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Suomalainen, Maarit ; Greber, Urs F

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Maarit Suomalainen[1] and Urs F. Greber[1,2]

[1] Institute of Molecular Life Sciences, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

[2] corresponding author:

urs.greber@imls.uzh.ch, telephone: +41 44 635 48 41, Fax: +41 44 635 68 17

Short title: Picornavirus, polyomavirus and adenovirus uncoating

Abstract

Non-enveloped viruses enclose their genome in capsids built of repetitive polypeptides interlinked with cementing proteins, divalent cations or disulphides. Interactions are broken in a stepwise manner during entry into cells leading to genome uncoating. Receptor or proteases induce conformational changes in case of rhinovirus, poliovirus or adenovirus, and thereby provide direct uncoating cues. Chemical cues from low endosomal pH activate rhinovirus or aphtovirus, and oxido-reductases mediate disulphide reshuffling of polyomavirus. Cellular motors provide a third class of cues as shown by adenoviruses. These examples highlight the diversity of cellular factors triggering virus uncoating, and offer new perspectives for the development of antivirals.

Introduction

Viruses coevolved with the host and have acquired properties of nano-machines for transport of viral genome from an infected cell to a new host cell [1]. During its passage from the plasma membrane to the site of genome replication, a virus finds its way through barriers such as cellular membranes or a crowded cytoplasm. Furthermore, since the encapsidated genome within a virion is inactive, it has to be freed from its protective coat during entry [2]. This is only possible if viruses are metastable, primed to undergo structural rearrangements, which let them acquire new properties.

The structural rearrangements must be spatiotemporally controlled since premature disassembly of the incoming virus particle could not only compromise targeting of the genome to its site of replication, but also expose the genome to host sensors detecting invading nucleic acids [3]. Downstream signalling from the sensors can severely restrict virus gene expression and progeny production. While it is fairly well understood, how enveloped viruses fuse their lipid membrane with the plasma membrane or an endomembrane, much less is known how non-enveloped viruses breach cellular membranes and dissociate the genome from the capsid.

In this review, we use members of three virus families, picornavirus, polyomavirus and adenovirus as examples to illustrate three principles of uncoating. We discuss how a stepwise engagement with host factors, exposure to different chemical cues or mechanical forces induce structural rearrangements in non-enveloped viruses that enable the particles to force membrane barriers or release their genome into a particular cellular compartment capable of supporting virus gene expression and genome replication. For other non-enveloped viruses, such as *reoviridae*, the reader is referred to the review from M. Nibert (pages xx in this issue).

Uncoating cues from receptors

Receptor-induced conformational changes have a strong impact on uncoating of picornaviruses. This virus family comprises a diverse range of agents, including the genus enterovirus with poliovirus (PV) or human rhinoviruses (HRV), or aphthoviruses. Picornaviruses are ~30 nm in size. They have a single-strand plus sense RNA genome, and a T=1 (quasi T=3) icosahedral symmetry with 60 copies of the viral proteins VP1, VP2, VP3 and VP4. VP1-3 share similar beta-barrel folds and are connected to each other by loops on the outside and inside of the capsid. Inside connections are particularly important for capsid stability and RNA attachment, and involve the N-termini of VP1 and VP2.

Enteroviruses and possibly aphtoviruses, such as equine rhinitis A virus or the closely related foot and mouth disease virus follow a stepwise uncoating program leading to empty and eventually disintegrated capsids [4]. A well-defined sequence of structural transitions of PV occurs when the virus binds the poliovirus receptor (PVR/CD155/nectin) and this enables the virus RNA to penetrate the membrane. PVR binds within a depression around the five-fold axis of the virus [5, 6]. This is similar to intercellular adhesion molecule (ICAM-1) receptor binding to the major group of HRV [7, 8]. In both cases, structural changes in the virus occur prior to or during virus uptake by endocytosis [9, 10]. It is not clear, however, if the internalization receptor is identical with the attachment receptor, since ICAM-1 lacks a known endocytosis signal [11, 12]. Both PVR and ICAM-1 induce externalization of the N-terminus of VP1 and release of the N-myristoylated VP4 from the virion [13, 14]. This depends on capsid breathing. Breathing can be blocked by uncoating inhibitors, such as pleconaril or WIN compounds, which bind to the receptor-docking cavity in the capsid, and block the release of VP4 and RNA from the virus [15]. In the absence of drug, the exposed VP1 tethers the virus to the limiting endosomal

membrane, and VP4 forms an aqueous pore in the membrane through which the viral RNA is thought to enter the cytosol. Whether the RNA exits from the virion through one of the 5-fold or 2-fold axes is currently debated. It is worth noting that human papilloma virus 16 (HPV-16) also uses receptor binding to initiate its uncoating program. This critically involves exposure of the virion internal protein L2 and cleavage of its N-terminal sequence by furin [16], which enables the altered virus to use low pH in late endosomes to access the cytosol [17].

