner et al. 1989). Gal-3 is found in many different tissues, expressed mainly in epithelial and myeloid cells, and has a variety
of different biological functions such as a role in inflammation, cell adhesion and cancer (Liu and Rabinovich 2005; Rabinovich and Toscano 2009).

In addition to the above mentioned cellular functions, Paz et al has shown that Gal-3 can also serve as a marker for ruptured phagosomes. Using immunofluorescence microscopy this group observed Gal-3 punctate structures accumulating around the bacteria in Shigella infection. These structures were not formed in cells infected with a mutant Shigella that fails to lyse the phagosome. Furthermore, Gal-3 puncta were observed by electron microscopy to bind membrane remnants and not the bacteria. Gal-3 labeling disrupted vacuoles was not specific to Shigella infection, since punctuate structures were seen in phagosomes ruptured by other bacteria. The cytosolic Gal-3 labels disrupted membranes by binding N-linked glycans found on the plasma membrane when these are exposed following membrane lysis (Fig. 1). Taken together these data suggest that Gal-3 can serve as a marker for vacuole lysis, and could potential be used to monitor membrane disruption by a nonenveloped virus.
Galectin 3 is a cytosolic protein that binds N-linked glycans. These glycans are present on the cell membrane, and are exposed to the cytosol only after vacuole lysis. Therefore, when a vacuole is lysed galectin-3 can bind N-linked glycans, and this binding can be visualized by immunofluorescence microscopy as punctuate staining.
ADENOVIRUS AS A MODEL SYSTEM FOR STUDYING NONENVELOPED VIRUS ENTRY

Adenovirus is an excellent model system to study nonenveloped virus entry. The adenovirus structure is well characterized, and the role of various cellular and viral proteins in adenovirus cell entry is known. In addition, a number of the viral capsid proteins can be produced as recombinant proteins in bacterial expression systems, allowing analysis of their function independent of the intact virion. Since many features of adenovirus cell entry are common to the entry mechanisms of other nonenveloped viruses, these studies will shed significant light on how these viruses enter cells.

Adenoviruses and disease

Human adenoviruses (Ads) were initially isolated more than 50 years ago by Hilleman and Rowe from patients with acute respiratory disease (Hilleman and Werner 1954, Rowe et al 1955). Currently there are at least 56 serotypes that are grouped in seven different species (A-G). In addition to acute respiratory syndrome, these viruses can also cause conjunctivitis, keratoconjunctivitis, and acute gastroenteritis. Most Ad infections occur early in life, and by the age of 10 most individuals have been infected with at least one serotype. In healthy individuals Ad infection is self-limited; however it can cause serious complications in immunocompromised individuals (Hierholzer 1992; Leen and Rooney 2005). Despite its association with human disease, Ad has also served as an important tool in understanding a number of biological processes (Chow, Gelinas et al. 1977; Berget, Moore et al. 2000). Furthermore Ad vectors are easy to purify, they allow for packaging of large genes and they have a wide tissue tropism, making them good candidates as vectors for gene therapy and vaccine development (Harvey, Kamphuis et al. 2002).
**Adenovirus structure**

Ad is among the largest non-enveloped viruses (90nm in diameter). Its structure is made up of an outer capsid, with icosahedral symmetry, surrounding the nucleocapsid core (Fig. 2). The outershell is primarily formed by 240 trimers of the capsid protein hexon, which forms the twenty facets of the icosahedrons (van Oostrum, Smith et al. 1987). Located at each of the 12 vertices is the penton complex, consisting of the homopentameric penton base non-covalently associated with the homotrimeric fiber protein (van Oostrum and Burnett 1985; Stewart, Fuller et al. 1993). The capsid is stabilized by four minor capsid proteins (IIIa, VI, IX and VIII) which act as cement proteins by interacting with each other as well as hexon and core proteins (Stewart, Fuller et al. 1993; Saban, Silvestry et al. 2006) The viral core contains the 36 kB double stranded DNA genome covalently bound to a terminal protein (TP) (Philipson 1995). This genome is also associated with the core proteins V, VII and Mu. In addition to the 11 structural proteins, the Ad capsid also contains a viral cysteine protease (23k) which is important for virus maturation (Cotten and Weber 1995).

**Adenovirus life cycle**

Ad infection begins with a high affinity interaction between the fiber protein and the coxsackie-adenovirus receptor (CAR) on target cells (Bergelson, Cunningham et al. 1997) (Fig. 3). A second lower affinity interaction between penton base and αv integrins triggers clathrin mediated endocytosis (Wickham, Mathias et al. 1993). Upon endosome acidification the capsid partially disassembles and pVI is released from the capsid interior
Fig. 2. Adenovirus structure

Adenovirus is a nonenveloped virus with icosahedral symmetry. The viral capsid is made up of the major proteins hexon, penton base and fiber as well as the minor capsid proteins (III, IX, VI, VIII). The virus core is made up of proteins V, VII, mu and surrounds the dsDNA genome. The viral terminal protein (TP) is covalently bound to the genome.
(Greber, Willetts et al. 1993). This protein has all of the in vitro membrane lytic activity of the virion (Wiethoff, Wodrich et al. 2005), suggesting that pVI disrupts endosomal membranes, allowing the virus to escape to the cytosol. The partially disassembled capsid then migrates on microtubules to the nucleus, docks at the nuclear pore, and releases its DNA genome into the nucleus, where viral transcription and DNA replication occurs (Greber, Willetts et al. 1993; Trotman, Mosberger et al. 2001). In addition to mediating Ad endosomal escape, pVI is also necessary for virus assembly. Ad assembly occurs in the nucleus, and requires pVI to bring hexon into the nucleus (Wodrich, Guan et al. 2003). Immature non-infectious virions are formed in the nucleus, and they contain the precursor forms of several structural proteins. These immature viruses become infectious when pVI activates the viral 23K protease, to cleave these structural proteins into their mature form (Anderson, Baum et al. 1973; Mangel, McGrath et al. 1993). The mature virions are then released usually upon destruction of the cell through mechanisms that are not well characterized (Jiang, White et al.).

**ADENOVIRUS CELL ENTRY**

Entry of human Ad into cells is a stepwise process. It can be divided into three separate events: attachment to the target cell, internalization, and endosomal escape (Fig. 3). These events are mediated by different viral proteins. Attachment occurs when the viral fiber protein binds its cellular receptor. An interaction between penton base and cellular integrins induce virus internalization via clathrin-mediated endocytosis. Finally the virus disrupts the endosomal membrane to gain access to the cytoplasm using protein VI.
Ad binds its target cell via a high affinity interaction between the fiber protein and the CAR receptor. A second interaction between penton base and $\alpha_v$ integrins triggers clathrin-mediated endocytosis. Upon acidification of the endosome, the virus partially disassembles. Protein VI is released from the capsid interior and disrupts the endosomal membrane facilitating Ad cytosolic translocation.
Adenovirus cell binding and internalization

Ad cell entry begins with the virus binding its target cells, followed by virus internalization via clathrin mediated endocytosis. Early studies by Silver and Anderson (1988) have shown that Ad2 binds but does not enter certain cells, suggesting that attachment and internalization are two separate events. These observations were confirmed by Wickham et al (1993) who showed that Ad2 entry into cells occurs upon engagement of αv integrins.

Ad cell attachment is mediated by the viral fiber protein. This fiber protein is a homotrimer, which projects from the virus surface at each of the 12 vertices. Each subunit of the fiber protein is made up of three domains: the amino terminal tail which associates with the viral penton base,(Devaux, Adrian et al. 1990), the shaft, and the knob which interacts with the cellular receptor (Philipson, Lonberg-Holm et al. 1968; Kirby, Davison et al. 2000) There are two main receptors that the fiber protein has been shown to bind: the coxsackie-adenovirus receptor (CAR) and CD46.

CAR is a 46-kDa transmembrane protein that was initially identified as a cellular receptor for coxsackie B viruses, Ad2 and Ad5. In addition to subgroup C Ad fibers, CAR binds fibers from subgroups A,D,E, F (Roelvink, Lizonova et al. 1998). Since its identification as a receptor for Ad2 and Ad5 this protein has been studied extensively. The normal cellular function of CAR is to mediate cell adhesion by forming homodimers (Honda, Saitoh et al. 2000; Philipson and Pettersson 2004). CAR is an immunoglobulin superfamily protein with two extracellular Ig-like domains (related to the immunoglobulin V (IgV) and C2 (IgC2) domain folds), a single membrane-spanning
sequence, and a significant cytoplasmic domain. The CAR region necessary for binding
the fiber protein is the N terminal IgV-related CAR domain (D1). (Freimuth et al 1999)
In humans CAR is expressed in a variety of different tissues, with the exception of
muscle cells and B and T cells (Freimuth, Philipson et al. 2008). Important to Ad
infection of the respiratory tract is the fact that CAR is expressed in respiratory epithelial
cells, and it is localized to the tight junctions and the basolateral side of polarized
epithelial cells (Cohen, Shieh et al. 2001). Thus CAR is not accessible to Ad, which
infects from the apical side. It has been suggested that infection starts in nonpolarized
cells that express CAR on the apical side, or in pre-existing lesions in the epithelium
(Meier and Greber 2003). Walters et al (Walters, Freimuth et al. 2002) have shown that
before cell lysis, an excess of fiber protein is released basolaterally from infected cells.
The affinity of fiber interaction with CAR is greater than the CAR- CAR interaction in
the tight junctions. Therefore binding of CAR to the excess fiber can disrupt the junctions
between the epithelial cells and promote viral release to the airway lumen, increasing
viral spread.

While CAR is the attachment receptor for most Ad subgroups, the fiber protein of
subgroup B viruses binds a different cell surface receptor. Several groups have identified
the membrane cofactor CD46 to be this receptor (Gaggar, Shayakhmetov et al. 2003;
Segerman, Atkinson et al. 2003; Marttila, Persson et al. 2005). CD46 belongs to a family
of proteins that regulate complement activation. This protein is expressed on all cell types
except erythrocytes and its biological role is to prevent complement activation by
autologous tissue (Post, Liszewski et al. 1991; Hsu, Dorig et al. 1997). The structure of
this protein consists of an extracellular region that contains four copies of a structural
motif named the short consensus repeat (SCR I-IV). The SCRs are then connected to a linker rich in serines, threonines and prolines (STP) followed by a single membrane-spanning domain and the C terminal cytoplasmic tail (Hsu, Dorig et al. 1997). The fiber protein of subgroup B viruses binds CD46 by interacting with SCRI and SCRII (Fleischli, Verhaagh et al. 2005; Persson, Reiter et al. 2007).

While virus attachment to cells is mediated by an interaction between fiber protein and the cellular receptors CAR or CD46, virus internalization requires a second interaction with a separate cellular receptor. Wang X et al (1999) have shown that the cytoplasmic tail of CAR is not required for virus infection, suggesting that signalling through a different receptor is needed for internalization. Additionally in the late 1950 studies have shown that a viral protein called “the toxic factor” is release from infected cells, causing cells to detach from glass or plastic surfaces (Everett and Ginsberg 1958; Pereira 1958) This “toxic factor” is in fact the viral penton base which Wickham et al (1993) has shown to bind a, integrins and mediate virus internalization. The cell detachment observed in infected cells can be explained by integrins binding released penton base instead of binding the extracellular matrix.

The adenoviral penton base is a 400 kD coat protein that binds noncovalently to the fiber protein, forming a penton complex at each of the 12 vertices of the Ad virion. Electron microscopy studies have shown that the N terminal domain of fiber inserts into the central cavity of the penton base (Stewart, Burnett et al. 1991; Fabry, Rosa-Calatrava et al. 2005). The penton base is then made up of five identical subunits that each contain an Arg-Gly-Asp (RGD) motif (Neumann, Chroboczek et al. 1988). This motif is important for binding integrins (Cheresh and Spiro 1987).
Integrins are heterodimeric transmembrane proteins composed of an α subunit and a β subunit. There are 20 different members of the integrin family and many can bind RGD motifs in host extracellular matrix proteins (Campbell and Humphries). Integrin interaction with these proteins results in signalling and mediate important cellular functions such as cell attachment, migration and differentiation (Hynes 2002). Binding of the adenoviral penton base to αv integrins results in activation of a signalling cascade that induces clathrin mediated endocytosis. Interfering with proteins that are involved in this process prevents Ad infection (Varga, Weibull et al. 1991; Rauma, Tuukkanen et al. 1999). In addition, EM studies have detected incoming virions in clathrin coated pits (Chardonnet and Dales 1970; Svensson 1985).

Adenovirus uncoating

Virus internalization triggers a process called viral uncoating, in which the protein coat that protects the Ad genome from the extracellular environment is partially shed. Ad uncoating has therefore been measured as an increase in the accessibility of the viral DNA to DNAs or DNA sensitive dyes. The current model based on studies with subgroup C and B viruses suggests that uncoating occurs in discrete steps. As a consequence of virus engaging its cellular receptor, the fiber protein is shed at the cell surface (Nakano, Boucke et al. 2000). Upon acidification of the endosomes, additional viral proteins such as the internal proteins IIIa, VIII and protein VI, and some of the hexon proteins are released (Greber, Willetts et al. 1993). This process is required for endosomal escape since a naked capsid containing the DNA, core proteins, and some hexon is translocated into the cytoplasm (Greber, Willetts et al. 1993).
Further evidence supporting a role for virus uncoating in endosomal lysis has been obtained from analysis of a temperature sensitive mutant Ad type 2 (Ad2ts1). When grown at the non-permissive temperature this virus fails to uncoat (Weber 1976; Mirza and Weber 1979). This defect is due to a point mutation (P137L) in the 23K protease. This mutation is linked to a decrease in protease incorporation into the virion (Rancourt, Keyvani-Amineh et al. 1995). The 23K protease functions inside the virion to cleave six structural proteins into their mature form (Webster, Russell et al. 1989; McGrath, Abola et al. 1996). A failure to incorporate the protease into the virion results in an immature virus, with increased stability (Silvestry, Lindert et al. 2009). Although Ad2ts1 can bind the CAR receptor and undergo internalization, it fails to escape the endosome, and is degraded in the lysosome (Greber, Webster et al. 1996) These observations suggest a link between capsid disassembly and endosomal lysis.

