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Review

DNA virus uncoating

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ABSTRACT

Virus genomes are condensed and packaged inside stable proteinaceous capsids that serve to protect them during transit from one cell or host organism, to the next. During virus entry, capsid shells are primed and disassembled in a complex, tightly-regulated, multi-step process termed uncoating. Here we compare the uncoating-programs of DNA viruses of the pox-, herpes-, adeno-, polyoma-, and papillomavirus families. Highlighting the chemical and mechanical cues virus capsids respond to, we review the conformational changes that occur during stepwise disassembly of virus capsids and how these culminate in the release of viral genomes at the right time and cellular location to assure successful replication.

Contents

Introduction

All viruses – enveloped and non-enveloped – protect their genome inside a proteinaceous shell referred to as a capsid or core. Its main function is to protect the genome during the extracellular phase of the virus life cycle. It thereby assists efficient cell-to-cell transmission, organ-to-organ spread, and host-to-host propagation. Each of these extracellular environments confront the virus particle and its precious cargo with a different subset of challenges. These might include UV irradiation, dehydration, non-physiological pH, proteases, complement, and factors of the innate and adaptive immune systems. In addition to providing protection, core structural proteins mediate many early host-pathogen interactions that occur during entry, such as receptor engagement, endocytic signaling, membrane penetration (for non-enveloped viruses), and intracellular capsid transport (Marsh and Helenius, 2006). In some cases,

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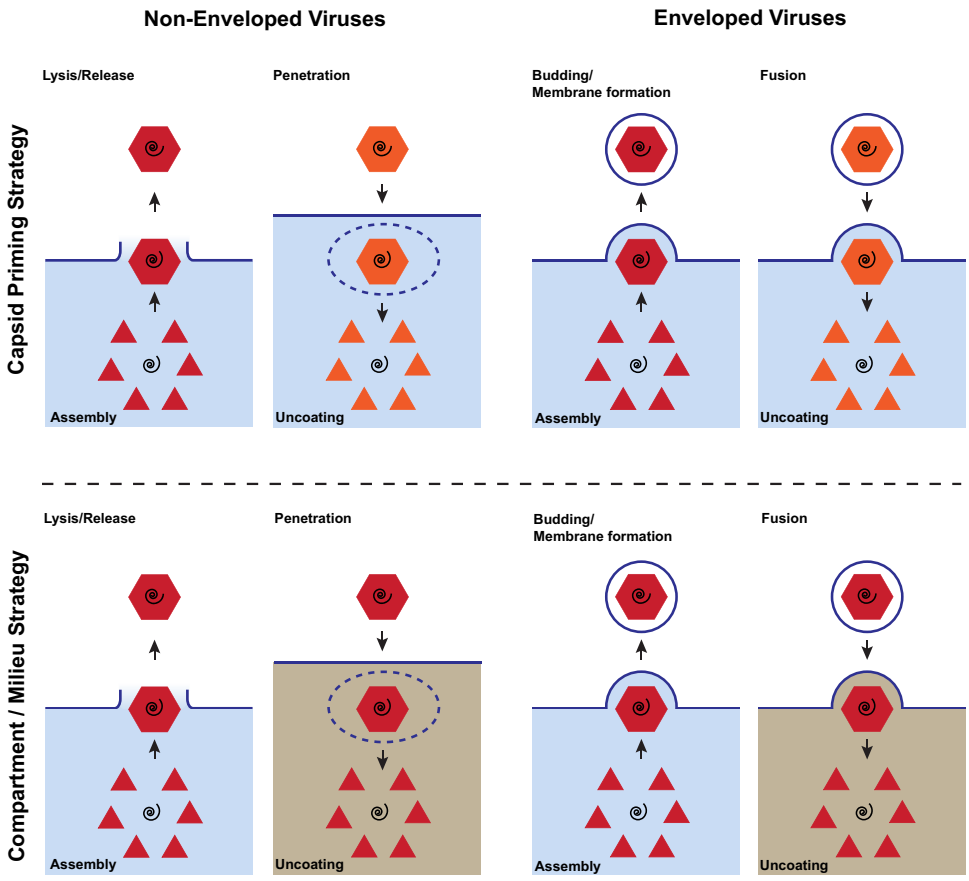

Fig. 1. Capsid priming *versus* compartment/milieu strategy. Conceptually, virion morphogenesis and uncoating are inverse reactions. The ability of viruses to disassemble their capsids during entry just hours before thousands of new stable particles are formed has dazzled virologists for years and is hence known as the "assembly-uncoating paradox". Viruses have evolved two strategies to avoid this convoluted situation: either a capsid priming strategy (top row) or a compartment/milieu strategy (bottom row). For each approach both non-enveloped (left) and enveloped (right) viruses are shown. The capsid priming strategy dictates that capsid properties or newly assembled viruses are altered prior to uncoating (indicated by change in capsid color from red to orange). Alternatively, viruses using a compartment/milieu strategy alter the cellular environment or resort to distinct cellular compartments (indicated by the change in cytoplasmic color from blue to beige). Thus uncoating and assembly programs occur in non-homologous cellular environments. Capsid proteins are displayed as triangles, intact capsids as hexagons, and vDNA as curly cues.

capsid proteins also mediate early effector functions to establish an intracellular environment to facilitate infection (Kelly et al., 2009).

Most virus capsids are assembled from regular capsomere units resulting in helical or icosahedral structures built from a limited number of capsid proteins. Inherently, such icosahedral capsids are highly symmetrical, a property described by the triangulation number (Caspar and Klug, 1962). In exceptional cases – such as poxviruses – capsids may be complex in structure being built from a large number of proteins and lacking symmetry. Regardless of their architecture, all virus capsids possess a built-in capacity for disassembly, referred to as structural metastability: assembled as stable structures during morphogenesis inside producer cells, capsids must be efficiently disassembled or disintegrated in newly infected cells. For some viruses, the environment in which assembly/egress and uncoating occur are homologous. For instance, herpesvirus uncoating which involves loss of tegument proteins and capsid transport towards the nucleus, occurs in the cytosol and at the nuclear pore (Sodeik et.al, 1997). During tegumentation and egress, newly assembled capsids are transported through the same cytoplasm away from the nucleus for secondary envelopment (Mettenleiter et al., 2009). The ability of viruses to perform such opposing processes in the same cell compartment has previously been described as the "assembly-uncoating paradox" (Greber et al., 1994).

In this review, we compare the uncoating and genome delivery programs of pathogenic DNA viruses of the adeno-, papilloma-, polyoma-, pox-, and herpesvirus families. The complex disassembly cascades these viruses undergo and the cellular cues to which they respond are discussed and compared. As genome release is a critical step in the life cycle of every virus and a prerequisite for genome replication, understanding the molecular details of viral capsid disassembly and genome delivery programs may reveal new drug targets and therapeutic opportunities.