Chemical cues

While PV uncoating is independent of low pH, HRV uncoating and infection are pH dependent [18]. This has been shown with drugs blocking the vacuolar ATPase or by neutralizing endosomes with a biophysical protonophore mechanism [19]. Chemical cues are confined to specific compartments, for example low pH in endosomes, oxido-reducing conditions in the ER, or the cytosol with very low calcium ions and reducing conditions. This compartmentalization provides specificity and timing for uncoating.

Remarkably, minor HRV such as HRV-2 do not receive sufficient uncoating cues from binding to their receptors, the low-density lipoprotein receptor (LDLR), very-LDLR or LDLR-related protein. Receptor binding occurs at a surface exposed structure near the 5-fold axis, distinct from the canyons used by major HRV for binding to ICAM-1 [20]. HRV-2 reaches late endosomes and undergoes antigenic conversion at pH 5.4 [21, 22]. This is thought to dissociate the virus from the receptor, and trigger release of the N-terminal amphipathic domain of VP1 and VP4 [23]. VP4 is thought to form ion permeable membrane channels, similar to VP4 channels from poliovirus [14]. Experiments using liposomes decorated with very-low density lipoprotein receptor fragments showed that the release of the viral genome occurs only under low pH conditions, thereby establishing that low pH and receptor binding are sufficient to

deliver the viral genome through the liposomal membrane [24]. After releasing the RNA, empty HRV particles remain in endosomes and get degraded in lysosomes [25]. Collectively, this suggests a model where the RNA of HRV-2 is released through a membrane pore formed by VP4 rather than by endosomal disruption.

Simian virus 40 (SV40) and murine polyoma virus (Py) from the family *Polyomaviridae* do not uncoat upon binding to the receptors. The receptors for these viruses are the gangliosides GM1 and GD1a, respectively [26, 27]. SV40/Py are composed of 72 pentamers of the main structural protein VP1. Inside the capsid are 72 copies of VP2/VP3 proteins and the viral double strand DNA genome of about 5 kb. C-terminal 'arms' of VP1 protrude from each VP1 monomer and extend into neighboring pentamers, thus linking the pentameric subunits together [28, 29]. The capsid is reinforced by VP1 interchain disulfide bonds and strategically positioned calcium ions [30]. The major infectious entry pathway of SV40 is not one of the 'classical' uptake pathways, such as clathrin-mediated endocytosis or macropinocytosis [31, 32]. Instead, multivalent interactions of capsid VP1 pentamers with the ganglioside glycolipid receptor induce an inward-directed membrane curvature on the plasma membrane, and a subsequent membrane fission reaction then releases a virus-carrying vesicle into the cytoplasm [32]. Virus particles travel via early and late endosomes to the ER, where they are activated for membrane penetration [33-35]. Members of the protein disulfide isomerase family are critical for the activation: ERp57 in the case of SV40, and ERp29, as well as ERp57, PDI and ERp72 in the case of Py [30, 36, 37]. These host factors catalyze partial disruption of VP1 disulfide bonds to produce structural alterations in the viral capsid that enable exposure of the VP2 hydrophobic N-terminus, which is the membrane active component of the virus [30, 36-38]. RNA interference studies further suggested that not only thiol-disulfide oxido-reductases, but also select members of the ER-associated degradation (ERAD) machinery are essential for membrane penetration of

SV40/Py, and productive infection [36, 38, 39]. When introduced into the ER membrane, a charged amino acid (glutamate 17) in the hydrophobic amino-terminus of SV40 VP2 interacts with a positively charged residue in the trans-membrane segment of the ERAD component BAP31. The amino-terminal residues, including glutamate 17 are highly conserved among polyomaviruses. As shown for SV40, the mimicry of misfolded membrane proteins recruits Bap29 and the ER chaperone Bip and leads to the formation of ER-associated foci, from which SV40 might penetrate the ER membrane [38]. The precise molecular mechanism by which the virus goes through these membrane domains is still unclear. It is interesting to note here that although SV40/Py undergo structural alterations in the ER, the particle that breaches the ER membrane is largely intact [40, 41]. This imposes special challenges on the membrane crossing reaction. In the cytosol, which contains reducing conditions and very low Ca2+, the capsids undergo further structural rearrangements, and this can be partly reproduced with isolated SV40 virions [42]. Cytosolic conditions most likely further destabilize the VP1 interchain disulfide-bond network and could induce detachment of calcium ions from the capsid, thus triggering disruptions in the capsid structure [36, 40, 41]. This enables the binding of nuclear import factors to nuclear localization sequences in capsid proteins, but it is not well established if capsid proteins or other factors mediate import of the virus genome into the nucleus [43].