Adenovirus endosomal escape

Adenovirus endosomal disruption has been demonstrated by the release of cointernalized molecules such as proteins or nucleic acids into the cytoplasm (FitzGerald, Padmanabhan et al. 1983; Yoshimura, Rosenfeld et al. 1993). Furthermore, Ad can induce the release of fluorescent dyes from liposomes (Blumenthal, Seth et al. 1986). These early studies have also shown that the membrane lytic activity of Ad is pH dependent, since agents that block endosomal acidification inhibit endosomal escape, but not virus internalization (Greber, Willetts et al. 1993). Membrane penetration also required capsid disassembly, however the membrane lytic factor was not known. Wiethoff et al. (2005) set out to determine the role of Ad capsid disassembly in endosomal lysis, and while doing so this group identified the Ad membrane lytic factor.
To determine the capsid protein mediating membrane disruption, the capsid was disassembled and the dissociated proteins were separated from the partially uncoated virus using a step gradient. Testing the membrane lytic activity of the different fractions revealed that the released proteins contained all of the membrane lytic activity of the virus. This lytic activity was abrogated when pVI was immunodepleted from the dissociated protein fractions. Furthermore a recombinant pVI could also disrupt membranes. Taken together these data suggest that pVI is the Ad membrane lytic factor.

Protein VI is a structural protein with a variety of different functions in the Ad life cycle. Electron microscopy studies propose that pVI is located within the cavity of all hexon trimers in the virion (Saban, Silvestry et al. 2006). This protein is suggested to be a trimer of dimers, with 360 monomers per virion (Stewart, Fuller et al. 1993). Upon virus maturation the viral protease cleaves pVI to the mature form. Although the crystal structure of pVI is not known, the N terminus of the mature pVI is predicted to be a four-helix bundle, with the rest of the protein predicted to be mainly unstructured. The first helix in this bundle is predicted to be an amphipathic α-helix (residues 36-53) (Fig 4A). This helix is important for the in vitro membrane lytic activity of pVI. Wiethoff et al (2005) has shown that a recombinant pVI that lacks this helix has decreased membrane lytic activity compared to full length pVI. This group has further shown that while Ad partial disassembly and release of pVI is pH dependent, the membrane lytic activity of pVI is pH independent. However the mechanism of pVI membrane lytic activity is not known.
CHAPTER II
MATERIALS AND EXPERIMENTAL METHODS

CELL LINES.
Tissue culture reagents were obtained from Mediatch and HyClone. HeLa cells and 293β5 cells were obtained from ATCC and maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 1 mg/ml streptomycin, 0.25 μg/ml amphotericin B, non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES buffer and 2 mM glutamine.

VIRUSES
The temperature sensitive mutant ts1 (Weber 1976), and Ad5-GFP (Wiethoff, Wodrich et al. 2005), an E1/E3-deleted adenovirus encoding EGFP under the control of a CMV promoter, were propagated in 293β5 cells. Viruses were purified from cellular lysates by double banding in cesium chloride gradients and dialyzed in 40 mM Tris, 150 mM NaCl, 10% glycerol, and 1 mM MgCl₂ (pH 8.2). For these studies, the ts1 virus was propagated at the nonpermissive temperature of 39.5 °C. (Cotten and Weber 1995). Viral concentrations were determined by Bradford assay (Bio-Rad Laboratories, Inc.) and aliquots were flash frozen in liquid nitrogen and stored at −80°C. Reovirus strain T3D was derived from lab stocks which were twice plaque purified. Virus was propagated on L929 cells and purified by cesium chloride density gradient centrifugation as previously described (Furlong, Nibert et al. 1988)
Generating the K45E virus

The K45E mutation was introduced into the Ad genome using a two step BAC recombination protocol (Tischer, von Einem et al. 2006) performed in GS1783 E. coli cells (a gift from GS lab). This strain encodes the lambda Red recombination proteins, and the I-SceI enzyme under inducible promoters (Tischer, Smith et al.) In the first step a kanamycin cassette was inserted in pVI, by a recombination between pAd5-BAC and the product of PCR amplifying kanamycin from the pEPKan-S2 plasmid (a gift from GS lab) using the following primers: 5’ATGAGCGGTGCGCGCTTCAGCTGGGCTCGCTG TGG TGG AGCGGCATT GAA ATTTCGGTTCCACC AGGATGACGACGATA AGTAGGG3’ and 5’GTTCCAGGCGCTTGCTGCCATAGTTCTTAACGGGTG GAACCGAAATTTCTAAATGCGGCTCCACAGCAACCAATTAACCAATTCTGATT AG 3’. The sequence homologous to pEPKan-S2 is underlined. The kanamycin cassette was replaced with a K45E PCR product through a second recombination event. The PCR product was generated by amplifying form pVIhis-K45E using the primers 5’ATGAGCGGTGCGCGCTTCAGCTGGGCTCGCTG TGG TGG AGCGGCATT GAA ATTTCGGTTCCACC AGGATGACGACGATAAGTAGGG 3’ and 5’GTTCCAGGCGCTTGCTGCCATAGTTCTTAACGGGTG GAACCGAAATTTCTAAATGCGGCTCCACAGCAACCAATTAACCAATTCTGATT AG 3’. The altered sequence is in bold. A third recombination between pAd5-BAC-K45E and PmeI linearized pADtrack introduced a GFP cassette in the K45E viral genome (pAd5-GFP- K45E). The mutant virus was then generated as described above.
Generating the K45EK52E virus

The K45EK52E mutation was introduced into pVI in the Ad genome using recombineering as previously described (Warming, Costantino et al. 2005). The mutant pVI was amplified from the pVIhis-K45EK52E plasmid using the following primers: pVIfor 5’ AGTCTGGACTCTCACG 3’ and pVIrev 5’ GGCGACATGGACGC 3’. The PCR product was incorporated into the Ad5 genome via lambda Red recombination in bacteria harboring pAd5-GFP ΔpVIgalK. This vector contains pVI replaced with the galactokinase (galK) cassette of pGalK, and was generated by lambda Red recombinant between pAd5-GFP and the product of PCR amplifying galK using the following primers: pVIgalK for 5’ GACATCAACTTTGCGTCTCTGGCCCCGC GACACGGCTCGCCCTGTTGAACAATTACATCGGGA 3’ and pVIgalK rev 5’TCAGAAGCATCGTCGGCGCTTCAGGGATTGCACCCCCAGAT CAGCACTGTCC TGCTCCTT 3’. The galK specific sequence is in italics and the pVI sequence is underlined. The final recombination products were sequenced and the virus was generated as described above. Briefly, Pac I linearized WT Ad5GFP and K45EK52E Ad5GFP viral genomes were transfected into 293β5 cells, followed by amplification in one additional passage. Infected cells from the second passage were collected at 48hrs post infection. The virus concentration from the cell lysates or supernatants was determined using qPCR analysis.

VIRUS INFECTIVITY

Virus infectivity was determined as previously described by measuring the expression of the GFP transgene (Wu, Fernandez et al. 2001). Briefly 100,000 HeLa cells were incubated for 24 hours with increasing concentrations of virus (gc/cell) in growth
medium. Cells were detached and analyzed by fluorescence-assisted cell sorting in an Accuri cytometer (Becton Dickenson, Franklin Lakes, N.J.). A threshold established by the fluorescence of uninfected cells was used to distinguish infected cells expressing GFP.

QUANTITATIVE PCR ANALYSIS

Viral DNA was isolated from supernatants using QIAamp DNA Mini kit (Qiagen) as directed by the manufacturer's instructions. The viral DNA was serially diluted and qPCR reactions that target the Ad hexon gene were performed using GoTaq qPCR master mix (Promega). Each reaction contained 10 μl of viral DNA and 40 μl of Master Mix. The Master Mix was made up of 1x GoTaq qPCR Master Mix, and 100nM of the RTHexon for primer 5’ CAGGACGCCTCGGAGTACCTGAG 3’ and RTHexon rev primer: 5’GGAGCCACCGTGAGGT 3’. Thermal cycle conditions consisted of initial denaturation incubation at 95°C for 10 minutes followed by 44 cycles of alternating 95°C incubations for 15 seconds, 50°C incubations for 2 minutes and 60°C incubations for 1 minute. Fluorescence was detected after every 60°C extension. For standard curves, qPCR was performed on a 10-fold dilution series of purified plasmid, hexon-pET15b, ranging from $1 \times 10^1$ to $1 \times 10^7$ copies/reaction.

SITE DIRECTED MUTAGENESIS

Generating pVI single tryptophan mutants

To generate pVI containing single tryptophan residues, mutations were introduced in pET15bVI-N, a construct encoding residues 34–114. This region of pVI has only 3 tryptophans, therefore to obtain single tryptophan mutants, 2 out of the 3 tryptophan residues were mutated to phenylalanine using the QuickChange II site-directed
mutagenesis kit (Strategene, La Jolla, CA). Three different mutants were generated using the following primers. Complementary primers are not shown. Altered nucleotides are indicated in bold.

W37 (W41/59F):

VIW59F 5’ GGCAGCAAGGCCCTTTAACAGCAGCACAGG 3’
VIW41F 5’ GCTGGGGCTCGCTGTTTAGCGGCATTAAAAATTTCG 3’

W41 (W37/59F):

VIW59F 5’ GGCAGCAAGGCCCTTTAACAGCAGCACAGG 3’
VIW37F 5’ ACAAGGCCTTCAGCTTTGGCTCGCTGTGGAGG 3’

W59 (W37/41F):

VIW3741F 5’ GCTTTGGCTCGCTGTTTAGCGGCATTAAAAATTTCG 3’

To obtain single tryptophan mutants in the 2nd, 3rd and 4th predicted helices, the 3 native tryptophans were first mutated to phenylalanine and various hydrophobic residues in these helices were then mutated to tryptophan using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The no tryptophan construct was generated using the following primers. Complementary primers are not shown. Altered nucleotides are indicated in bold.

VIW59F 5’ GGCAGCAAGGCCCTTTAACAGCAGCACAGG 3’
VIW41F 5’ GCTGGGGCTCGCTGTTTAGCGGCATTAAAAATTTCG 3’
VIW37F 5’ ACAAGGCCTTCAGCTTTGGCTCGCTGTGGAGG 3’

The single tryptophan mutants were generated using the following primers:

L67W 5’ ACAGGCCAGATGGAGGGATAGTTGAAAG 3’
V80W 5’ AAAATTTCAACAAAAAGTGTAAGATGGCCTG 3’
Generating the L40Q, W37L40Q, W41L40Q, and W59L40Q mutants

To generate L40Q single tryptophan mutants, the L40Q mutation was introduced in the single tryptophan mutant (W37, W41 and W59) constructs previously described using the QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Three different mutants were generated using the following primers. Complementary primers are not shown. Altered nucleotides are indicated in bold.

VIW37FL40Q 5′ GC\text{**TT**}TGGCTCGCAGTGGAGC\text{**G**}GCATTAAAAATTTCG 3′

VIW37FL40QW41F 5′ GC\text{**TT**}TGGCTCGCAGTT\text{**T**}AGCGGCATTAAAAATTTTCG 3′

VIW41FL40Q 5′ GCTGGGGCTCGCAGTT\text{**T**}AGCGGCATTAAAAATTTTCG 3′

The single point mutation L40Q was generated using the following primers:

L40Q 5′ GCTGGGGCTCGCAG\text{**T**}GGAGC\text{**G**}GCATTAAAAATTTTCG 3′

Generating the K45E, K52E, K45EK52E, W37AK45E and W37AK52E mutants:

The K45E and the K52E mutations were introduced into pET15bVIhis, containing the mature form of pVI, using the following primers. Complementary primers are not shown. Altered nucleotides are indicated in bold.

K45E 5′ GTGGAGCGGCATT\text{**G**}AAATTTTCGGTCCAC 3′

K52E 5′ CGGTTCCACCGTT\text{**G**}AAACTATGGCAGCA 3′

To generate the W37AK45E and W37AK52E the primers listed above were used to insert the K45E and the K52E mutations in the pVIhis-W37A. The double lysine mutant was generated by inserting the K45E mutation into the K52E-pVI plasmid.

Generating the mutants with altered topology
The S1LT15L, S1QG8QS14Q and S1IG3IF12S were generated by introducing the mutations as described above in the pVI-his plasmid with the following primers. Altered nucleotides are indicated in bold.

**S1L**
5’ AGCGGTGGCGCCTTCCTCTTGGGGCTCGCTGTGG 3’

**T15L**
5’ AAAAAATTTCGTTCCTCCTCGTTAAGAACTCTGGCAGC 3’

**G8QS14Q**
5’ GCTGTGGAAGCCAGATTAAAAATTTTCGGTCAGACCGTTAAGAA 3’

**S1Q**
5’ AGCGGTGGCGCTCCTCAAGGTTGGGCTCGCTGTGG 3’

**F12S**
5’ CATTTTTAATTTCGGTTCCACCG 3’

**S1IG3I**
5’ GCCTTCATCTGGGCGACCTGCTGTGG 3’

The mutations were confirmed by sequencing, and the plasmids were used to overexpress proteins in *E. coli*.