General principles of uncoating

Given the complexity of the genome delivery program we think it is beneficial to discuss the terminology used when describing it. The term "Priming" details the changes to the capsid that occur prior to productive genome deposition. "Uncoating" on the other hand specifies the loss of structural proteins from the capsid. However, both the loss of structural proteins from the capsid as well as structural rearrangements within it may be considered priming events, given that they are a prerequisite for successful genome release. Therefore, "uncoating steps" and "priming steps" are not mutually exclusive terminologies. Finally, the term "genome delivery" describes the last step of the entry program when the genetic information is deposited into the host cell cytosol or nucleus.

In general, two strategies are used by viruses to mediate genome release and overcome the "assembly-uncoating paradox". The capsid priming strategy and the compartment or milieu strategy (Depicted in Fig. 1).

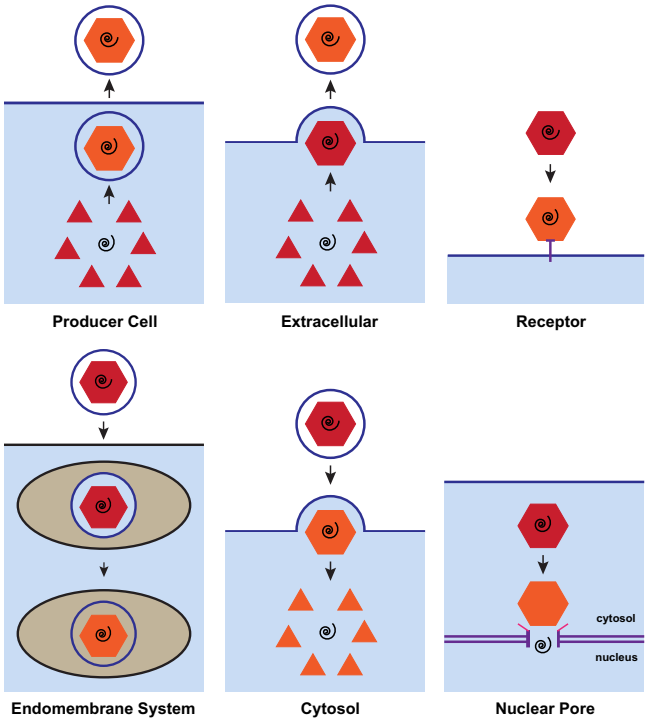

Fig. 2. Location of capsid priming. "Capsid priming" describes changes that occur to the capsid prior to successful genome deposition. These changes activate the capsid to respond to specific uncoating cues and induce structural meta-stability. Priming strategies are diverse, and depending on the virus can occur at any time point from virus assembly to release of the virus genome. Thus priming can occur in many different locations as displayed: these include priming inside the producer cell during morphogenesis, in the extracellular space, during interaction with cell-surface receptors, within the endomembrane system during endocytic trafficking, in the cytosol, or at the nuclear pore. Priming may involve the loss of structural capsid proteins or simply rearrangements within or between capsid proteins. Capsids that have been primed in the various cell locations are shown in orange.

Capsid priming strategy

The capsid priming strategy relies on the capsids undergoing structural modifications prior to genome delivery. These can occur during morphogenesis, when the virus exits the cell, or during entry before the capsid reaches the preferred site of genome delivery (Illustrated in Fig. 2). These modifications increase the capsids ability to receive specific cellular uncoating cues, or facilitate disassembly (structural metastability) of the capsid once it reaches the environment where genome release is to occur.

Compartment or milieu strategy

Alternatively, viruses using a compartment or milieu strategy remain structurally sound, but cause a change in the cellular environment. Altering the environment of the producer cell during morphogenesis or entry is necessary for viruses that uncoat and assemble in the same compartment. However, it does require that the virus devotes a subset of genes for this purpose. Alternatively, morphogenesis and uncoating may be spatially separated *i.e.* require distinct organelles.

In practice, most viruses use a combination of these strategies. Genome uncoating is a complicated multi-step process that depends on a series of consecutive cues. These often take place in different subcellular compartments or depend on diverse host-factors. The nature of these cues can be very diverse and have recently been classified as "receptor driven", "chemical", or "mechanical" (Suomalainen and Greber, 2013) (Table 1).

Non-enveloped virus uncoating

The capsids of non-enveloped viruses constitute the outermost layer of the virion. Thus they are likely to receive uncoating cues within the extracellular space or upon contact with binding factors and/or receptors on the cell surface. These capsids protect the viral genome, determine host tropism, entry pathways, and penetration mechanisms.

Adenoviruses

Adenoviruses feature icosahedral capsids with distinct morphology: capsid faces and edges consist of homotrimeric hexon protein units, and the 12 vertices are built from penton base proteins from which fibers extend. In addition, there are four minor capsid proteins and inner core factors – mostly associated with the dsDNA genome (reviewed by Russell, 2009).

With regard to genome uncoating of DNA viruses, adenoviruses – in particular human adenovirus type 2 and type 5 (Ad2/5) – are the best-studied. These viruses go through a step-wise uncoating program that begins during virus assembly and proceeds through distinct disassembly intermediates during the entry process (Greber et al., 1993). The first capsid-priming step occurs during virion morphogenesis. It is mediated by the virus-encoded protease that facilitates the transition from immature to mature virions through proteolytic cleavage (Anderson, 1990; Mangel et al., 2003; Weber, 1976; Webster et al., 1989a, 1998b). Adenovirus particles generated in the absence of functional protease package the viral genome and appear morphologically normal (Imelli et al., 2009; Perez-Berna et al., 2009; Weber, 1976). However they display strong defects in uncoating and membrane penetration (Greber et al., 1996). This indicates that proteolytic cleavage serves to prime the virus for subsequent uncoating cues encoutered during entry. Thus protease activity is the trigger of virus metastability and must be tightly regulated. To this end, protease packaged into mature virions is activated by DNA binding and interaction with the C-terminal peptide of the protein VI precursor (pVI) (Gupta et al., 2004; Honkavuori et al., 2004; Mangel et al., 2003, 1993; Webster et al., 1993). Fully activated protease moves along viral DNA in a one-dimensional diffusion reaction (sledging) to meet its substrates, all of which are DNA-associated (Graziano et al., 2013; Mangel and San Martin, 2014). Later, the protein is inactivated by disulfide oxidation when these virions exit the cell (Greber et al., 1996).

During the entry phase, uncoating of the proteolytically activated virions begins immediately. At the cell-surface Ad2/5 fiber knobs contact the coxsackie-adenovirus receptor (CAR) (Bergelson et al., 1997) and Ad2 virion penton-base proteins bind the adenovirus co-receptor αv-β3/5 integrin (Chiu et al., 1999; Wickham et al., 1993). Engagement of αv-β3/5 integrin untwists the pentamer possibly weakening the capsid vertex from which fibers protrude (Lindert et al., 2009), while the virus-induced clustering of CAR triggers actin and myosin-2 mediated drifting-motions (Burckhardt et al., 2011). The coordinate action of CAR drifting and αv-β3/5 integrin confinement facilitates fiber-shedding. This initial uncoating step, likely mediated by mechanical forces, results in the exposure of the membrane lytic portion of protein VI during virion endocytosis (Burckhardt et al., 2011; Wodrich et al., 2010).