Mechanical cues

Molecular motors transport viruses at the cell surface, in endosomes or the cytosol and thereby enhance infection [for recent reviews, see 44, 45, 46]. Recent imaging and tracking experiments with adenoviruses have shown that motors not only overcome diffusion barriers, but are key for virus uncoating as well [47, 48]. Adenoviruses are widespread pathogens employing a stepwise uncoating programme during entry [49, 50]. Their structure closely relates to bacteriophage PRD1 from the family of *tectiviridae* with a unique T=25 geometry secured by cementing proteins [51-

53]. The major structural protein hexon makes up the outer shell of the ~90 nm capsid, and pentameric penton base proteins localize to the vertices of the capsid acting as anchors for protruding trimeric fiber proteins. The inside of the capsid is stabilized by protein VI, which has an amphipathic N-terminal helix and has been implicated in membrane disruption during virus entry [54, 55]. Protein VI also links to the viral double-strand DNA of about 36 kb, which is condensed by proteins, VII, V and X. Following replication and assembly in the nucleus, a subset of proteins in the capsid is processed by a viral protease [56, 57]. This maturation step is key for virus infectivity, as it brings the particle into a metastable configuration capable of responding to the cellular uncoating cues.

The uncoating cues are best characterized for two closely related species C serotypes, human adenoviruses type 2 (HAdV-2) and type 5 (HAdV-5) [47, 58-61]. HAdV-2/5 initially interacts with the coxsackievirus adenovirus receptor (CAR) via fiber proteins, and subsequently engages αvβ3 or αvβ5 integrin receptors via penton base proteins [62, 63]. The two receptors have different motion patterns: CAR can engage in actomyosin-2-dependent drifts, whereas integrins are more immobile [47]. Simultaneous engagement of virus with drifting CAR molecules and immobile integrins creates a mechanical strain that leads to detachment of the fibers and exposure of protein VI [47]. The shedding of fibers and protein VI exposure are actomyosin-2-dependent, which indicates that binding of CAR or integrins to the virus *per se* is not triggering fiber or protein VI uncoating steps. Integrins are important for uncoating in two ways. Since they are immobile they provide a holding force against the drifts exerted by CAR, and second, they induce conformational changes in the virus observed as untwisting of the penton base, which could loosen fiber-penton base interactions and facilitate fiber loss [64]. Although it is not known if protein VI exits through the 5-fold axis or elsewhere in the capsid, the externalization of protein VI is necessary for escape of the virus from endosomes [47, 59, 65]. In addition to

the direct roles of αvβ3 or αvβ5 integrins in uncoating, integrin signalling may expose the virus to endosomal cues through the NPXY (asparagine-proline-any amino acid-tyrosine) motive in β3 or β5, which recruits the adaptor complex AP-2 and facilitates endocytic uptake of virus [50, 66-68]. The precise nature of the endosomes, or host and further viral factors facilitating membrane disruption are presently unknown.

Regardless of the mechanism of penetration, the cytosolic HAdV-2/5 is still largely intact, and unable to diffuse over large distances to reach the nucleus. It engages microtubule-associated motors for movement to the nuclear envelope [69-72]. Virus docks at the cytoplasmic side of the nuclear pore complex (NPC) by binding to the nucleoporin Nup214, and undergoes final disassembly [58, 73]. This step is mediated by the microtubule motor kinesin-1 [48]. The virus recruits the light chain of the kinesin motor through its minor capsid-associated protein IX. The motor gets activated upon binding to Nup358, a binding partner of Nup214 [74]. Nup358 contains a microtubule-binding domain and positions a track proximal to kinesin [75]. Strunze et al. [48] suggested that kinesin is pulling on the virus capsid against a holding force from the NPC, and thereby disrupts the capsid. This disruption process also dislodges Nup214, Nup358 and Nup62 from the NPC, and transiently increases the permeability of the NPCs for influx of solutes. It is possible that the increased permeability of the NPC promotes import of the viral genome into the nucleus [76].