**RECOMBINANT PROTEIN PURIFICATION**

Recombinant proteins were expressed in BL21(DE3) cells. Cultures inoculated with overnight culture were grown at 37 °C, until they reached an optical density at 600 nm of 1.0. The NaCl concentration was then increased by adding an additional 0.9 g NaCl/L, and protein expression was induced by adding 1 mM IPTG (isopropyl-α-D-thiogalactopyranoside) for 1 h. Cells were pelleted, resuspended in cell lysis buffer (1% Triton X-100, 25mM phosphate, 150 mM NaCl pH 7.5, 0.5 mg/ml lysozyme, 0.1 mg/ml DNAsel and 1 mM PMSF (phenylmethylsulfonylfluoride)), and soluble protein was isolated by centrifugation at 13,000×g for 15 min at 4 °C. Recombinant proteins were purified with Talon cobalt resin using the manufacturer’s protocol (BD Biosciences). Proteins were extensively dialyzed into 25mM Phosphate, 150 mM NaCl, and 10% (v/v)
glycerol pH 7.5 before flash freezing in liquid nitrogen. Aliquots were stored at −80 °C until use.

**LIPIDS**

1-Palmitoyl,2-oleoylphosphatidylcholine (POPC), 1-palmitoyl, 2-oleoylphosphatidylserine (POPS), 1-palmitoyl,2-oleoylphosphatidylethanolamine (POPE), 1-palmitoyl-2-stearoyl(6′,7′-dibromo)-snglycero-3-phosphocholine 1-palmitoyl-2-stearoyl (9′,10′-dibromo)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-stearoyl(11′,12′-dibromo)-sn-glycero-3-phosphocholine, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (DOGS- NTA-Ni) were purchased from Avanti Polar Lipids. α-Lysophosphatidylcholine (lysoPC) , N-fluoresceinyl-1, 2-ndihexadecylphosphatidylethanolamine (FITC-DHPE) and α-Lysophosphatidylcholine (lysoPC) were from Sigma, and N-fluoresceinyl-1, 2-sndihexadecylphosphatidylethanolamine (FITC-DHPE) were obtained from Invitrogen.

**pVI IN VITRO MEMBRANE LYTIC ACTIVITY**

Liposomes were prepare as previously described (Blumenthal, Seth et al. 1986) by mixing POPC and POPS (75:25 mol%), or POPC, POPS and DOGS-NTA-Ni (70:25:5 mol%) in chloroform. A thin lipid film was then generated on a glass tube by evaporating the chloroform with a stream of nitrogen gas. Residual chloroform was removed by placing the tube under vacuum for 2 hours. The film was rehydrated with 100 mM sulforhodamine B (SulfoB) (Molecular Probes) Liposomes containing entrapped SulfoB were separated from free dye using a Sephadex G-75 column, pre-equilibrated
with 25mMHEPES, 150mMNaCl bufferpH7.5 (HBS). The liposome concentration was determined using a phosphate assay as previously described (Fiske and Subbarrow, 1925). Membrane lytic activity of recombinant pVI was determined by measuring SulfoB fluorescence dequenching upon release from liposomes. The liposomes were diluted in HBS to a final concentration of 10 μM. Different concentrations of pVI were then added to the liposomes and incubated for 20 min at 37 °C. Fluorescence intensity was measured using the Cary Eclipse fluorescence spectrophotometer (Varian) with the excitation wavelength of 575 nm and emission wavelength of 590 nm. One hundred percent dye release was determined by adding Triton X-100 to the liposomes at a final concentration of 0.5% (w/v). The percentage of SulfoB released was calculated using the formula:

\[
\text{% SulfoB released} = 100 \times \frac{F_{\text{meas}} - F_0}{F_{\text{tx100}} - F_0},
\]

where \( F_{\text{meas}} \) is the maximum fluorescence intensity measured, \( F_0 \) is fluorescence intensity in absence of protein, and \( F_{\text{tx100}} \) is the fluorescence intensity in the presence of 0.5% Triton X-100.

**PVI MEMBRANE BINDING**

Protein VI contains 4 tryptophans at residues 37, 41, 59 and 229. Binding to liposomes was assessed by monitoring changes in pVI intrinsic tryptophan fluorescence upon titration with increasing amounts of liposomes (POPC:POPS 75:25 mol% or POPC:POPS:DOGS-NTA-Ni 70:25:5 mol%). This approach is routinely used for monitoring interactions between proteins and ligands, membranes or other proteins and relies on the assumption that the fractional spectral change in tryptophan fluorescence correlates directly with the amount of protein bound to its substrate (Eftink and Ramsay 1997). PVI fluorescence emission spectra from 300–480 nm in HBS and at 37 °C was obtained by selective excitation of tryptophan at 295 nm. Increasing amounts of
liposomes were added to pVI with mixing for 3 min and additional spectra were obtained. Spectra of buffer or an equivalent amount of liposomes alone were subtracted from the spectra of each protein/lipid mixture to obtain corrected spectra. The spectral center of mass, $I_\lambda$, for the emission spectra were determined using the Carey Eclipse software. Assuming that this spectral change in tryptophan fluorescence correlates with the amount of protein bound, the fractional saturation of binding sites, $\theta$, was calculated using the following equation: 

$$\theta = \frac{I_\lambda(\text{obs}) - I_\lambda(0)}{I_\lambda(\text{max}) - I_\lambda(0)},$$

where $I_\lambda(\text{obs})$ is the spectral center of mass for each protein/lipid ratio and $I_\lambda(0)$ and $I_\lambda(\text{max})$ is the spectral center of mass for protein alone and the protein in the presence of saturating amounts of liposomes, respectively. Plotting $\theta$ versus protein/lipid molar ratios yielded the resulting binding isotherms.

**ANALYSIS OF PVI MEMBRANE PENETRATION USING GIANT LIPID VESICLES (GLV)**

GLV were generated as described previously (Akashi, Miyata et al. 1996) by mixing POPC, POPS and FITC-DHPE (70:25:5 mol ratio) or POPC, POPS, DOG-NTA-Ni, FITC-DHPE (65:25:5:5 mol ratio) in chloroform. A thin lipid film was then generated on a glass tube by evaporating the chloroform with a stream of nitrogen gas. Residual chloroform was removed by placing the tube under vacuum for 6 h. The lipid film was then prehydrated with a stream of water saturated nitrogen gas for 25 min, followed by rehydration in 6 ml of HBS containing 0.1 M sucrose. The tube was then sealed with parafilm and incubated overnight at 37 °C. GLVs were harvested as a flocculate near the top of the solution the next day and quantified by phosphate assay as described above. Typical preparations of GLVs are polydisperse with vesicle diameters.
ranging from 5 to 50 μm. To visualize pVI membrane lytic activity, recombinant pVI was incubated with GLV at a 1:100 (lipid:protein) ratio in HBS with 0.1 M glucose, on a glass slide. After 15 min the samples were analyzed by using an epifluorescence microscope.

**TRYPTOPHAN DEPTH OF MEMBRANE PENETRATION**

Quenching of tryptophan fluorescence by brominated phospholipids was used to determine the depth of tryptophan penetration into the lipid bilayer (Chattopadhyay and London 1987; Ladokhin, Selsted et al. 1997). Liposomes containing 25 mol% POPS, 25 mol% POPC and 50 mol% brominated phosphatidylcholine (Br2-PC) were made as described above. Recombinant pVI single tryptophan mutants were incubated for 10 min at 37 °C with brominated liposomes at a 1:100 (protein:lipid) ratio in HBS pH 7.5. The intensity of tryptophan fluorescence was measured at 325 nm upon excitation at 295 nm. The differences in quenching tryptophan fluorescence by the (6,7)-, (9,10)-, (11,12)-Br2-PC was used to calculate the location of the residue in the bilayer using two methods: the parallax method (Chattopadhyay and London 1987) and distribution analysis (Ladokhin, Selsted et al. 1997). In the parallax method, the depth of the tryptophan residue was calculated using the formula:

\[
Z_{cf} = L_{cl} + \left[\frac{-\ln(F_1/F_2)}{\pi C - L_{21}^2}/2L_{21}\right]
\]

\(L_{cl}\) is the distance of the shallow quencher from the center of the bilayer, \(L_{cl}\) is the distance between the shallow and deep quencher, \(F_1\) is the fluorescence intensity in the presence of the shallow quencher, \(F_2\) is the fluorescence intensity in the presence of the deep quencher, and \(C\) is the concentration of quencher in molecules/Å². In the
distribution analysis the depth of tryptophan residue was calculated by fitting the data to the equation:

\[
\ln \left( \frac{F_0}{F_h} \right) \times c(h) = [S/(\sigma 2\pi)^{1/2}] \times \exp \left[ -\frac{(h-h_m)^2}{2\sigma^2} \right]
\]

where \( F_0 \) represents the fluorescence intensity in the absence of the brominated phospholipids, \( F_h \) is the intensity measured as a function of the distance from the center of the lipid bilayer to the quencher \( h \), \( c(h) \) is the concentration of the different quenchers, \( S \) is the area under the curve (measurement of quenching efficiency), \( \sigma \) is the dispersion (a measure of the distribution of the depth in the bilayer), \( h_m \) is the most probable position of the fluorophore in the membrane, and \( h \) is the average bromine distances from the center of the bilayer, based on X-ray diffraction and taken to be 10.8, 8.3, and 6.3 Å for (6,7)-, (9,10)- and (11,12)-Br2-PC respectively. When equal concentrations of the Br-lipids are used, the \( c(h) \) value is unity.

**IMMUNOFLUORESCENCE MICROSCOPY**

A total of \( 1 \times 10^5 \) HeLa cells were plated on glass coverslips. The next day the cells were infected with \( 3 \times 10^4 \) vp/cell of Ad5gfp or tsI or with 3000 pfu/cell of reovirus on ice for 1 hour after which the cells were shifted at 37°C to allow for virus internalization. At different times post virus internalization the cells were washed with PBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 min. The cells were then permeabilized with 0.5% (v/v) Triton X-100 (Sigma-Aldrich) for 2 min, washed with PBS and blocked for 1 hr in 10% FBS. Staining with specific mono- or polyclonal antibodies was done in 10% FBS for 1 hr. Galectin-3 was immunostained using a specific mouse monoclonal anti-Galectin-3 antibody (BD Transduction 556904) used at 1/100
dilution. Reovirus was immunostained using polyclonal reovirus antibody used at 1/200 dilution, and pVI was detected with anti-pVI serum at 1/1000 dilution. For the K45EK52E galectin 3 experiment, virions were visualized using DyLight 488 labeled anti-hexon antibody at a 1/1000 dilution. Secondary Alexa Fluor 568-conjugated and DyLight 649-conjugated antibodies were used to visualize galectin-3 and pVI, respectively. Ad5gfp, and ts1 were prelabeled with Dylight 488 NHS-Ester Fluorophores, according to the manufacturer's protocol prior to use. Hoechst stain (ImmunoChemistry Technologies) was used to counterstain nuclei before coverslips were mounted on glass slides with ProLong Gold (Invitrogen). Z-stack images were acquired with a DeltaVision microscope (Applied Precision) using a CoolSnap HQ digital camera (Photometrics) with a 1.4-numerical aperture (NA) 100× objective lens, and deconvolved with SoftWorx deconvolution software (Applied Precision). Images were assembled using IMARIS and ImageJ softwares. The percent galectin-3 colocalizing with virus and/or pVI was determined using IMARIS software by first quantifying galectin 3 puncta as any punctuate signal in the TRITC channel above background. Any signal above background in the virus (GFP) or pVI (Cy5) channel was counted as galectin 3 association. A similar approach was used to determine the pVI percentage colocalizing with virus.
CHAPTER III
EXPERIMENTAL RESULTS

THE MECHANISM OF pVI IN VITRO MEMBRANE LYTIC ACTIVITY

The N-terminal amphipathic α-helix is important for pVI membrane lytic activity. Currently there is no structural information regarding pVI. Secondary structure predictions suggest that the N-terminal 80 residues of mature pVI (residues 34–114) form a stable α-helical domain (Fig. 4A) (Wiethoff, Wodrich et al. 2005). The C-terminal domain is mostly disordered and contains a mixture of α-helical and β-structures. The membrane lytic activity of pVI was previously shown to be pH independent and strongly dependent on an N-terminus amphipathic α-helix (Wiethoff, Wodrich et al. 2005). To examine the importance of specific pVI domains for the observed membrane lytic activity, I and others in the lab had generated truncated versions of pVI. I then measured the ability of these different proteins to disrupt membranes, by monitoring the release of an entrapped fluorophore (SulfoB) from the interior of liposomal membranes (Maier, Galan et al. 2010) (Fig 4B.). Wild type pVI caused a dose dependent release of SulfoB from liposomes. The truncated 80 residue N-terminal domain (VI114Δ) had similar membrane lytic activity as the full length pVI, suggesting that the C-terminal 125 residues contribute significantly less to this process. Equivalent membrane lytic activity was also observed for a 24 residue peptide corresponding to the amphipathic α-helix.
Fig. 4. Adenovirus protein VI disruption of and binding to liposomes.

A) The predicted secondary structure of pVI with α-helices (cylinders) β-sheets (arrows) and unstructured (lines) secondary structures are displayed. Recombinant forms of pre-pVI, pVI, VIΔ54, VI114Δ and VI34–54 were used in studies of membrane binding and permeabilization. B) PVI membrane lytic activity was measured by quantifying the release of SulfoB from liposomes after treatment with increasing concentrations of the different pVI constructs. C) Membrane binding of the different pVI constructs. Increasing lipid concentrations were added to pVI constructs and changes in tryptophan fluorescence were used to determine the fractional saturation (θ) of pVI binding capacity as described in the Materials and methods. Error bars represent the standard error of the mean for a minimum of 3 replicates (■) Protein VI (◆) VI34–54 (▲) VIΔ54 (○) VI114Δ. (△) BSA
(residues 34–54) with an additional C-terminal tetralysine tag to enhance aqueous solubility, suggesting that this helix was sufficient for \textit{in vitro} membrane disruption. Removing the amphipathic α-helix (VIΔ54) greatly reduces membrane lytic activity, requiring \(~400\)-fold higher protein concentrations to elicit similar SulfoB release as the mature form of the protein. Since equivalent amounts of the irrelevant protein, bovine serum albumin, do not induce significant SulfoB release, our data with the truncated VIΔ54 protein suggest that additional residues within pVI may also facilitate membrane interactions.