Although, low pH was believed to play a key role in the activation of protein VI-mediated endosomal escape (Blumenthal et al., 1986; Prchla et al., 1995), recent *in-vivo* quantification of cytosolic particles suggests that penetration does not depend on endosomal acidification (Suomalainen et al., 2013). Thus the molecular cue that times Ad penetration from endosomes remains to be determined.

Upon penetration, the virus re-encounters reducing conditions within the cytosol activating the packaged protease. Protease then

Table 1
Comparison of priming and uncoating strategies used by DNA viruses.

	Priming/ Uncoating	Location	Trigger/Cue	Structural changes/Enzyme activation	References
Adenovirus	Priming	Cytosol/ Producer cell	Cofactor activation of protease	Cleavage of protein precursors, minor structural changes, destabilization of the capsid	(Anderson, 1990; Gupta et al., 2004; Honkavuori et al., 2004; Mangel et al., 2003, 1993; McGrath et al., 2003; Perez-Berna et al., 2009; Perez-Berna et al., 2012; Rancourt et al., 1995; Weber, 1976; Webster et al., 1993, 1989)
	Priming/ Uncoating	Cell surface	Receptor and co-receptor engagement	Fiber-shedding, Protein VI exposure	(Burckhardt et al., 2011; Lindert et al., 2009)
	Priming	Cytosol	Reducing environment	Activation of protease, cleavage of inner capsid protein VI	(Greber et al., 1996; Ruzindana-Umunyana et al., 2002)
	Uncoating	Nuclear pore	Kinesin recruitment to capsid and Nup358 and activation	Mechanical disruption of the capsid and nuclear pore components	(Strunze et al., 2011)
Polyomavirus	Priming	ER	Protein disulfide isomerases	Disulfide rearrangement, exposure of VP2/3	(Gilbert et al., 2006; Schelhaas et al., 2007; Walczak and Tsai, 2011)
	Uncoating	Cytosol/ Nucleus?	Reducing environment, low calcium, chaperones	Loss of pentamers, NLS exposure, genome exposure?	(Chromy et al., 2006; Inoue and Tsai, 2011; Kuksin and Norkin, 2012a, b; Li et al., 2009)
Papilloma virus	Priming	Cell surface	Binding to GAGs of HSPG	Exposure of a cleft between capsomers (L1)	(Cerqueira et al., 2013)
	Priming	Cell surface	Cyclophilin B interaction with L2	Exposure of L2 N-terminus including furin cleavage site	(Bienkowska-Haba et al., 2009; Day et al., 2008)
	Priming	Cell surface	Furin	Cleavage of L2 N-terminus	(Kines et al., 2009; Richards et al., 2006)
	Priming/ Uncoating	Endosome	Acidification/ Cyclophilin B	Segregation of L1 from L2/vDNA complex, exposure of L2 C-terminus	(Bienkowska-Haba et al., 2012; Campos et al., 2012; Kamper et al., 2006; Smith et al., 2008)
	Priming?	TGN / ER	Unclear, possibly disulfide reduction/ rearrangement	membrane insertion and penetration?	(Campos et al., 2012; Day et al., 2013; Zhang et al., 2014)
Poxvirus	Priming	Immature virion/ Producer cell	Disulfide oxidation/ rearrangement. Possibly phosphoregulation?	Regulation of transcription machinery?	(Liu et al., 1995; Schmidt et al., 2013; Shuman and Moss, 1989)
	Priming	Endosome	Acidification	Activation of the core?	(Schmidt et al., 2013; Townsley and Moss, 2007)
	Priming/ Uncoating "Activation"	Endosome/ cytoplasm	Membrane fusion	Loss of LBs, release of immunomodulators	(Schmidt et al., 2013)
	Priming/ Uncoating "Activation"	Cytoplasm	Reducing environment, other?	Capsid expansion, reduction of capsid proteins, initiation of transcription within capsid	(Cyrklaff et al., 2007; Dales, 1963; Locker and Griffiths, 1999; Schmidt et al., 2013)
	Uncoating	Cytoplasm	Viral early gene expression, Cytosolic proteasomes	D5-recruitment to capsid, D5- and proteasome-mediated genome release, Core disintegration or disassembly	(Kates and McAuslan, 1967; Kilcher et al., 2014; Mercer et al., 2012; Yang and Moss, 2009)
Herpes virus	Priming/ Uncoating	Cytosol	Membrane Fusion, Phosphorylation?	Tegument release	(Granzow et al., 2005; Maurer et al., 2008; Morrison et al., 1998a; Morrison et al., 1998b)
	Priming	Nuclear pore	Interaction with Nups	Docking and positioning of capsid	(Copeland et al., 2009; Liashkovich et al., 2011; Trotman et al., 2001)
	Priming	Nuclear pore	Protease	Proteolytic cleavage of pUL36 at the NPC	(Jovasevic et al., 2008)
	Uncoating	Nuclear pore	Unknown	DNA translocation (requires pUL36 and pUL25)	(Jovasevic et al., 2008; Liashkovich et al., 2011; Preston et al., 2008)

cleaves inner capsid protein VI-, which sits underneath the vertex region together with other cementing proteins (Reddy and Nemerow, 2014). This event is thought to free the genome from the inner capsid wall (Russell and Precious, 1982; Ruzindana-Umunyana et al., 2002). Thus, protein VI cleavage, the cytosolic uncoating step, is a pre-requisite for uncoating at the nuclear pore (Greber et al., 1996).

During the course of this cytosolic uncoating step Ad capsids are transported along microtubules (MTs) to the nucleus (Suomalainen et al., 1999). Particles dock to the nuclear pore complex (NPC) via Nup214 (Cassany et al., 2014; Trotman et al., 2001). Capsids are disassembled and the genomes imported. Like fiber shedding, capsid disassembly is an active process that relies on mechanical force (Strunze et al., 2011). Kinesin 1 is recruited to the sub-viral particles via kinesin-light-chain binding to pIX, while kinesin heavy chain binds nucleoporin Nup358 and activates the motor (Cho et al., 2009). Consequently, NPC components and sub-viral particles are pulled towards the plus-end of NPC-proximal microtubules. This results in mechanical disruption of the subviral particle and breakdown of NPC integrity thereby increasing the permeability of the nuclear envelope (Strunze et al., 2011).

By this mechanism the viral genome is mechanically uncoated and nuclear import facilitated. Although a role for classical import machinery (Greber et al., 1997; Saphire et al., 2000) and molecular chaperones has been demonstrated (Saphire et al., 2000), the mechanism of vDNA and associated protein nuclear import remains incompletely understood.