Conclusions and outlook

Non-enveloped viruses are metastable and undergo conformational changes upon entry into cells leading to the uncoating of the genome. The examples from different animal virus families discussed here illustrate that viruses employ a range of different uncoating programs. Invariably, however, uncoating is a stepwise process triggered by cellular factors. Uncoating can thus be controlled in time and space with two important consequences. One is that the virus changes its outfit during the entry

process. This leads to display of proteins from the particle interior towards its surface, and empowers virus to break cellular barriers, such as membranes. A second key advantage of a stepwise uncoating process is that the virus avoids untimely exposure of innate immunity triggers, such as cytosolic DNA or RNA genomes. Avoiding the detection by innate sensors is an important aspect in the arms race between viruses and their host cells. We envision that a range of additional cellular cues will be discovered in the future for the uncoating of both non-enveloped and enveloped virus capsids. We also expect that increasingly cellular structures will be dismantled by virus infections, such as P-bodies, lipid storage granules or promyelocytic leukemia bodies. This will extend the well documented dismantling of the nuclear envelope upon adenovirus, picornavirus, rhabdovirus, parvovirus or human immune deficiency virus infections [for a recent review, see 77].

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* important conceptual contribution for non-enveloped virus uncoating

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Figures

Figure 1: Example of an uncoating program for non-enveloped viruses.

A non-enveloped virus is made up of major and minor capsid proteins and a DNA or RNA genome depicted in red, orange and black curved lines, respectively. It enters a host cell and undergoes structural changes, such as shedding of ~~sheds~~ some of its minor stabilizing proteins. This is controlled by host factors in time and space. Uncoating cues and the shedding of minor proteins expose hidden proteins from the virus (grey shapes). This leads to a gain of function for the virus, for example enabling the passage through cellular barriers. The final step of uncoating separates the genome from the capsid enabling viral transcription and replication.

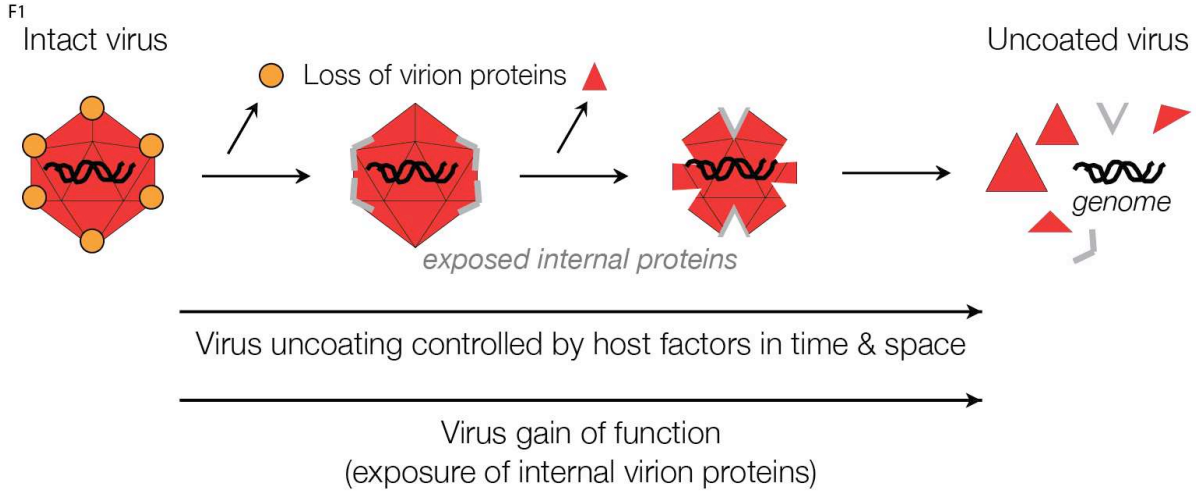

Figure 2: Schematic depiction of three classes of uncoating cues for non-enveloped viruses.

Cartoon in (a) shows an intact virus with binding sites (dark orange) for cellular proteins, for example receptors, the protein shell (red), stabilizing proteins inside of the shell (light orange) and a DNA or RNA core, which can be condensed with proteins to a nucleo-(protein) core (grey). Cartoons (b-d) depict uncoating cues from cellular receptors (b), chemicals or enzymes, such as protons, proteases or thioloxidases (c), and motors acting against holding receptors (d).