To further assess the role of the amphipathic α-helix in pVI membrane disruption, I examined the relative affinity of each protein construct and peptide for the same liposomal membranes (Maier, Galan et al. 2010). The interaction between pVI and liposomes was determined using changes in intrinsic tryptophan fluorescence upon membrane association. By assuming that the relative change in tryptophan fluorescence upon binding liposomes was directly related to the amount of membrane bound pVI, I generated binding isotherms by plotting the fractional saturation of binding sites versus increasing lipid concentrations (Fig 4C.). While pVI, VI114Δ and VI34–54 all possessed similar affinities as evidenced by dissociation constants between 2–4 \(\mu\)M, VIΔ54 membrane affinity was \(~600\)-fold lower. These results suggest that the N-terminal amphipathic α-helix is important for membrane binding. Additionally, there is a strong correlation between the affinity of pVI for membranes and the \textit{in vitro} membrane lytic activity.
**PVI amphipathic α-helix membrane topology**

The pVI N-terminal amphipathic α-helix membrane topology is also of interest and could provide considerable insight into the mechanism of membrane disruption. Using the well documented distance-dependent quenching of tryptophan fluorescence by bromine atoms (Markello, Zlotnick et al. 1985), I determined the depth of the conserved W37, W41 and W59 residues in lipid membranes. These bilayers contained brominated lipid with bromine atoms covalently attached at specific positions on the lipid alkyl chains. Single tryptophan mutants of the 80 residue VI114Δ construct were made by mutating 2 out of the 3 tryptophans to phenylalanine since phenylalanine residues do not possess the fluorescent properties of tryptophan but have membrane binding properties most similar to tryptophan (Wimley and White 1996). Using this approach W37 and W41 were found to be 9.6 and 10.6 Å from the center of the bilayer by parallax analysis (Fig. 5A.) (Maier, Galan et al. 2010). The distance from the center of the bilayer \( Z_{cf} \), was determined both via parallax and distribution analyses (Chattopadhyay and London 1987; Ladokhin, Selsted et al. 1997). Interestingly, W59, which is outside the predicted N-terminal amphipathic α-helix was also found to interact with the membrane, being 10.3 Å from the center of the bilayer. As a control for the assay, I found that the single tryptophan of melittin was 10.9 Å from the center of the bilayer, which is in good agreement with previously published reports which positions this tryptophan 10.8 Å from the bilayer center (Ghosh et al., 1997). Since W37 and W41 would be ~6 Å apart in an α-helix, yet they are positioned at depths which differ by only ~1 Å in the membrane, it is likely that this helix is positioned in an oblique orientation relative to the membrane surface and does not traverse the apolar region of the lipid bilayer. The angle between
A.  

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<th>Zcf (Å)</th>
<th>Parallax</th>
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<td>W37</td>
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<td>W41</td>
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<td>W59</td>
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B.  

The distances of W37, W41 and W59 from the center of the lipid bilayer (Z_{cf}) were calculated by distance-dependent quenching of tryptophan fluorescence. The Zcf values were calculated for each tryptophan residue using Parallax and Distribution analyses. (B) Residues 34–54 of Ad5 protein VI were modeled as an α-helix using swisspdb viewer and overlaid on the three dimensional structure of POPC lipids in the L_{ca} phase (POPC128a.pdb, downloaded from http://people.ucalgary.ca/tieleman/download.html) such that the distance of W37 and W41 (green) were positioned at the distance from the center of the bilayer (Z_{cf}) presented in A.

**Fig 5.** PVI amphipathic α-helix membrane topology. (A). The distances of W37, W41 and W59 from the center of the lipid bilayer (Z_{cf}) were calculated by distance-dependent quenching of tryptophan fluorescence. The Zcf values were calculated for each tryptophan residue using Parallax and Distribution analyses. (B) Residues 34–54 of Ad5 protein VI were modeled as an α-helix using swisspdb viewer and overlaid on the three dimensional structure of POPC lipids in the L_{ca} phase (POPC128a.pdb, downloaded from http://people.ucalgary.ca/tieleman/download.html) such that the distance of W37 and W41 (green) were positioned at the distance from the center of the bilayer (Z_{cf}) presented in A.
W37 and W41 would correspond to 10° from the bilayer–water interface. A model for the orientation of this helix on membranes is shown in Fig. 5B.

_PVI fragments membranes by inducing positive membrane curvature_

Since the above data suggest a superficial oblique orientation for the N-terminal α-helix, the key determinant of pVI membrane lytic activity, this protein may be inducing curvature stress in membranes leading to membrane disruption. To examine this possibility, I determined the effects of pVI on membrane morphology using giant fluorescent lipid vesicles (GLV). GLV membranes were labeled with 5 mol% fluoresceinylated lipid and observed by epifluorescence microscopy (Maier, Galan et al. 2010). Vesicles appear 5–50 μm in diameter and this morphology is unchanged upon addition of PBS (Fig. 6, top). Vesicles incubated with VIΔ54 (1:100 protein: lipid) appeared similar to PBS treated vesicles (Fig. 6, middle). Upon addition of pVI VI114Δ to GLVs at a protein:lipid molar ratio of 1:100, the vesicles were fragmented into smaller structures with an increased radius of curvature (Fig. 6, bottom). Of note, reorganization of lipid membranes into tubular structures (arrows) appears to result from pVI addition. This data supports the hypothesis that pVI fragments membranes by inducing significant membrane curvature stress.

The observation that pVI fragments membranes by inducing membrane curvature is in line with one proposed mechanism of membrane disruption by cationic antimicrobial peptides (Brogden 2005). These peptides have been shown to fragment membranes either by inducing positive membrane curvature or negative membrane curvature (Campelo, McMahon et al. 2008) (Fig. 7) To determine the type of membrane curvature pVI induces, Debra Galan in our lab prepared liposomes entrapping SulfoB and containing
increasing amounts of lipids which have a preference to adopt either positively (lysophosphatidylcholine, lysoPC) or negatively (phosphatidylethanolamine PE) curved membranes. If pVI membrane lytic activity involves the induction of positive membrane curvature, then we would expect the presence of lysoPC to enhance pVI membrane lytic activity while PE would inhibit pVI membrane lytic activity. Since she observed exactly these effects of lysoPC and PE on pVI membrane lytic activity, we concluded that pVI disrupts membranes by inducing positive membrane curvature (Maier, Galan et al. 2010).

In further support of this observation, the primary sequence of the N-terminal amphipathic α-helix of pVI fits recently identified criteria for helices which induce or associate with positively curved membranes (Drin, Casella et al. 2007). These helices typically possess a relatively small hydrophobic surface, few if any charged residues, and a hydrophilic surface which is composed mostly of short hydrophilic amino acids such as serine, threonine or glycine.

Although results above demonstrate that pVI can fragment lipid membranes by inducing positive membrane curvature, they do not rule out the possibility that at lower pVI to lipid ratios, pore structures are formed. In fact, the toroidal pore model for peptide induced membrane permeabilization involves amphipathic α-helix induced positive curvature in membrane lipids such that the pore channel is lined both by peptide and lipid headgroups (Epand and Vogel 1999; Brogden 2005). To determine whether pVI induced size selective pores in lipid membranes, Debra compared the release of the 0.5 kDa SulfoB and 70 kDa FITC-dextran from liposomes incubated with increasing amounts of
Fig. 6. Protein VI fragments giant lipid vesicles.

Fluorescein-DHPE labeled giant compared to those previously described for lipid vesicles were incubated with PBS (TOP), VIΔ54 (MIDDLE) or protein VI (BOTTOM) for 15 min before visualization by epifluorescence microscopy. Protein–lipid ratios were 1:100 (mol/mol). Arrows indicate tubular lipid structures formed in the presence of protein VI.
Fig. 7. Membrane curvature induction by amphipathic α-helices.

Amphipathic α-helices that have a shallow membrane topology induce positive membrane curvature by bending the helix containing monolayer away from the protein. Negative curvature is induced by helices with a deeper membrane penetration, and this is mediated by bending the protein containing monolayer towards the protein.
pVI at an overall lower ratio of protein to lipid. Previous studies have demonstrated that 70 kDa FITC dextran has a hydrodynamic radius of 100 Å (Bohrer, Deen et al. 1979) and is unable to diffuse through membrane channels less than 50 Å in diameter (Ladokhin, Selsted et al. 1997). If a size selective pore were formed at these lower ratios of pVI to lipid, then we would expect that a greater release of sulfoB compared to FITC-dextran would be observed. Since Debra observed comparable degrees of fluorophore release from vesicles by pVI, we concluded that pVI does not form pores capable of discriminating between 6 and 100 Å diameter molecules (Maier, Galan et al. 2010).

To summarize my in vitro data characterizing the mechanism used by pVI to lyse membranes, I propose the following model described in Fig 8. An N-terminal amphipathic α-helix in pVI is necessary and sufficient for membrane lytic activity. This helix binds membranes with an oblique orientation and a shallow membrane insertion, inducing positive membrane curvature stress. This curvature stress results in membrane fragmentation.

**PVI DOMAINS IMPORTANT FOR POSITIVE MEMBRANE CURVATURE INDUCTION**

Data described above suggests that pVI induces positive membrane curvature, however the pVI-lipid interactions necessary for generating this type of curvature are not known. I was first interested in understanding what pVI domains are involved in this process. Three possibilities exist: the amphipathic α-helix alone induces the observed curvature, the helix cooperates with additional residues in this process, or the helix merely anchors pVI to membranes while the rest of the protein bends membranes.
Fig. 8. Proposed model for pVI endosomal membrane lysis.

PVI is released from the capsid interior during virus uncoating in the endosome. An amphipathic α-helix in pVI (yellow) anchors the protein to membranes by binding the lipid bilayer with an oblique shallow orientation. This binding fragments the endosomal membrane by inducing positive membrane curvature stress.
VIΔ54 has increased membrane lytic activity with DOGS–NTA–Ni liposomes

Although the N-terminal amphipathic α-helix in pVI is largely responsible for membrane lytic activity, removing this helix does not completely abrogate the protein’s lytic activity. A construct that lacks this helix, VIΔ54, can still disrupt membranes but with much lower efficiency (Maier, Galan et al. 2010). This decreased lytic activity can be either due to inefficient membrane binding, or due to an inability to induce positive membrane curvature. Therefore, if residues in VIΔ54 participate in curvature induction, then enhancing this construct’s membrane affinity should increase lytic activity. We artificially enhanced VIΔ54 membrane affinity by appending an N-terminal 6×His-tagged tag to the recombinant protein and using liposomes containing 5 mol% of the Nickel-chelating phospholipid, DOGS–NTA–Ni. The binding of His-tagged pVI constructs to liposomes was assessed as previously described using changes in intrinsic tryptophan fluorescence upon association to membranes (Maier, Galan et al. 2010). Compared to untagged VIΔ54, binding of 6×His-tagged VIΔ54 to DOGS–NTA–Ni containing liposomes displayed an apparent affinity much closer to that observed for pVI (Fig. 9A). Addition of a 6×His tag to pVI did not significantly enhance membrane affinity. To determine if this increased membrane binding correlates with increased membrane lytic activity, VIΔ54 was added to SulfoB-loaded DOGS–NTA–Ni liposomes and dye release was then measured. Addition of VIΔ54 to SulfoB-loaded DOGS–NTA–Ni liposomes resulted in increased membrane lytic activity compared to liposomes without the nickel-chelating lipid (Fig. 9B). To control for the influence of the N-terminal 6×His tag on observed membrane lytic activity, similar titrations with purified 6×His-tagged penton base were performed. Tagged penton base did not induce significant SulfoB release over
Fig. 9. The membrane affinity and lytic activity of VIA54 are enhanced by DOGS–NTA–Ni phospholipids. (A) Membrane binding of the different pVI constructs. Increasing lipid concentrations were added to pVI constructs and changes in tryptophan fluorescence were used to determine the fractional saturation (θ) of pVI binding capacity. (B) Protein VI membrane lytic activity was determined by measuring the release of SulfoB from liposomes after incubation with increasing protein concentrations. 6×His-pVI incubated with PC:PS:DOGS–NTA–Ni (70:25:5 mol%) (■) or PC:PS(70:25 mol% ) (□). 6×His-VIA54 incubated with PC:PS:DOGS–NTA–Ni (70:25:5 mol%) (Δ) or PC:PS (75:25 mol%) (▲). 6×His- penton base incubated with PC:PS:DOGS–NTA–Ni (70:25:5 mol%) (●).
the same concentration range, suggesting that the His-tag was not responsible for the enhanced membrane lytic activity of VIΔ54 on DOGS–NTA–Ni liposomes. These results suggest that while the N-terminal amphipathic α-helix greatly enhances pVI membrane affinity, the remainder of the protein can also contribute to efficient membrane lysis (Maier and Wiethoff). It is therefore possible that residues in VIΔ54 contribute to the induction of positive membrane curvature by pVI.