In sum, adenovirus uncoating relies on multiple priming steps during morphogenesis, in the extracellular space, upon binding of their receptors, within endosomes, inside the cytosol, and at the nuclear pore. For successful genome delivery, such a complicated process must be highly coordinated both spatially and temporally, perhaps explaining recent single-genome tracking experiments which indicate that nuclear import is inefficient with misdelivery of vDNA to the cytosol being common (Wang et al., 2013a).

Polyoma- and Papillomaviruses

Polyomaviruses (PYs) and Papillomaviruses (PVs) are structurally related non-enveloped viruses with icosahedral capsids ($T=7$) built from 72 pentameric capsomers (Cerqueira and Schelhaas, 2012). Pentamer units are stabilized by hydrophobic interactions between major capsid proteins PY VP1 or PV L1, and strengthened by invasion of the terminal arms of neighboring pentamers. Disulfide bonds between the pentamers are likely formed after lysis and exposure to the oxidizing extracellular space. Minor capsid proteins PV VP2/VP3 or PY L2 are located in the central cavity of the capsomer and are required to incorporate DNA during morphogenesis. For successful uncoating, these many capsid-stabilizing interactions need to be altered or broken.

Polyomaviruses

Crystallographic data suggests that PYs do not change their structure upon contact with receptors (Stehle and Harrison, 1997; Stehle et al., 1994). This suggests that these viruses, unlike adenoviruses, are not primed for uncoating at the cell surface. Upon endocytosis, PYs are routed to the endoplasmic reticulum (ER) in a receptor-dependent fashion (Engel et al., 2011; Kartenbeck et al., 1989; Qian et al., 2009). During this journey, PYs encounter low pH, proteases, and additional endosomal cues involved in the uncoating of other viruses. However PYs do not undergo proteolytic cleavage (Jiang et al., 2009; Schelhaas et al., 2007) and with the exception of BK virus(Jiang et al., 2009), remain structurally unchanged until reaching the ER.

Most information on the initiation of uncoating within the ER has been obtained by studies of mouse polyomavirus (mPY) and simian virus 40 (SV40). In the case of SV40, the protein disulfide isomerase (PDI) ERp57 isomerases disulfide bonds that link the vertex of adjacent pentamers (c9–c9). The isomerization reaction results in c9–c104 intrachain disulfides, thereby destabilizing the vertex–pentamer interaction (Schelhaas et al., 2007). Mouse PY uses a similar strategy but different PDIs (Gilbert et al., 2006; Walczak and Tsai, 2011). Generally, PYs engage ER chaperones such as ERp57, ERp29, PDI, BiP, BAP31, and DNAJ family members (Daniels et al., 2006; Geiger et al., 2011; Goodwin et al., 2011; Magnuson et al., 2005; Schelhaas et al., 2007; Walczak and Tsai, 2011).

The thiol-disulfide oxido-reductases serve to expose the minor capsid proteins VP2/VP3 whose hydrophobic N-terminus (VP2) binds to and inserts into the ER membrane (Daniels et al., 2006; Magnuson et al., 2005). VP2 membrane insertion is required for initiation of membrane penetration. It was demonstrated that SV40 recruits the ERAD component BAP31 via a positively charged residue in the VP2 transmembrane domain that mimics misfolded membrane proteins (Geiger et al., 2011). The process of translocation requires both ERAD factors BiP, Bap29, and BAP31 in the case of SV40 (Geiger et al., 2011; Schelhaas et al., 2007), derlin-2 for mPY, (Lilley et al., 2006), and cytosolic chaperones (Walczak et al., 2014). While the mechanism of translocation remains elusive, interestingly, SV40 has been reported to reach the host cell cytosol largely intact (Inoue and Tsai, 2011).

Once there, a second major uncoating step occurs during which the capsid structure is disrupted (Inoue and Tsai, 2011; Kuksin and Norkin, 2012a). This cytosolic disassembly step culminates in destabilization of VP1 interchain-disulfides and detachment of calcium from the capsid. These changes are thought to be assisted by the reducing conditions and low calcium concentration within the cytosol. Partial capsid disruption exposes nuclear localization signals (NLS) in capsid proteins and leads to nuclear targeting of SV40 subviral particles (Nakanishi et al., 1996).

It has been suggested that polyomaviruses enter the nucleus as a more or less intact entity (Clever et al., 1991; Yamada and Kasamatsu, 1993). Yet it is unclear how a 50 nm particle can enter the nucleus through the NPC that has a size restriction of ~39 nm (Pante and Kann, 2002). Recent data suggests that cytosolic capsid disassembly is more substantial than previously thought (Inoue and Tsai, 2011), and that vDNA might enter the nucleus as a subviral entity devoid of VP2/VP3 proteins (Kuksin and Norkin, 2012a; Kuksin and Norkin, 2012b). As the mechanisms of nuclear translocation and the configuration/composition of the subviral structure inside the nucleus is not known, further research will be required to clarify this last step of polyomavirus uncoating and nuclear translocation of the genome.

Papillomaviruses

In contrast to PYs, PVs initiate their uncoating program at the plasma membrane. For HPV-16 – the most prevalent cancerogenic PV (Bosch and de Sanjose, 2003) – priming is initiated at the cell surface. Binding to heparan sulfate proteoglycan (HSPG) receptors initiates the first structural changes within the capsid resulting in the exposure of an L1 epitope sitting between capsomers (Cerqueira et al., 2013). The functional consequence of this rearrangement is currently unclear. However, interaction of the virus capsid with cyclophilin B – a cell surface peptidylprolyl isomerase – exposes the N-terminus of L2 (Bienkowska-Haba et al., 2009; Day et al., 2008) that contains a furin cleavage site (Richards et al., 2006). Subsequent furin-mediated cleavage of the L2 N-terminus is required for infection possibly by allowing binding to a secondary, HSPG-independent receptor. These early priming events have been shown to occur both in cell culture and *in-vivo* (Kines et al., 2009; Richards et al., 2006).

After endocytic uptake, PV capsids accumulate in late endosomes (Bergant Marusic et al., 2012; Schelhaas et al., 2012). Low pH serves as an important trigger for additional structural changes required for uncoating: First, HPV-16 and HPV-33 expose an epitope normally buried within the capsid (Sapp et al., 1994; Spoden et al., 2008). Second, the putative membrane-destabilizing L2 C-terminus (Kamper et al., 2006; Smith et al., 2008) and the viral genome are

exposed (Campos et al., 2012). This is the most dramatic uncoating step in the life cycle of PVs as it leads to substantial segregation of L1 from the L2/vDNA complex. However, capsids do not appear to be completely disassembled at this stage, as they can be visualized in lysosomal compartments late during infection (Schelhaas et al., 2012). Low pH is likely the key player in this process as exposure of PV virions to low pH *in-vitro* increases the accessibility of the genome to DNAseI (Smith et al., 2008) and endosomal proteases such as the cathepsins do not seem to play a role in uncoating (Dabydeen and Meneses, 2009; Richards et al., 2006).