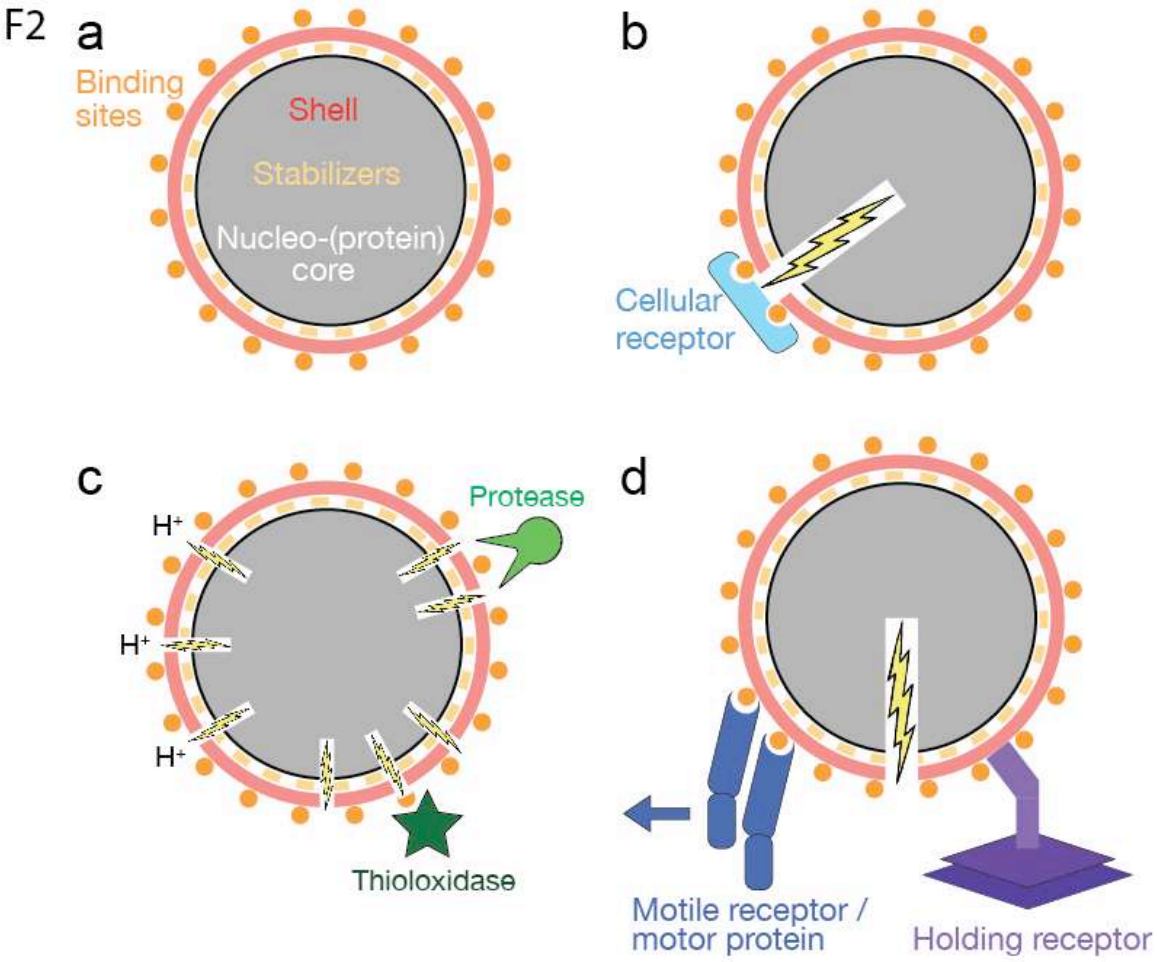

Table 1: Summary of uncoating cues used by picornaviruses, polyomaviruses and adenoviruses.

Details and abbreviations are given in the main text.

Virus	Receptor cue	Chemical cue	Mechanical cue
Poliovirus	PVR	not low pH	?
Human rhinovirus 14 / 16	ICAM-1	low pH	?
Human rhinovirus 1A / 2	(LDLR)	low pH	?
Simian virus 40	no	oxido-reductions, ERAD, low Ca^{++}	?
Mouse polyoma virus	no	oxido-reductions, ERAD, low Ca^{++}	?
Human adenovirus 2 / 5	integrin	?	acto-myosin dependent CAR drifts microtubule dependent kinesin-1 motions