Mapping membrane interacting domains within pVI

An N-terminal 80 amino acid domain in pVI (residues 34–114) possesses 87% α-helical content as assessed by circular dichroism (Maier, Galan et al. 2010). This domain is predicted to contain 4 α-helices (Fig 4A.), and I have previously shown that the first amphipathic α-helix has an oblique membrane orientation. Since residues outside this N-terminal α-helix contribute to pVI lytic activity, I determined if additional predicted α-helices in this 80 residue domain associate with membranes. Helical wheel diagrams suggest that each of these putative α-helices have considerable amphipathy (Fig 10A). In addition, predictions of the free energy of interfacial membrane partitioning, ΔG_{if}, suggest that each of these helices would spontaneously associate with membranes although the magnitude of ΔG_{if} for these 3 additional α helices is considerably smaller than that for the N-terminal α-helix (Fig 10A).

To determine whether these α-helices interact with membranes, single tryptophan (Trp) mutations were introduced individually at 1 or 2 sites for each helix. These Trp residues replaced residues L67, L71, V80, L84 or V91 in the plasmid encoding a no Trp VI114Δ (the 3 native Trp residues were replaced by phenylalanine). The depth of Trp penetration into lipid bilayers was determined as described earlier by measuring
Fig 10. α-Helices in the N-terminal 80 residue domain of pVI interact with the membrane. Helical wheel diagrams of the different helices in the N-terminal 80 residue domain of pVI are shown, with the hydrophilic residues in white and hydrophobic residues in black. The residues in each helix mutated to tryptophan have been boxed. The free energy of partitioning into the lipid membrane interface, ΔGif, calculated using the Membrane Protein Explorer software are shown for each helix with units of kilojoules per mole. (B) Depth of protein VI tryptophan residues penetration into the membrane quenching of Trp fluorescence by brominated lipids.
To measure the depth of Trp membrane penetration, the single Trp mutants were incubated with PS:PC:PC–Br liposomes (25:25:50 mol%) at a 1:100 ratio (protein–lipid), and Trp fluorescence was then measured. Tryptophan fluorescence of each of these mutants was quenched upon mixing with brominated liposomes suggesting that these mutants interact with the lipid bilayer (Maier and Wiethoff). The distance from the center of the bilayer was calculated using both the parallax method and the distribution analysis (Fig 10B). Since the W67 and W71 residues in the 2nd helix, and W80 and W84 in the 3rd helix would be ~6 Å apart in an α-helix, yet they are positioned at depths which differ by only ~1 Å in the membrane, it is likely that the 2nd and 3rd helices are positioned in an oblique orientation relative to the membrane surface. These helices do not traverse the apolar region of the lipid bilayer similar to the orientation reported for the N-terminal amphipathic α-helix.

*Induction of positive membrane curvature by the N-terminal amphipathic α-helix and VIΔ54*

The shallow and oblique membrane orientation observed with the α-helices within the N-terminal 80 residue domain is indicative of a protein that induces positive membrane curvature stress (Drin et al., 2007; Epand and Epand, 2000; Zimmerberg and Kozlov, 2006). Therefore, I hypothesized that these helices are contributing to the membrane curvature induced with full length pVI. To determine this contribution, I examined the influence lipids with a propensity to adopt positive (lysoPC) or negative (POPE) membrane curvature have on the membrane lytic activity of the N-terminal amphipathic α-helix or VIΔ54. If the membrane lytic activity of either the N-terminal amphipathic α-helix or VIΔ54 involves the induction of positive membrane curvature,
then I would expect the presence of lysoPC to enhance membrane lytic activity while POPE would inhibit membrane lytic activity. Similar to previous observations with pVI, the N-terminal amphipathic α-helix membrane lytic activity is significantly enhanced in the presence of increasing amounts of lysoPC while this activity is reduced in the presence of POPE (Fig. 11A). The membrane lytic activity of VIΔ54 is much less influenced by the inclusion of increasing amounts of lysoPC in liposomes and only slightly influenced by the inclusion of POPE (Fig. 11B). These data suggest that the residues 54–114 contribute more significantly to protein VI induction of positive membrane curvature than the rest of the protein.

The N-terminal amphipathic α-helix and VIΔ54 cooperate to induce membrane tubule formation

Since the amphipathic α-helix and residues in VIΔ54 can induce membrane curvature, I wanted to determine the latter’s contribution to pVI membrane lysis. As mentioned earlier, pVI membrane lytic activity involves the fragmentation of target membranes (Wiethoff, Wodrich et al. 2005; Maier, Galan et al. 2010). When added to fluorescently labeled giant lipid vesicles (GLV), this fragmentation also leads to the formation of tubular structures which likely possess significant membrane curvature stress. It was also shown that the ability of protein VI to induce tubule formation requires only the N-terminal 80 residues of pVI (Maier, Galan et al. 2010). To determine what domains in pVI are responsible for tubule formation I investigated the effects of N-terminal amphipathic α-helix and VIΔ54 on GLV morphology. The GLV membranes with or without 5 mol% DOGS–NTA–Ni were labeled with 5 mol% fluoresceinylated lipid and observed by epifluorescence microscopy. Vesicles appear 5–50 μm in
Fig. 11. The amphipathic α-helix peptide and VIΔ54 induce positive membrane curvature. Increasing concentrations of VI34–54 (A) or VIΔ54 (B) were incubated with POPC:POPS (75:25 mol%) (A) or POPC:POPS:DOGS–NTA–Ni (70:25:5 mol%) (B). * indicates liposomes entrapping SulfoB and in which some POPC was replaced with 5 (■) or 10 (□) mol% lysoPC or 5 (▲) or 25 (Δ) mol% of POPE. The % SulfoB released was determined as described in the Material and Methods section.
diameter and this morphology is unchanged upon addition of PBS (Fig. 10A). The addition of full length pVI to GLVs at a protein:lipid molar ratio of 1:100 fragments vesicles into smaller structures, (Fig. 10B) including tubules (arrows). However, although the N-terminal amphipathic α-helix is able to disrupt these GLVs, it fails to form the highly curved tubular structures observed with full length protein (Fig. 10C). Incubating VIΔ54 with GLVs containing DOGS–NTA–Ni also results in membrane fragmentation without tubulation (Fig. 10D). These data suggest that although the N-terminal amphipathic α-helix is sufficient to induce positive membrane curvature and lyse membranes, the highly curved membrane tubules observed upon pVI membrane lysis require additional elements within residues 54–114 of pVI to interact with membranes. However, at this time the significance of pVI membrane tubulation during Ad entry is not understood.

**PVI AMPHIPATHIC α-HELIX MEMBRANE TOPOLOGY IS IMPORTANT FOR POSITIVE MEMBRANE CURVATURE INDUCTION**

Once I determined how the different domains in pVI contribute to positive membrane curvature induction and tubule formation, I was interested in understanding what additional protein-lipid interactions are important for this process. Studies with amphipathic α-helical peptides have shown that membrane topology dictates the type of curvature these peptides generate. Peptides that penetrate deeper into the membrane induce negative membrane curvature, while those that have a shallow membrane insertion induce positive curvature (Fig. 7) Protein VI amphipathic α-helix has a shallow bilayer insertion is necessary for producing positively curved membranes.
Fig. 12. P VI domains involved in membrane tubule formation.

Fluorescein-DHPE labeled POPC:POPS (75:25 mol%) (A–C) or POPC:POPS:DOGS–NTA–Ni (70:25:5 mol%) (D). Giant lipid vesicles were incubated with PBS (A), pVI (B), VI_{34-54} (C) or VIΔ54 (D) for 15 min. Tubule formation was visualized by epifluorescence microscopy. Arrows indicate tubular lipid structures formed in the presence of pVI.
To determine if amphipathic α-helix membrane topology dictates the type of membrane curvature pVI generates, we altered the depth of membrane penetration by two different approaches. In the first approach we increased the helix hydrophobic sector by mutating hydrophilic residues at the polar/nonpolar interface to hydrophobic residues (S1L/T15L mutant) (Kiyota, Lee et al. 1996). The other approach we used was to widen the amphipathic α-helix polar sector by mutating polar residues that have smaller side chains to polar residues with longer side chains (S1Q/G8Q/S14Q mutant). This allows the helix to penetrate deeper into the membrane due to the “snorkelling” effect of the longer side chains of Glutamine (Gln) residues. The bulk of the van der Waals surface area of these residues is hydrophobic, and can insert into the membrane while the polar moiety can extend into the aqueous environment. The presence of the longer hydrocarbon side chain of Gln at the polar/nonpolar interface, allows the peptide to penetrate deeper into the membrane (Mishra and Palgunachari 1996; Zelezetsky, Pag et al. 2005).

To determine if these mutations in the amphipathic α-helix decrease pVI positive curvature induction, I measured membrane lytic activity in the presence of lysoPC (lipids that favour positive membrane curvature) or PE (lipids that favour negative membrane curvature) as described earlier. If amphipathic α-helix membrane topology dictates the type of curvature pVI induces, then a pVI mutant that penetrates deeper in the lipid bilayer should have decreased membrane lytic activity in the presence of lysoPC lipids, and increased activity in the presence of PE lipids. The results indicate that while the S1Q/G8Q/S14Q mutant is not impaired in positive curvature induction, the S1L/T15L mutant has diminished capacity to induce positively curved membranes (Fig 13).
Fig 13. Membrane curvature induction by pVI mutants with increased hydrophobic sector. (A) Liposomes containing different concentrations of lyso-PC or PE, and entrapping SulfoB are made. Increasing concentrations of (■) WT (○)S1Q/G8Q/S14Q (●)S1L/T15L are incubated with 10uM of these liposomes, and SulfoB release is then measured. A positive on this graph is indicative of a protein that induces positive curvature, while a negative slope correlates with a protein that induces negative curvature. (B) Helical wheel diagrams S1Q/G8Q/S14Q and S1L/T15L mutants depicting the altered nucleotides. Hydrophobic sector is the area under the lines.
Studies with fusion peptides have shown that a tilted helix membrane topology is necessary to induce negative curvature and subsequent fusion (Epand, Epand et al. 2001). The tilt angle of the fusion peptide in its inactive state (at neutral pH) is 23°, and in its active form (acidic pH) is 38° (Han, Bushweller et al. 2001). The deeper membrane penetration observed at lower pH is necessary for negative curvature induction (Lai and Tamm; Epand, Epand et al. 2001; Lai, Park et al. 2006). My in vitro studies suggest that pVI has an oblique membrane topology, with a tilt angle of 10°, however it is not known if this orientation is necessary for producing positive membrane curvature. To change the amphipathic α-helix membrane orientation, I introduced mutations that resulted in a more hydrophobic N terminus and a less hydrophobic C terminus (S1I/G3I/F12S). This would allow the N terminus to penetrate deeper in the membrane while the C terminus of the amphipathic α-helix would have a shallow membrane insertion, thereby tilting the helix.

Once I generated this mutant protein I determined its ability to induce positive membrane curvature as described earlier. If pVI amphipathic α-helix membrane topology dictates the induction of positive membrane curvature, then a mutant that has a tilted membrane orientation should have a defect in inducing this type of curvature. As a control I used the influenza fusion peptide which is known to induce negative membrane curvature. The results indicate that the tilted helix mutant has a defect in inducing positive curvature (Fig 14.). The same results were also observed with a peptide that contains the S1I/G3I/F12S mutant helix. Taken together these data suggest that the orientation of the amphipathic α-helix on membranes is important for positive membrane curvature induction.
Fig. 14. Membrane curvature induction by tilted helix mutant pVI. (A) Liposomes containing different concentrations of lyso-PC or PE, and entrapping SulfoB are made. Increasing concentrations of (■) WT (×) Influenza FP (△) S1I/G3I/F12S protein and (▲) S1IG3IF12S peptide are incubated with 10uM of these liposomes, and SulfoB release is then measured. The slope for each line is shown in the same colour as the line. A positive on this graph is indicative of a protein that induces positive curvature, while a negative slope correlates with a protein that induces negative curvature (B) S1IG3IF12S helical wheel diagram indicating the altered residues
My *in vitro* data suggests that pVI amphipathic α-helix is the key determinant of pVI membrane binding and subsequent lysis. However the role of this helix in Ad endosomal escape is not known. I was therefore interested in understanding what helix-membrane interactions are important for Ad endosomal escape. To address this question, I had to first determine what residues in the amphipathic α-helix are important for *in vitro* membrane binding. Protein attachment to membranes can be mediated by electrostatic interactions between positive residues on the protein and the negatively charged lipid head groups, as well as interaction between hydrophobic residues and the hydrocarbon core of the bilayer. Preliminary data in our lab indicates that mutations in the amphipathic α-helix that affect the hydrocarbon sector thickness (W37A,W41A) decrease pVI membrane lytic activity. I predict that this decreased membrane lytic activity is due to decreased membrane affinity, suggesting that hydrophobic residues are important for helix membrane binding. Furthermore, Debra Galan in our lab has shown that electrostatic interaction between pVI and membranes is important for pVI lytic activity. This electrostatic interaction is potentially mediated by the interaction of two positively charged lysine (Lys) residues in the amphipathic α-helix with the negatively charged lipid head groups. Based on these preliminary studies I hypothesized that hydrophobic and charged residues in the amphipathic α-helix are necessary for pVI membrane binding. To test this hypothesis I generated a series of mutants that have positively charged Lys residues replaced with negatively charged glutamate (Glu) (K45E, K52, K45E,K52E), or mutants that affect both the charge and helix hydrophobicity (W37A,K52E and
W37AK45E). I then determined the membrane lytic activity of these pVI mutants by monitoring the release of SulfoB from liposomes as described earlier. If hydrophobic and positively charged amino acids in the helix are important for pVI membrane binding, then mutations that alter the charge and hydrophobicity should decrease pVI membrane affinity, and subsequent membrane lytic activity. As predicted, I observed a decrease in membrane lytic activity with these mutants (Fig 15A). This decrease in lytic activity correlates with a decrease in membrane affinity (Fig 15B).