While the mechanism of L1/L2 vDNA segregation is not known, the viral DNA and L2 (as well as small amounts of L1 (DiGiuseppe et al., 2014)) are transported to the trans-golgi network (TGN) (Day et al., 2013). Golgicide A treatment blocks infection and leads to the accumulation of the pseudogenome in late endosomal compartments (Day et al., 2013). It was recently shown that the endosome to TGN transition requires the activity of the cellular membrane-associated protease γ-secretase (Karanam et al., 2010; Zhang et al., 2014). Interestingly, γ-secretase does not cleave structural proteins of the capsid, but rather an unknown cellular substrate required for endosome-to-TGN transport. Hence this protease is required for proper trafficking rather than uncoating.

How and where penetration of PY viruses occurs is unclear. Experimental evidence suggests that a recently identified NLS and the putative membrane lytic c-terminus of L2 are required (DiGiuseppe et al., 2014; Kamper et al., 2006; Mamoor et al., 2012). Another model suggests that an N-terminal L2 transmembrane domain facilitates membrane insertion and penetration (Bronnimann et al., 2013). The requirement of a cellular reductase and/or PDIs for penetration has been demonstrated (Campos et al., 2012) and an intramolecular disulfide-bond important for penetration is in proximity of the L2 N-terminal motif (Campos and Ozbun, 2009). In support of this, proximity ligation assays of incoming L2 suggest that the subviral complex enters the ER and interacts with ER-resident BiP and PDI (Zhang et al., 2014). Even though the molecular mechanism of penetration is unclear, it is likely that the subviral particle undergoes additional structural changes within the TGN or ER that render it competent for L2 membrane insertion and subsequent translocation of the L2/vDNA complex. Thus, it is likely that TGN – which is absolutely required - and ER-resident cellular proteins are involved in this step of uncoating explaining the need to pass through these compartments. Such a mechanism would share conceptual similarity with the ER-mediated uncoating of polyomaviruses.

Once in the cytoplasm, vDNA and L2 are transported into the nucleus (Day et al., 2004). This requires host cell progression through early mitosis as the L2/vDNA complex can only access its final destination during nuclear envelope breakdown (NEB) explaining why only mitotic cells are infected (Aydin et al., 2014; Pyeon et al., 2009). While transport through nuclear pores is not involved (Aydin et al., 2014), direct transport through the ER to the nucleus has been suggested as an alternative model (Zhang et al., 2014).

In sum, PV are primed for uncoating through a number of conformational changes at the PM followed by substantial endosome-mediated uncoating. Upon transport of the L2/vDNA complex to the TGN and ER, additional undefined disassembly steps or rearrangements may occur leading to translocation of the subviral complex to the cytosol. It is not clear whether further uncoating steps occur in the cytoplasm or during nuclear entry. What is clear is that in contrast to PYs, PVs undergo major uncoating steps before accessing the cytoplasm (Merle et al., 1999). Such a strategy is somewhat surprising – especially considering the fact that the viral DNA may be required to wait for NEB for a considerable amount of time without its protective capsid. It will be of interest to address how the L2/vDNA complex can avoid detection by cytosolic innate DNA-sensors prior to gaining access to the nucleus. Direct penetration from the ER into the nuclear compartment during NEB would be an attractive model but has yet to be experimentally addressed.

Enveloped virus uncoating

Unlike non-enveloped virus capsids, enveloped virus capsids cloaked by a lipid membrane are not directly exposed to uncoating cues during spread from one cell to another or during transit within the endosome system. Thus, capsids of enveloped viruses have fundamentally different roles from those of non-enveloped viruses in that they mostly respond to uncoating cues after fusion.

Poxviruses

Pox virions are large, barrel-shaped particles that contain a complex, proteolytically-processed, disulfide-linked, dumbell-shaped capsid – generally referred to as the virus core. It is flanked by two proteinaceous lateral bodies (LBs) which fill the concavities between core and membrane. The core contains the 200 kb dsDNA genome and is composed of at least 47 structural proteins and core enzymes (Condit et al., 2006; Moss, 2007). Vaccinia virus (VACV), the model poxvirus and variola vaccine has been studied most intensively and we will therefore focus exclusively on VACV uncoating.

As poxvirus genome-release and replication occur in the cytoplasm, these viruses face particular challenges related to innate DNA-sensing and antiviral response. As a consequence, they have evolved a highly regulated, multi-step core-disassembly program. Elegant biochemical studies in the 1960s demonstrated that poxvirus uncoating is a two-step process (Joklik, 1964a, 1964b): The first stage commences immediately after membrane fusion and is referred to as “core activation”, the second stage, cytoplasmic genome release, occurs after a lag-period due to the requirement for expression of a virus-encoded uncoating protein (Joklik, 1964a, 1964b; Kates and McAuslan, 1967; Prescott et al., 1971).

Structural changes within the core prior to the fusion of the VACV membrane have not been described. However, there is indirect evidence that the capsid may be modified as macropinosomes are acidified during endocytosis (Schmidt et al., 2012): Pretreatment of virus with pH 5 prior to adsorption enhances the kinetics of infection without by-passing the need for endosomal acidification and fusion (Townsley and Moss, 2007). This suggests that VACV entry involves two acid-dependent steps. More recently, the presence of a proton channel in the mature virion (MV) membrane was proposed based on the observation that fluorescence of EGFP-cores in intact MVs was reversibly quenched when exposed to low pH (Schmidt et al., 2013). The identity of this channel is unknown and its existence therefore speculative. However, it is conceivable that pre-fusion acidification might prime the core for efficient uncoating or early transcription. Such a function has recently been described for the proton channel of influenza virus (Stauffer et al., 2014).

Immediately upon fusion cores undergo dramatic morphological changes (Dales, 1963; Schmidt et al., 2013). Cores expand and change from biconcave to oval shape, leaving behind the two LBs at the endosomal membrane where fusion occurred (Cyrklaff et al., 2007; Dales, 1963; Hollinshead et al., 1999; Schmidt et al., 2013). During this “core activation” step, disulfide-bonds within and between core structural proteins 4a, 4b, possibly VP8, and the LB scaffold protein F17 are reduced. *In vitro* studies have identified additional disulfide-bonded core proteins that may be reduced *in-vivo* as well (Locker and Griffiths, 1999; Schmidt et al., 2013).

VACV cores can be generated *in-vitro* by treating MVs with NP-40 and reducing agent. With the exception of a lack of LB detachment, this treatment leads to similar morphological changes as observed during activation *in vivo*. This suggests that entry into the reducing cytoplasm is the trigger for activation. However, that *in vitro* lipid-solubilization and reduction is not sufficient to detach LBs suggests their removal depends on fusion (Easterbrook, 1966; Schmidt et al., 2013).