We identified additional helix-membrane interactions necessary for pVI membrane lytic activity, when our collaborators recovered a mutant Ad with a 10 fold decrease in endosomal escape (Moyer, Wiethoff et al.). This virus has a mutation (L40Q) in pVI amphipathic α- helix which changes the hydrophobic residue leucine (Leu) to the hydrophilic residue glutamine (Gln). Since this mutation would decrease pVI overall hydrophobicity, I hypothesized that the defect in endosomal escape is due to decreased pVI membrane affinity. Using purified recombinant protein and liposomal membranes, I found that the L40Q mutant has a 10 fold decrease in membrane lytic activity compared to WT pVI (Fig 15A). This decrease in membrane lytic activity correlates with a 10 fold decrease in membrane affinity (Fig 15B)(Moyer, Wiethoff et al.). These data confirm my findings that hydrophobic residues in the amphipathic α-helix are important for pVI membrane lytic activity. Furthermore, these results also show that hydrophobic
Fig. 15. Hydrophobic and positively charged residues are important for pVI activity.

(A) Increasing concentrations of the different pVI constructs was added to liposomes and SulfoB release was measured. (B) Membrane binding of the different pVI constructs. Increasing lipid concentrations were added to pVI constructs and changes in tryptophan fluorescence were used to determine the fractional saturation (θ) of pVI binding capacity. Error bars represent the standard error of the mean for a minimum of 3 replicates.

(■) WT (◆) K45E (◇) K52E (▲) W37AK45E (▲) W37AK52E (○) K45EK52E (★) VIΔ54
interactions between this helix and the endosomal membrane are important for Ad endosomal escape.

The amphipathic α-helix has an oblique membrane orientation, and this orientation is important for the type of curvature stress pVI induces. I wanted to determine if replacing the hydrophobic Leu residue with the hydrophilic Gln alters pVI membrane topology. The L40Q mutation was introduced in the single Trp constructs W37, W41 and W59, and the depth of membrane penetration was determined as previously described, by measuring Trp fluorescence quenching by brominated lipids. Interestingly, I found that these lipids do not quench the fluorescence for residues W37 and W41, while the depth of W59 membrane penetration is similar to WT (Fig 17) These data suggest that residues in the N terminus of the helix fail to interact with the membrane (Moyer, Wiethoff et al.). This was seen at protein concentration where L40Q is maximally bound to membranes, suggesting that amphipathic α-helix membrane topology might also contribute to pVI membrane lytic activity and Ad endosomal escape.

GALECTIN-3 AS A MARKER FOR ADENOVIRUS-PERMEABILIZED ENDOSONMES

*Galectin-3 is forms punctate structures during Ad entry.*

Non-enveloped viruses have developed different mechanisms of permeabilizing their limiting membrane. Viruses such as reovirus induce the formation of size selective pores in membranes (Agosto, Ivanovic et al. 2006), while Ads use a different mechanism (Prchla, Plank et al. 1995). My *in vitro* data suggests that the membrane lytic activity of
Fig. 16. The L40Q mutation attenuates membrane lysis and affinity. (A) SulfoB-entrapped liposomes were incubated with increasing concentrations of purified (●) WT, (□) L40Q, or (▲) VIA54 and the released dye was measured. (B) Membrane binding of various VI constructs was determined by monitoring changes in intrinsic tryptophan fluorescence following incubation with increasing amounts of lipid. The data shown are reported as the means ± SEM from three independent experiments.
Fig 17. The L40Q mutation alters amphipathic α-helix membrane topology.

(A) Insertion depths of three tryptophan residues (W37, W41, and W59) inserted into brominated phospholipids were measured independently via tryptophan fluorescence quenching. BSA was included as a negative control. (B) Depth of membrane penetration for the different tryptophan residues ($Z_{cf}$)
pVI involves the fragmentation of target membranes. This gross membrane reorganization observed in my *in vitro* studies is consistent with previous observations that Ad can facilitate the cytosolic translocation of 70kd dextrans or whole virions (Brabec, Schober et al. 2005; Farr, Zhang et al. 2005). Although these data suggest that Ad disrupts the endosomal membrane, virus translocation from the internal to the external side of the disrupted endosomal membrane has not been documented.

Recently, a marker for vacuole lysis during bacterial infection has been identified. This marker is the cytosolic protein galectin-3 (Gal-3) (Paz, Sachse et al.). Gal-3 labels disrupted vacuolar membranes by binding cell surface N-linked glycans when these are exposed following vacuole lysis. Binding of Gal-3 to membrane fragments can be visualized by immunofluorescence microscopy as punctate structures. To determine if Gal-3 labels disrupted endosomal membranes during Ad entry, I infected HeLa cells with fluorescently labeled virus, and 30 minutes post infection the cells were fixed and stained for Gal-3. A separate set of HeLa cells were infected with reovirus, which has been shown to form size selective membrane pores, or *tsI*, a temperature-sensitive mutant Ad which fails to disrupt the endosome. Gal-3 accumulates in punctate structures in Ad infected cells (Fig 18B), and is diffusely distributed in the cytoplasm and nucleus of uninfected cells (Fig 18A). The diffuse staining was also observed in cells infected with *tsI* or reovirus (Fig 18C and D). When I quantified the number of Gal-3 puncta per cell I saw a statistically significant difference in the number of puncta per cell in Ad infected cells compared to cells infected with *tsI* and reovirus, or uninfected cells. These data suggest that Gal-3 labels disrupted endosomal membranes during Ad entry.
Fig. 18. Galectin 3 accumulates in punctate structures in Ad5 infected cells.

HeLa cells were either uninfected (A) or infected with fluorescently labeled Ad5 (B) Ad2ts1 (C) or reovirus (D) and at 30min post infections the cells were fixed and stained for galectin-3 or virus (reovirus). The number of galectin 3 puncta per cell was then counted. Bar represents 5μm
Fig. 19. The number of galectin 3 puncta increases with increasing virus concentration. HeLa cells were infected with increasing concentrations of fluorescently labeled Ad5, and at 30 min post infections the cells were fixed and stained for galectin-3. The number of galectin-3 and virus puncta per cell was then counted.
If Gal-3 accumulates on disrupted endosomal membranes during Ad infection, increasing the virus concentration should increase the number of disrupted endosomes, and therefore the number of Gal-3 puncta. A similar experiment as described earlier was performed, by infecting cells with increasing virus concentrations and monitoring the accumulation of Gal-3 puncta. The number of Gal-3 punctate structures increased with increasing virus concentrations, confirming our initial findings that this marker labels disrupted endosomes during Ad infection (Fig. 19).

*Time course for Gal-3 accumulation on membranes*

Cryo-electron microscopy studies suggest that pVI is buried within the capsid interior inside the hexon cavity (Saban, Silvestry et al. 2006). During virus uncoating in the acidified endosome, pVI is released from the capsid (Greber, Willetts et al. 1993). The release of pVI was previously shown to be a requirement for *in vitro* membrane lytic activity of the Ad virion (Wiethoff, Wodrich et al. 2005). Therefore I hypothesize that the Gal-3 structures form only after protein VI is released from the capsid.

To determine the timing of Gal-3 accumulation on disrupted endosomal membranes I synchronously infected HeLa cells by bind fluorescently labeled virus at 4°C and, after washing away unbound virus, allowed for virus internalization by warming cells to 37°C. At different times post virus internalization the cells were fixed, and using immunofluorescence microscopy I visualized the release of pVI from internalized virions as well as the formation of Gal-3 puncta. Protein VI release from capsids begins at 10 min post virus internalization which is consistent with published observations of adenovirus uncoating during cell entry (Fig 20A) (Wodrich, Henaff et al. 2010). Consistent with our previous data the Gal-3 positive structures only form at 20 min post
virus internalization after pVI has already been released from the endosome (Fig 20A). These data confirm our findings that Gal-3 labels disrupted endosomal membranes, since the membrane lytic factor has to be released from the capsid interior in order for the Gal-3 structures to form.

After endosomal escape the partially disassembled virion travels on microtubules toward the nucleus where it docks at the nuclear pore to deliver its genome into the nucleus (Strunze, Trotman et al. 2005; Smith, Cassany et al. 2008). If Gal-3 labels Ad lysed endosomal membranes, then early during infection Gal-3 structures should contain virus that is escaping from the endosome. However after 1 hr when the virus docks at the nuclear pore, these structures should no longer colocalize with virus. Furthermore, the disrupted Gal-3 positive membranes should also contain pVI throughout the infection. I used image analysis software to determine the percentage of Gal-3 which colocalizes with virus or pVI at different times post virus internalization in the time course experiment described above. When the Gal-3 structures first form, 70% of these structures colocalize with virus and this colocalization decreases to 50% at later times in infection. (Fig. 20B). These data suggest that the virus is moving away from the Gal-3 positive membranes. While the colocalization of Gal-3 with virus decreases, the percentage of Gal-3 colocalizing with pVI alone remains similar throughout the infection (Fig 20B). Furthermore, when I determined the number of pVI and Gal-3 puncta per cell, I observed a similar trend with a peak at 30 minutes and decrease by 1 hr for both Gal-3 and pVI (Fig 20C). Taken together these data suggest that pVI and Gal-3 decorate the same membrane fragments.
Fig. 20. **Time course of galectin 3 accumulation during Ad entry.** Fluorescently labeled Ad5 was bound to HeLa cells for 1 hr after which the cells were washed and shifted to 37°C to allow for virus internalization. A) Cells were fixed and stained for pVI and galectin 3 at different time post virus internalization. Inserts represent an enlarged view of galectin 3 puncta. Virus is in green, pVI in blue and galectin 3 in red. B) Using image analysis software we determined the percent galectin 3 colocalizing with Ad5, pVI, both or galectin 3 alone for each time point. C) The number of (♦) galectin 3 and (■) pVI puncta per cell was counted for each time point.
Ad endosomal escape visualized in real time

Labeling disrupted endosomes with Gal-3, gave us the opportunity to visualize Ad endosomal escape in real time. Until now, this process has been measured as the translocation of membrane impermeable toxins (Moyer, Wiethoff et al.; Wiethoff, Wodrich et al. 2005) and antibody–toxin conjugates (FitzGerald, Padmanabhan et al. 1983) across the endosomal membrane. Our collaborator, Harry Wodrich, infected Gal-3-RFP expressing cells with a fluorescently labeled Ad, and 10 minutes post infection he visualized the cells by live cell imaging. He observed a number of virions escaping Gal-3 positive endosomes, indicative of virions that are in the process of escaping the lysed endosome. Virions lysing the endosomal membrane were also captured, and visualized as Gal-3 puncta forming around virus. These data confirm our findings that Gal-3 can serve as a marker for Ad endosomal escape. Our studies are the first to directly show a nonenveloped virus translocating across disrupted membranes.

ELECTROSTATIC INTERACTIONS BETWEEN PVI AND MEMBRANES ARE IMPORTANT FOR ADENOVIRUS ENDOSONAL ESCAPE

Currently, the mechanism used by Ad to disrupt the endosomal membrane during cell entry is not clear. Identifying a marker for disrupted endosomal membranes, can serve as a tool not only for visualizing Ad endosomal escape in real time, but also to determine what pVI-membrane interactions are important for this process. Viruses with mutations in pVI that decrease membrane lytic activity can be generated, and a defect in endosomal lysis can be evaluated by measuring Gal-3 accumulation.

My in vitro studies suggest that positively charged residues in the amphipathic α-helix are important for pVI membrane binding. Together with our collaborators, we have
also shown that an interaction between this helix and the membrane is important for Ad endosomal escape (Moyer, Wiethoff et al.). Therefore, I hypothesized that an electrostatic interaction between pVI amphipathic α-helix and the endosomal membrane is necessary for Ad endosomal escape. If my hypothesis is correct, then recombinant viruses with Lys to Glu mutations in pVI amphipathic α-helix should have a defect in endosomal escape. Recombinant viruses with Lys to Glu mutations in pVI amphipathic α-helix have decreased infectivity.

To test this hypothesis, I generated the K45E and K45EK52E recombinant viruses and determined their infectivity. To generate these mutants, I introduced the mutations in Ad5GFP, a replication defective virus construct that lacks the E1, E3 genes and has a GFP expression cassette under the control of a CMV promoter. The mutant viral genomes were transfected into 293 cells, and passaged a number of times, after which the recovered virus was purified using a CsCl gradient as previously described (Wu, Trauger et al. 2004). Interestingly, during the first few passages I observed that the double Lys mutant (K45EK52E) was growing slower than WT, however this defect was not observed at later passages. When I sequenced pVI from the purified viral genome I found that the K45EK52E virus had reverted by the third passage to a single K45E mutation. This initial observation suggested that the two positive charged Lys residues are important for virus spread. To prevent the virus from reverting I collected the supernatants containing virus on passage two and quantified virus using quantitative PCR.

To determine if the K45E virus has decreased infectivity, I infected HeLa cells with varying virus particles/cell of either WT or mutant pVI, and 24 hrs post infection I quantified the percent GFP positive cells. Since the virus cannot replicate in this cell line,
this experiment is measuring only virus entry. Therefore if the amphipathic $\alpha$-helix membrane binding is necessary for Ad cell entry, I should see a decrease in specific infectivity with the K45E virus compared to WT. Our results however suggest that the K45E infectivity is similar to WT (Table 1), suggesting that a more significant decrease in membrane binding may be required to observe decreased Ad entry. Therefore I performed a similar experiment using the K45EK52E virus since membrane affinity is decreased about 40 fold when both Lys 45 and Lys 52 are mutated to Glu. The results indicate that at the same virus concentration there is a decrease in the percent GFP positive cells in cells infected with mutant virus compared to WT. Using the endpoint dilutions, I quantified infectivity and determined that the K45EK52E supernatants have a 4 log decrease in infectious virus compared to WT, suggesting that the mutant virus has an entry defect (Table 2).