Released VACV cores have the unique ability to produce early viral transcripts prior to their disassembly. Early gene transcription and mRNA export from cores is initiated immediately after fusion via the action of viral RNA polymerases and transcription factors that are pre-bound on early promoters inside the core (Kates and McAuslan, 1967; Yang and Moss, 2009). As early viral gene expression is an absolute pre-requisite to initiate the second phase of uncoating, the activation of the transcription machinery must be considered a priming step.

Two RNA polymerase subunits are disulfide-bonded in MVs (Schmidt et al., 2013) making it tempting to speculate that disulfide reduction during activation triggers early transcription. Supporting this, early transcription from VACV MVs can be induced artificially in the presence of NP-40, dithiothreitol (DTT), and nucleotides (Shuman and Moss, 1989). In addition to reduction, experimental evidence suggests that the early transcription machinery may be regulated by phosphorylation. VACV packages both a kinase (F10) and a phosphatase (H1)(Liu et al., 1995; Szajner et al., 2004), and phosphatase-deficient particles are defective for early gene transcription (Liu et al., 1995).

As a consequence of activation, a set of early viral transcripts are released from the core and translated on host ribosomes. How viral transcripts leave the core to initiate the second phase of uncoating is not known. Cryo-electron tomography suggests the presence of pore-like structures with a diameter of ~7 nm through which the viral mRNA could potentially escape (Cyrklaff et al., 2005). Without these early gene products, the uncoating program arrests, cores accumulate in the cytoplasm (Dales, 1965), and the viral DNA remains inaccessible to DNAseI in cell lysates (Joklik, 1964b).

Collectively, this early data from the 1960s suggested the existence of a viral "uncoating protein" (Joklik, 1964a, 1964b). Recently, the primase/helicase protein D5 was identified as the viral uncoating factor (Kilcher et al., 2014). In the absence of newly synthesized D5 the viral genome remains inaccessible to cytosolic enzymes and does not leave the cores, which remain in the cytosol as stable structures. The molecular mechanism of D5-mediated genome release remains unclear. However, super-resolution microscopy showed that D5 localizes to distinct lateral subdomains of incoming cores suggesting a defined exit portal for the viral DNA exists. As the replicative helicase, D5 is also required for DNA replication (Boyle et al., 2007; De Silva et al., 2007; Evans et al., 1995) albeit with different enzymatic requirements: the C-terminal AAA+ ATPase domain – a putative SFIII helicase – is required for both uncoating and replication of the genome, whereas the N-terminal primase is dispensable for uncoating (Kilcher et al., 2014). The involvement of a hexameric ring-like AAA+ ATPase (Boyle et al., 2007; Sele et al., 2013) in uncoating suggests that mechanical force or major structural rearrangements are required for genome release. Whether D5 destabilizes the capsid structure or actively removes the genome from a portal remains to be determined.

Previously, it was assumed that expression of the viral uncoating protein would be sufficient to release the genome. Thus, a possible contribution of cellular proteins was not addressed. Recent studies demonstrated that host proteasome activity was also required for core degradation and genome release. In the presence of proteasome inhibitors, the uncoating factor D5 is expressed. However, viral cores are not disassembled (Mercer et al., 2012), genomes remain protected, and do not disassociate from cores (Kilcher et al., 2014). As new ubiquitination is not required and core proteins are packaged in an ubiquitinated state, we speculate that proteasomes act directly on core proteins (Mercer et al., 2012). As both proteasomes and D5 are necessary, but not sufficient, to mediate uncoating, investigation of the interplay between D5 and proteasomes will hopefully shed light on these final steps of poxvirus genome release.

Herpesviruses

Herpesviruses are a family of large, enveloped, linear dsDNA viruses with distinct morphology. An icosahedral capsid containing the genome is surrounded by a layered multi-protein assembly, the tegument, and enclosed within the virus proteolipid membrane. Most information on herpesvirus uncoating was obtained using the alphaherpesvirus, herpes simplex virus 1 (HSV-1). Given the diversity of HSV-1 cell-type and virus-specific entry pathways and that no changes to the tegument or capsid prior to fusion have been reported, only post-fusion events will be discussed here.

Concomitant with fusion, tegument proteins are released into the cytosol (Maurer et al., 2008). The mechanism by which this occurs is not understood. Although not essential in cell culture (Coulter et al., 1993; Purves et al., 1987), *in-vitro*, tegument dissociation depends on the kinase activity of tegument resident kinases pUL13 and pUS3 (Morrison et al., 1998a, 1998b). Uncoated capsids and remaining associated inner tegument are transported along microtubules (MTs) in a dynein-dependent manner (Sodeik et al., 1997). The outer capsid protein pUL35 (Desai et al., 1998; Douglas et al., 2004), inner tegument proteins (Wolfstein et al., 2006), tegument protein pUL14 (Yamauchi et al., 2008) and ICP0 (Delboy and Nicola, 2011) facilitate targeting to the nuclear pore, where nucleocapsid uncoating occurs.

For HSV-1, binding to the NPC requires imortin β and a functional RanGTP/GDP cycle (Ojala et al., 2000). Capsid proteins pUL25 (Pasdeloup et al., 2009) and pUL36 (Copeland et al., 2009) have been implicated in mediating the interaction of nucleocapsids with nuclear pore protein Nup214 and NPC filament protein Nup358.

While some of the players involved in genome uncoating at the nuclear pore are known, the mechanism is poorly understood. Temperature-sensitive mutants of pUL25 (Preston et al., 2008) and pUL36 (Jovasevic et al., 2008) dock to the NPC, but fail to release their genome. This suggests that their role in NPC docking and uncoating can be separated.

Although proteolytic cleavage of pUL36 at the NPC seems to be required for uncoating *in-vivo* (Jovasevic et al., 2008), the trigger of DNA release is currently unknown (Liashkovich et al., 2011). *In-vitro*, genome release can be provoked in the presence of guanidine-hydrochloride (Newcomb and Brown, 1994) or by heating of isolated capsids (Newcomb et al., 2007). Capsids also uncoat in the presence of isolated nuclei, cytosolic extract, and an ATP regenerating system (Ojala et al., 2000). Similar to phage DNA ejection, the genome is likely translocated in a polarized fashion (Newcomb et al., 2009), although other mechanisms have been proposed (Shahin et al., 2006).

The driving force for DNA translocation is not currently known, but several models have been suggested (reviewed in Liashkovich et al., 2011). These include: pressure-driven release, passive ratchet-like diffusion, crowding effects, and a pull-in mechanism involving transcribing RNA-polymerases, akin to T7 bacteriophage (Kemp et al., 2004). *In vitro* studies indicate that the genome exits the capsid through the packaging portal (Newcomb et al., 2009), which is likely to hold true *in-vivo* as well. To this end, the portal protein pUL6 that sits at one defined capsid vertex (Cardone et al., 2007; Chang et al., 2007), may be important for DNA translocation. In addition to providing the translocation channel, it could also

provide for proper orientation of the capsid on the NPC (Liashkovich et al., 2011).

In sum, the combination of *in vitro* and *in vivo* studies have provided insight into viral and cell factors required for herpesvirus uncoating. Further work should seek to define the role of tegument, if any, additional cell factors, and the mechanism by which the genome is translocated from the capsid.

Uncoating and innate sensing of DNA

Not surprisingly, the host immune system has evolved numerous strategies to interfere with the process of virus genome uncoating. TRIM5α, for example, was discovered as an innate restriction factor that counteracts infection of Old World Monkeys by HIV-1. TRIM5α binds incoming capsids directly and leads to their premature fragmentation in a proteasome-dependent or independent manner (Malim and Bieniasz, 2012; Nakayama and Shioda, 2010).

Proper timing of DNA release is an important aspect of the virus disassembly program. Premature DNA release not only leads to abortive infection due to genome misdelivery, but also exposes viral nucleic acids to pattern recognition receptors (PRRs) of the innate immune system.

Recently, many DNA-specific PRRs have been identified and characterized. These include members of the Toll-like receptor family that survey the endosomal space (reviewed by Wu and Chen, 2014). Cytosolic detection of DNA is mediated by several pathways including: RNA polymeraseIII/RIG-I, cGAS/STING/TBK1, or the other putative cytosolic sensors DAI, IFI16, DDX41, and DNA-PK (Wu and Chen, 2014). Many of these sensor-pathways converge upon activation and nuclear translocation of interferon regulatory factors IRF-3, IRF-7, and of NF-κB. Subsequent transactivation of type I interferons and other interferon-inducible genes promote an antiviral state. In some cell types including macrophages, cytosolic DNA is sensed by AIM2 leading to inflammasome assembly. Subsequently, interleukins (IL-1β and IL-18) are activated and released (Hornung and Latz, 2010; Wu and Chen, 2014).

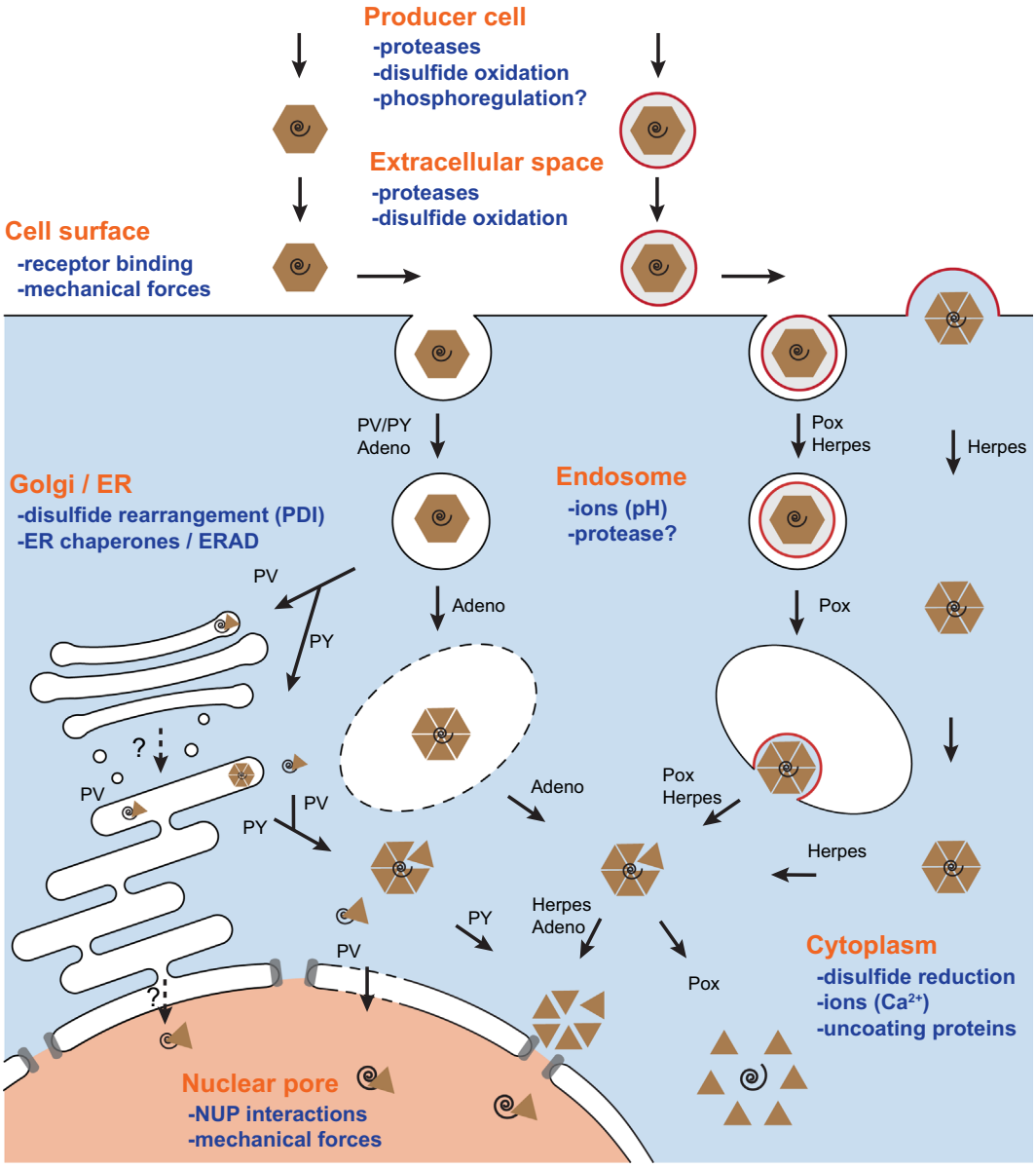

Fig. 3. Capsid priming and genome uncoating of DNA viruses. Priming and uncoating pathways of the non-enveloped and enveloped viruses discussed throughout this review are summarized. The location of capsid priming (orange text) and the molecular cues to which capsids respond at these sites (bold navy text) are indicated. The routes taken by specific virus families (black text) are mapped. See text for details.

In turn, many viruses have evolved effector proteins to block the function of key players within these pathways. It reasons, that the earlier viruses counteract innate immune signaling, the more likely they are to avoid induction of an antiviral state. Thus, most viral innate effectors are expressed during an early phase of infection.

However, the ideal strategy would be to counteract innate sensing prior to genome release. This can be achieved when capsid or capsid-associated proteins that are released from incoming virions exert immediate effector function. In such a scenario, the uncoating program and the subversion of innate sensing pathways would be mechanistically linked. There is currently no evidence supporting such a mechanism for any of the non-enveloped DNA viruses. For enveloped viruses however, immediate effector functions have been demonstrated and we will therefore focus on herpesviruses and poxviruses in the next section.