*The K45EK52E recombinant virus has a defect in endosomal escape*

To determine if the decreased infectivity is due to a defect in endosomal escape, I monitored the accumulation of the vacuole lysis marker, Gal-3, to disrupted endosomal membranes in cells infected with either K45EK52E or WT virus. Gal-3 binding membrane fragments was visualized by immunofluorescence as punctate staining. My results show punctate Gal-3 staining in cells infected with WT virus, and diffuse staining in uninfected cells or cells infected with the K45EK52E virus, suggesting that this virus has a defect in disrupting the endosome (Fig 21). From these data I concluded that an
Table 1. Infectivity for the K45E virus

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<td><strong>WT</strong></td>
<td>$172 \pm 50 \text{ vp/GTU}$</td>
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<tr>
<td><strong>K45E</strong></td>
<td>$198 \pm 17 \text{ vp/GTU}$</td>
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Table 2. Infectivity for the K45E/K52E virus

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<tr>
<td><strong>WT</strong></td>
<td>$0.35 \pm 0.001 \text{ gc/GTU}$</td>
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<td><strong>K45E/K52E</strong></td>
<td>$1041.7 \pm 18.3 \text{ gc/GTU}$</td>
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Fig. 21. Ad5 K45EK52E virus has a defect in endosomal lysis. (A) HeLa cells were infected with Ad5 WT or Ad5 K45EK52E and at 30min post infections the cells were fixed and stained for galectin-3 or the viral protein hexon. (B) The number of galectin-3 puncta per cell was then counted.
electrostatic interaction between the pVI amphipathic α- helix and the endosomal membrane is important for Ad endosomal escape.

Since pVI can also function to stabilize the capsid, an observed defect in Ad endosomal escape could also be attributed to a defect in pVI release from the capsid interior during uncoating. To rule out an uncoating defect with the K45EK52E virus, I infected cells with either WT or mutant virus and at 30min post virus internalization, I fixed and stained the cells for pVI and hexon. If the mutant virus has an uncoating defect pVI, staining should not be detected. However, a similar amount of pVI is released by both WT and K45EK52E (Fig 22). These data confirm that the K45EK52E pVI is released from the capsid but has a defect in endosomal lysis. Furthermore we observed that in cells infected with the mutant virus, 70% of pVI colocalized with the virus, while in cells infected with WT virus only 30% of pVI colocalized with virus (Fig 22B). These data further support our hypothesis that pVI is released from the capsid but is defective in endosomal escape.
Fig. 22. Ad5 K45EK52E virus does not have an uncoating defect.

(A) HeLa cells were infected with Ad5 WT or Ad5 K45EK52E and at 30min post infections the cells were fixed and stained for pVI or hexon. (B) The percentage of pVI colocalizing with virus was quantified using IMARIS image analysis software.
CHAPTER IV
DISCUSSION

ADENOVIRUS MEMBRANE DISRUPTION IS DISTINCT FROM OTHER NONENVELOPED VIRUSES

The molecular mechanisms of cell membrane disruption by capsid proteins of nonenveloped viruses are still poorly defined. While studies have demonstrated that reovirus μ1 protein (Ivanovic, Agosto et al. 2008) and picornavirus VP1/VP4 (Tosteson and Chow 1997) form pores in membranes, much less is known regarding the mechanisms used by other nonenveloped viruses to penetrate cell membranes. While it has been clearly demonstrated that Ad, a non-enveloped virus, disrupts the endosomal membrane during cell entry, the mechanism has not been characterized (Meier and Greber 2003). Recent studies suggest that Ad pVI, which is released from the interior of the capsid during cell entry, has all of the in vitro membrane lytic activity of the virion (Wiethoff, Wodrich et al. 2005). These data suggest that pVI is involved in Ad escape from the endosome. Work presented in this dissertation supports a role for pVI in Ad endosomal escape, and proposes a mechanism for this process that is different from other nonenveloped viruses.

A common theme in nonenveloped virus entry is membrane disruption by small hydrophobic proteins or peptides. The membrane lytic factor for Flock House virus, an insect virus, is a 4kD γ peptide. This peptide contains an N terminal amphipathic α-helix
called $\gamma_1$, which is sufficient to disrupt membranes \textit{in vitro} (Maia, Soares et al. 2006). Reovirus was also shown to use a hydrophobic peptide $\mu1$ to disrupt membranes. An N terminal myristolated cleavage product, $\mu1N$ is sufficient to permeabilize the membranes of red blood cells. Similar to these nonenveloped viruses, the major determinant of pVI lytic appears to be a 20 residue putative N-terminal amphipathic helix (Wiethoff, Wodrich et al. 2005). Removing this helix greatly decreases pVI membrane binding and lytic activity (Wiethoff, Wodrich et al. 2005). I confirmed this finding, and further determined that a peptide containing this helix is sufficient for \textit{in vitro} membrane lytic activity (Maier, Galan et al. 2010).

However unlike the FHV and reovirus lytic peptides which are thought to form size selective membrane pores, Ad pVI disrupts membranes through a different mechanism. In addition to pore formation, lipid bilayers can be lysed by the induction of significant membrane curvature stress. The amphipathic helix of pVI possesses a conserved primary sequence found in many membrane associated proteins which correlates with their association with positively curved membrane surfaces (Drin, Casella et al. 2007). This helix binds to membrane surfaces in an oblique orientation, a characteristic of proteins that induce positive membrane curvature. In addition, my data and those of others in the lab demonstrate that pVI membrane lytic activity is enhanced by membranes with an increased propensity to form positively curved membranes and is decreased by membranes with a greater propensity to adopt negatively curved structures (Maier, Galan et al. 2010). Thus, these observations support a model in which pVI lyses membranes by inducing positive curvature in target membranes. Parvoviral capsids deploy a phospholipase A2 enzyme which hydrolyzes phospholipids to release the
positive curvature inducing-lysolipids, although a role for curvature stress has yet to be reported for paroviral escape from endosomes (Zadori, Szelei et al. 2001; Farr, Zhang et al. 2005; Lupescu, Bock et al. 2006).

Induction of membrane curvature stress by lytic proteins results in a detergent-like membrane fragmentation. Previously, it was proposed that Ad5 pVI disrupts membranes by fragmentation (Wiethoff, Wodrich et al. 2005). The oblique orientation of the N-terminal amphipathic helix, as well as pVI ability to induce positive membrane curvature support these initial findings (Epand and Vogel 1999; Epand and Epand 2000). This more severe reorganization of membranes via pVI could facilitate the translocation of a 90 nm diameter capsid across endosomal membranes. Additionally, Ad has been shown to facilitate the cytosolic translocation of other viruses such as a 25 nm diameter Minute Virus of Mice further supporting a gross reorganization of endosomal membranes by Ad (Farr, Zhang et al. 2005). My results suggest that pVI disruption of membranes does not likely involve the formation of pores with diameters less than 100 Å and it is unlikely that a 22 kDa or perhaps a 20 residue peptide would be able to induce stable pores with larger diameters (Maier, Galan et al. 2010).

Using epifluorescence microscopy to examine the ability of pVI to reorganize fluorescently labeled giant lipid vesicles, I and others in the lab confirmed previous observations that pVI fragments lipid membranes as seen by negative stain TEM (Wiethoff, Wodrich et al. 2005). However, negative stain TEM involves substantial dehydration of lipid membranes leading to potentially artifactual structures. Our current study employed fully hydrated membranes in which only the lipid membranes and not potential protein aggregates were visualized, therefore providing greater confidence in
the observation that pVI fragments membranes. Additionally, examination of pVI membrane lysis in solution has allowed us to visualize novel tubular structures possessing highly curved surfaces not previously observed by TEM (Maier, Galan et al. 2010).

Although my data indicate that the pVI amphipathic α-helix is sufficient to lyse liposomal membranes, I determined that this helix is not sufficient to generate these highly curved tubular structures (Maier and Wiethoff). I was therefore interested in understanding what additional domains in pVI contribute to tubule formation.

My work has identified that the in vitro lytic activity of pVI is contained in an N-terminal 80-residue helical domain (VI114Δ) (Maier, Galan et al. 2010). In addition to the N-terminal amphipathic α-helix, three additional α-helices are also predicted for this domain. These α-helices are also expected to spontaneously associate with membrane interfaces as evidenced by the negative ΔG_{if} values. These predictions were confirmed by my observation that these 3 helices associate with membranes, binding in shallow oblique orientations in the lipid bilayer. The magnitude of these ΔG_{if} are considerably less than that for the N-terminal amphipathic α-helix which is in agreement with the severe reduction in membrane affinity observed for VIΔ54 (a construct that lacks this helix) compared to pVI. To examine the contributions of these additional α-helices to pVI membrane lytic activity, the affinity of VIΔ54 for membranes was artificially enhanced using Ni2+-NTA containing lipids in the target membrane and a 6×His tag on the protein. The increased affinity of 6×His–VIΔ54 for membranes correlated with an increase in membrane lytic activity which was more comparable to that observed for pVI (Maier and Wiethoff). Although the affinity of the various forms of pVI for membranes correlates with membrane lytic activity this relationship appears to be non-linear since the
6×His–VIΔ54 binding to Ni2±–NTA liposomes is less than pVI yet possesses similar membrane lytic activity.

The ability of residues outside the N terminal amphipathic α-helix to interact with and disrupt membranes, suggested that these helices might contribute to positive membrane curvature induction and tubule formation. Unlike pVI and a peptide corresponding to the N-terminal amphipathic α-helix, the membrane lytic activity of 6×His-tagged VIΔ54 does not appear to be as strongly influenced by the inclusion of lipids which alter the propensity for positive or negative membrane curvature (Maier and Wiethoff). Thus, it is possible that VIΔ54 is able to more strongly induce positive membrane curvature in the absence of lysolipids. Furthermore I determined that as seen with the amphipathic α-helix peptide, the 6×His-tagged VIΔ54 alone also cannot induce membrane tabulation. However my studies indicate that VI114Δ containing the 4 α-helices generates tubular membrane structures (Maier and Wiethoff; Maier, Galan et al. 2010). Taken together these data suggest that although both the N-terminal amphipathic α-helix and VIΔ54 alone can induce positive membrane curvature, an intact VI114Δ membrane interacting domain is necessary to form membrane tubules. One possible explanation could be that an intact domain from residues 34–114 can stabilize the highly curved structures induced upon pVI membrane binding, either through cooperative influences on membrane curvature or through protein–protein interactions which would not occur in either the N-terminal peptide or VIΔ54 alone. Further studies are required to gain a better understanding of this phenomenon.

Cooperativity between an N-terminal amphipathic α-helix which induces positive membrane curvature and additional helical domains which stabilize these curved
membranes is not unprecedented. A similar cooperativity is observed with the COPII proteins Sar1p, and scaffolding proteins Sec23/24p and Sec13/31p (Lee, Orci et al. 2005). During COPII vesicle formation an N-terminal amphipathic α-helix in Sar1p induces positive membrane curvature, which is then recognized and stabilized by the additional coat proteins Sec23/24p and Sec13/31p. The same is true for other proteins involved in membrane fission events such as those involving the N-BAR domain containing proteins, endophilin and amphiphysin (Masuda, Takeda et al. 2006; Low, Weininger et al. 2008). Topologically, membrane fission through induction of positive membrane curvature is very similar to the membrane fragmentation performed by Ad pVI. This similarity appears to be only conceptual, as pVI does not share any obvious sequence similarity with these proteins.

Although the importance of membrane tubulation during Ad cell entry has yet to be defined, overexpression of the mRFP–pVI1–239 in mammalian cells was recently shown to result in the protein associating with dynamic tubular membrane structures (Wodrich, Henaff et al. 2010). Furthermore, studies with proteins which deform membranes have shown that the formation of tubular structures in vitro can be used as a marker for a protein's ability to mediate membrane fission in vivo (Lee, Orci et al. 2005). Therefore the cooperativity observed between the N-terminal amphipathic α-helix and the 3 additional helices to form tubular structures in vitro might be important for pVI to fragment the endosomal membrane during cell entry. Further experiments will be required to define a role for these pVI domains in endosomal escape of Ad during cell entry.
As a whole, my data shed new light into the mechanisms of membrane disruption by a nonenveloped virus. Ads appear to possess a novel method for disrupting endosomal membranes during cell entry compared to those previously described for reoviruses, picornaviruses or parvoviruses.

PROTEIN VI –MEMBRANE INTERACTIONS IMPORTANT FOR AD ENDOSONMAL ESCAPE

Although my *in vitro* studies suggest that the N-terminal amphipathic α-helix is important for pVI lytic activity, the helix-lipid interactions necessary for Ad endosomal escape are not known. Amphipathic α-helical peptides can bind membranes via two different interactions: hydrophobic and electrostatic (Dathe, Wieprecht et al. 1997; Dathe, Nikolenko et al. 2001). The protein VI amphipathic α-helix contains two Lys at positions 45 and 52. Mutating these residues to the negatively charged Glu (K45E, K52E, K45EK52E) decreased pVI membrane binding and lytic activity. These data suggest that electrostatic interaction between the helix and membranes are important for pVI activity. Additionally, hydrophobic interactions are also important for this activity since mutating Trp residues to Ala (a residue with decreased hydrophobicity) (W37AW41A) greatly decreased pVI membrane binding and subsequent lysis.