DNA sensing of herpesviruses involves TLR9 (Krug et al., 2004a, 2004b; Lund et al., 2003), IFI16, and STING. Sensing can occur in both the cytosol and in the nucleus of infected cells (Horan et al., 2013; Li et al., 2012). Alphaherpesvinae package around 20 tegument proteins (Kelly et al., 2009; Loret et al., 2008), eight of which have ascribed immune-modulatory functions. HSV-1 tegument proteins pUL13, pUL48 (VP16), and ICP0 all interfere with IRF-3 function through direct binding, which leads to sequestration of nuclear IRF3 (Orzalli et al., 2012) or inhibition of IRF3 interaction with its transcriptional co-activator CBP (Hwang et al., 2009; Xing et al., 2013).

Tegument proteins ICP34.5 and pUL36 target TBK1. ICP34.5 disrupts the interaction of TBK1 with IRF-3 to prevent STING dependent type I interferon responses (Abe et al., 2013; Verpooten et al., 2009), whereas pUL36, deubiquitinates TRAF3 preventing the recruitment of TBK1 (Wang et al., 2013b).

NF-κB activation is targeted by tegument proteins pUL41 (Cotter et al., 2011; Suzutani et al., 2000) and pUL48 (Xing et al., 2013) and US3 was shown to interfere with NF-κB and IRF-3 activation downstream of TLR signaling (Peri et al., 2008; Sen et al., 2013). An additional HSV tegument protein targets the RIG-I pathway: US11 physically interacts with endogenous RIG-I and MDA5 to prevent interaction with MAVS. Thereby it blocks downstream activation of IRF-3 and subsequent IFNβ production (Xing et al., 2012). For these experiments, innate sensing was activated by Sendai virus infection. It remains unclear whether this mechanism also contributes to HSV pathogenesis. Lastly, ICP0 appears to interfere with the IFI16 DNA-sensing pathway preventing HSV-1-induced IFNβ induction. While the exact mechanism is still controversial it seems to involve IRF-3 and IRF-7 (Lin et al., 2004; Melroe et al., 2004; Orzalli et al., 2012; Paladino et al., 2010).

The presence of such a large number of immune-modulators indicates that DNA sensing is an important hurdle for herpesviruses to overcome. As such, these tegument proteins are not only delivered by incoming virus, but produced early during viral infection. It is generally assumed that incoming tegument protein is not a major contributor to immune evasion since cells infected with UV-inactivated herpesvirus, or in the presence of cycloheximide or actinomycinD induce the expression of interferon-responsive genes (Nicholl et al., 2000; Preston et al., 2001).

While this data demonstrates the need for sustained expression of these effectors, it does not exclude the importance of their delivery as components of the tegument. Immediate effector delivery may serve as a first line of defense, used to hold back the innate immune response until immediate early genes are expressed. To assess this experimentally, the innate responses to UV-inactivated virus in the presence or absence of specific tegument proteins would need to be compared.

An interesting recent example of this is the tegument protein pUL83 of human cytomegalovirus (HCMV). UL83 interacts with IFI16, inhibits its oligomerization, and thereby prevents antiviral response via STING-TBK1-IRF-3. UV-inactivated ΔUL83 virus induces a three-fold increase in the production of IFNβ compared to wt UV-inactivated virus, demonstrating a direct role of this tegument protein in supressing cellular innate immune responses (Li et al., 2013).

In contrast to herpesviruses, poxviruses release and replicate their genome in the cytosol. Given this exposure of a large amount of DNA, it comes as no surprise that poxviruses encode a handful of proteins to counteract DNA sensing, TLR-, and interferon-signaling pathways. The VACV strain western-reserve strain encodes no less than 31 immune modulators (recently reviewed by Smith et al., 2013). With regards to cytosolic DNA sensing, they target IRF-3, NF-κB, RIG-I/MAVS, and DNA-PK (Smith et al., 2013).

As we have discussed in the uncoating section, poxviruses are unique in their ability to express early genes from the intact viral core. By expressing both the viral uncoating protein and the majority of immune modulators from early promoters, VACV ensures that genomes are not exposed before the encoded immune evasion factors disable immune signaling pathways. Uncoating and immune evasion are therefore synchronized by the early gene-transcription machinery.

In addition to these early effectors, the virus has evolved an immediate-early strategy analogous to the herpesvirus tegument. The virion-associated phosphatase, VH1, was shown to dephosphorylate p-STAT1 and mitigate interferon-γ responses (Mann et al., 2008; Najarro et al., 2001). While VH1-mediated immune modulation was found to depend on virus uncoating, it was independent of early gene expression (Najarro et al., 2001). This finding suggested that VH1 associated with incoming virions was responsible for this action. Recently, the mechanism was solved: VH1 is packaged inside the VACV lateral bodies (LBs) as an effector protein, which is released upon degradation of LB scaffold protein F17 (Schmidt et al., 2013). Since LB disassembly and VH1-mediated inhibition of STAT1 nuclear translocation depend on proteasome function, the authors hypothesize that VH1 is activated by proteasome-mediated release from LBs. Whether VACV delivers additional immune effectors by this mechanism remains to be determined.

Given their large size and unusual virion structure, herpes- and poxviruses are able to store a considerable amount of protein outside the capsid that can be deposited into the host cytosol immediately after fusion. Whether smaller, non-enveloped DNA viruses package and subsequently deliver viral immune effectors remains to be determined.

Conclusions and outlook

The primary goal of all viruses is to deliver a replication competent genome to the right cellular location at the right time. The examples discussed here highlight the various spatial/temporal strategies employed by adeno-, papilloma-, polyoma-, pox-, and herpesvirus families to assure successful infection (Illustrated in Fig. 3). As metastable particles, viruses are like puzzle boxes, in which each step of the uncoating program is required for the next. The cell unwillingly and unknowingly solves the puzzle as cellular endocytosis and trafficking machineries move the virus closer to its preferred site of genome release.

While much is known about the uncoating programs used by these pathogens, there remains a major gap in our mechanistic understanding of genome translocation from the capsid. In light of the assembly-uncoating paradox, it will also be important to begin investigation of the link between viral morphogenesis and uncoating. Not only to uncover additional cell and viral factors that contribute to the solution of this paradox, but to define how capsid metastability is 'built in' to virus capsids.

Finally, amantadine – a selective inhibitor of the influenza virus M2 channel – blocks uncoating of certain influenza strains and has long been used as an FDA approved anti-influenza drug before

most strains developed resistance (Ison, 2011; Maugh, 1976). Thus, given the number of steps involved in the uncoating of each of these viruses, it will be of interest to explore novel antiviral strategies that target virus uncoating.

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