Work performed with antimicrobial peptides suggests that these two types of interactions are important for peptides that disrupt membranes though a detergent-like mechanism. An electrostatic interaction between basic residues in the peptide and the phospholipid head groups is needed for initial membrane binding. Hydrophobic residues binding the hydrophobic core of the lipid bilayer then generates a curvature stress in membranes resulting in fragmentation (Shai 1999). In addition to my study, these types
of interactions are also shown to be important for other nonenveloped viruses. The papillomavirus membrane disrupting peptide is located at the C terminus of the minor capsid protein L2 (Kamper, Day et al. 2006). This peptide contains hydrophobic and basic residues which are important for membrane binding and lytic activity. Reversing the positive charge of these residues or deleting hydrophobic residues decreases membrane binding and lysis (Kamper, Day et al. 2006). Although the high peptide concentration required for membrane disruption suggests that L2 functions via a detergent-like mechanism, the membrane lysis mechanism is not yet known.

Together with collaborators we show that hydrophobic and electrostatic interactions between pVI amphipathic α-helix and membranes are also important for Ad endosomal escape. Using a random mutagenesis approach, our collaborators identified a Leu to Gln mutation at position 40 in pVI amphipathic α-helix that decrease virus infectivity and endosomal escape. I determined that a L40Q recombinant protein has decreased membrane binding and lytic activity. Taken together these data suggest that hydrophobic helix-membrane interactions are important for Ad endosomal escape (Moyer, Wiethoff et al.).

Interestingly I found that in addition to decreased membrane affinity the L40Q mutant protein also has altered topology. At protein concentrations where lipid binding is saturated, I found that residues in the N terminus of the amphipathic α-helix are not interacting with membranes. These data suggest that membrane topology contributes to pVI lytic activity. However my data at this point do not distinguish between a defect in membrane affinity and membrane topology. To determine if the altered membrane topology contributes to the defect observed with L40Q, the membrane affinity of this
construct could be artificially enhanced using nickel chelating lipids (DOGS-NTA-Ni) and a His tag on the protein. If a defect in membrane lytic activity is observed when L40Q affinity is similar to WT pVI, then I can conclude that the altered membrane topology contributes to the observed defect in lytic activity.

At the same time, I found that a recombinant virus bearing the K45EK52E mutation also has a defect in endosomal escape. Electrostatic interactions between the amphipathic α-helix and membranes are therefore also important for Ad endosomal escape. Interestingly, viruses that have a single Lys mutated to Glu (K45E, K52E) do not have an entry defect, although these mutations do slightly decrease in vitro pVI membrane lytic activity. This discrepancy could be explained by the protein concentration present in our in vitro system (100nM) vs the endosomal compartment (~22mM). The increased pVI concentration in the endosome could therefore compensate for the slight decrease in pVI membrane lytic activity.

MEMBRANE FRAGMENTATION DURING AD ENTRY

Although numerous studies indirectly document a membrane lytic event during nonenveloped virus entry, there has not been any direct evidence showing a virus transported across the disrupted limiting membrane. Since these studies are mainly done using fixed cells, one factor that contributes to the difficulty of capturing the lytic event is the speed at which virions cross the membrane. For example, electron microscopy studies of Ad infected cells have depicted virus at the cell surface, in an intact endosome or in the cytoplasm (Imelli, Ruzsics et al. 2009). Therefore, although my in vitro data indicate that Ad pVI fragments membranes, this process has not yet been documented during virus
entry. Work presented in this dissertation identifies galectin 3 (Gal-3) as a marker for disrupted endosomal membranes during Ad entry. Using this marker, together with our collaborators, we were able to see for the first time Ad endosomal escape in real time using live cell imaging.

Gal-3 was first identified as a marker for vacuole lysis during bacterial infection (Paz, Sachse et al.). This cytosolic protein labels disrupted membranes by binding N-linked glycans present on the exo-domains of membrane proteins when these are exposed to the cytoplasm following vacuole lysis. Similar to what was observed during bacterial phagosomal lysis, I found Gal-3 punctate structures in WT Ad infected cells but not in cells infected with a Ad2ts1, a mutant virus that fails to escape the endosome (Weber 1976; Greber, Webster et al. 1996). Furthermore, these structures were not formed during infection with reovirus, a virus which forms size selective membrane pores (Agosto, Ivanovic et al. 2006). These data confirm our findings that Ad does not form size selective pores in membranes, and allows the passage of a 30kD Gal-3 protein from the cytosol to the lumen of the disrupted endosome.

Identifying a marker for disrupted endosomal membranes allowed me to determine the fate of these membranes in the course of Ad infection. Ad membrane disruption occurs only after the capsid partially disassembles and pVI is released from the capsid interior (Wiethoff, Wodrich et al. 2005) In line with these observations, using immunofluorescence microscopy I found that the Gal-3 structures only form after pVI is released from the capsid interior and can be detected using anti-pVI antibodies. Furthermore at 20 min post virus internalization, the majority of these structures contain both virus and pVI. These structures represent a virus that has recently lysed the
endo
somal membrane. However as the virus moves towards the nucleus, later during infection, fewer Gal-3 structures contain virus. These observations suggest that the virus moves away from a disrupted endosome.

Preliminary data from our lab suggests that after endosomal disruption pVI is degraded by an unknown mechanism. My studies confirm these findings, as I observed a decrease in the number of pVI puncta by 4 hrs post virus internalization. The same trend is observed for the number of Gal-3 structures as well. Furthermore although the total number of Gal-3 structures decreased with time, the percentage of Gal-3 that colocalizes with pVI remains the same. These data further confirm that Gal-3 labels pVI disrupted endosomal membranes during Ad entry, and these membranes are degraded by an unknown mechanism.

Dupont et al. proposed that phagocytic membrane fragments labeled with Gal-3 are degraded by autophagy. This group showed that during Shigella infection, proteins on ruptured phagosomal membrane are ubiquitinated. Ubiquitinated proteins then recruit the autophagy marker LC3 and are targeted to autophagic degradation (Dupont, Lacas-Gervais et al. 2009). Recently data from our lab suggest that LC3 is recruited to Gal-3 positive membranes during Ad infection, suggesting that these membrane remnants might also be degraded by autophagy. Further studies need to be performed to determine how pVI and Gal-3 positive membranes are being degraded.

Since disrupted endosomes can be labeled with Gal-3, Ad endosomal escape could be visualized in real time. Until now this process has been measured as the translocation of membrane impermeable toxins (Moyer, Wiethoff et al.; Wiethoff, Wodrich et al. 2005; Wodrich, Henaff et al. 2010) and antibody–toxin conjugates
across the endosomal membrane. By infecting cells overexpressing RFP-Gal-3 with a fluorescently labeled virus, our collaborators visualized Ad escaping a Gal-3 positive disrupted endosome. Additionally, they found Gal-3 structures forming around the virus, indicative of a virus that just lysed the endosomal membrane. Visualizing Ad endosomal escape in real time allowed us to make an observation that could not be made in fixed cells. We observed that Ad seems to struggle to escape the endosome. A delay in this process could be in part explained by a need for additional factors to be recruited to the endosomal membrane.

One factor that might be important for Ad endosomal escape is pVI interaction with microtubules. Studies have shown that many cellular organelles use microtubule movement to generate or maintain their structure. One such example is the extension of tubular structures from the ER (Terasaki, Chen et al. 1986; Lee and Chen 1988). Microtubule motors are thought to bind integral membrane proteins in this organelle, and the actual movement on microtubules is thought to drive tubule formation (Allan and Vale 1994). Tubule formation is indicative of membrane curvature stress, which in the case of pVI ultimately results in membrane lysis (Maier, Galan et al. 2010). Protein VI was shown to travel on microtubules (Wodrich, Henaff et al. 2010). Furthermore, preliminary data from our lab suggests that a virus with a mutation in pVI that decreases microtubule movement is less efficient at endosomal escape. This mutant virus colocalizes with Gal-3 structures longer then WT virus, suggesting that it takes longer for this virus to escape the endosome. Our collaborators confirmed these findings by live cell microscopy. Therefore pVI movement on microtubule might enhance the initial membrane curvature stress induced by pVI leading to membrane disruption. Another
possibility is that pVI initiates this process, and interaction with microtubules motors allows for the endosomal membrane to be “peeled” away, allowing the virus to escape. However, the role of pVI microtubule movement in endosomal escape is not yet known. The membrane curvature induced by pVI could also be enhanced by a family of proteins known as the sorting nexins (SNX). These are cytosolic proteins that bind membranes either through protein-protein interactions, or through a lipid binding motif. SNX are involved in protein sorting and regulation of vesicle trafficking (Worby and Dixon 2002). One of the membrane binding motifs found in SNX is a bin-amphiphysin-RVS (BAR) domain (Pylypenko, Lundmark et al. 2007; Shin, Ahn et al. 2008). This α-helical domain has a concave shape, and has been shown to sense as well as induce membrane curvature (Habermann 2004). Our collaborators have observed that when overexpressed in cells, pVI colocalizes with SNX 1/2, although the significance of this colocalization has not been documented. However it is possible that pVI initiates endosomal lysis by inducing membrane curvature which is then recognized by the BAR domain of SNX. In support of this observation, in vitro studies in our lab with purified pVI and the BAR-domain of SNX1 show that pVI fragmentation of liposomal membranes increases the amount of membrane-associated BAR-domain. Thus, these BAR-domain containing proteins could be recruited to the endosomal membrane and enhance membrane curvature which could help fragment endosomal membranes and release virus into the cytoplasm. Further investigation is required to determine if SNX play a role in Ad endosomal escape. The data presented in this dissertation supports a gross reorganization of the endosomal membrane by pVI. However the extent of membrane fragmentation is still not known. Using Gal-3 as a marker for disrupted endosomal membranes, immuno-electron
microscopy could be performed and these membrane fragments could be visualized. This would be the first time that membrane fragments would be observed during entry of a nonenveloped virus.

THE ROLE OF POSITIVE MEMBRANE CURVATURE IN AD ENDOSONAL ESCAPE

The recruitment of additional factors to the endosomal membrane could explain the need for pVI induced positive membrane curvature induction during Ad endosomal escape. My in vitro studies as well as others in the lab suggest that pVI induces positive membrane curvature (Maier, Galan et al. 2010). Furthermore, the N terminal amphipathic α-helix membrane topology dictates the type of curvature induced by pVI. A series of recombinant pVI constructs with mutations in the amphipathic α-helix that should either alter the depth of membrane penetration (S1L/T1L), or the angle of membrane insertion (S1I/G3I/F12S), are defective in positive curvature induction. An important question that remains unanswered then is whether viruses bearing these mutations are defective in endosomal escape. Answering this question would help us understand the significance of pVI induced positive membrane curvature during Ad infection.

Membrane curvature stress is relieved by a distortion of the membrane shape, forming highly curved membranes. BAR domain containing proteins, such as SNX described earlier, sense and bind these membranes, further enhancing membrane curvature (Worby and Dixon 2002). An excess amount of curvature strain then fragments membranes by the detachment of patches from the membrane surface. Therefore pVI induced positive membrane curvature can either be important for membrane fragmentation or for recruiting additional proteins to the endosomal membrane.
While we determined that Ad pVI fragments membranes by inducing positive membrane curvature, studies with peptides that produce negative membrane curvature have shown that these can also lyse membranes. Influenza hemagglutinin fusion peptide induces negative membrane curvature, and at certain concentrations it can facilitate the release of fluorescent dyes from liposomal membrane (Epand, Macosko et al. 1999). Furthermore, the cell toxicity of certain amyloidogenic peptides is dependent on their ability to fragment membranes by inducing negative membrane curvature strain (Smith, Brender et al. 2009). Thus, it is interesting to consider whether Ad pVI fragmentation of membranes through induction of positive vs negative membrane curvature is important for virus cell entry.

It is interesting to speculate, based on preliminary studies described earlier, that additional cellular or viral proteins might be important for Ad endosomal escape. It may be possible that the positively curved membranes generated by pVI are recognized by these proteins. Future studies using viruses that bearing the mutations described above could determine what role pVI positive membrane curvature induction plays in Ad endosomal escape.

**ADENOVIRUS MEMBRANE FRAGMENTATION ACTIVATES THE INNATE IMMUNE RESPONSE**

In addition to mediating Ad endosomal escape, pVI membrane lysis can also contribute to Ad-induced proinflammatory response. Ads have numerous qualities that make them excellent vectors for gene therapy, however their use has been greatly limited by the potent inflammatory response generated upon systemic administration (Volpers and Kochanek 2004). Activation of the immune response results in rapid Ad vector
elimination, as well as tissue damage (Worgall, Leopold et al. 1997; Muruve, Barnes et al. 1999). This response is dominated by infected macrophages secreting the proinflammatory cytokine IL1β (Shayakhmetov, Li et al. 2005). Recent studies demonstrate that macrophages are stimulated by Ad to secrete this cytokine by activating a multi-protein complex called the Nalp3 inflammasome (Muruve, Petrilli et al. 2008). Ad inflammasome activation is in part mediated by pVI membrane lytic activity, and requires release of endosomal cathepsins (Barlan, Griffin et al. 2010). Interestingly, reovirus, a nonenveloped virus that also escapes from the endo-lysosomal compartment, does not facilitate cathepsin B release or activation of the Nalp3 inflammasome (Barlan, Danthi et al.). As mentioned earlier, reovirus disrupts membranes by forming size selective pores (Agosto, Ivanovic et al. 2006). The 28kD cathepsin B is not able to escape through these pores, but it can escape following the gross membrane reorganization induced by pVI.

Furthermore, the membrane fragments that are potentially generated during Ad endosomal escape can also serve as a danger signal that can activate the immune response. Phagosomal membrane remnants during Shigella infection act as danger associated molecular patterns that activate different components of the innate immune system (Dupont, Lacas-Gervais et al. 2009). Therefore the mechanism used by pVI to disrupt membranes contributes to innate immune activation. Elucidating the mechanism used by Ad to escape endosomes has therefore led not only to a better understanding of nonenveloped virus membrane penetration, but how this process stimulates an immune response.
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