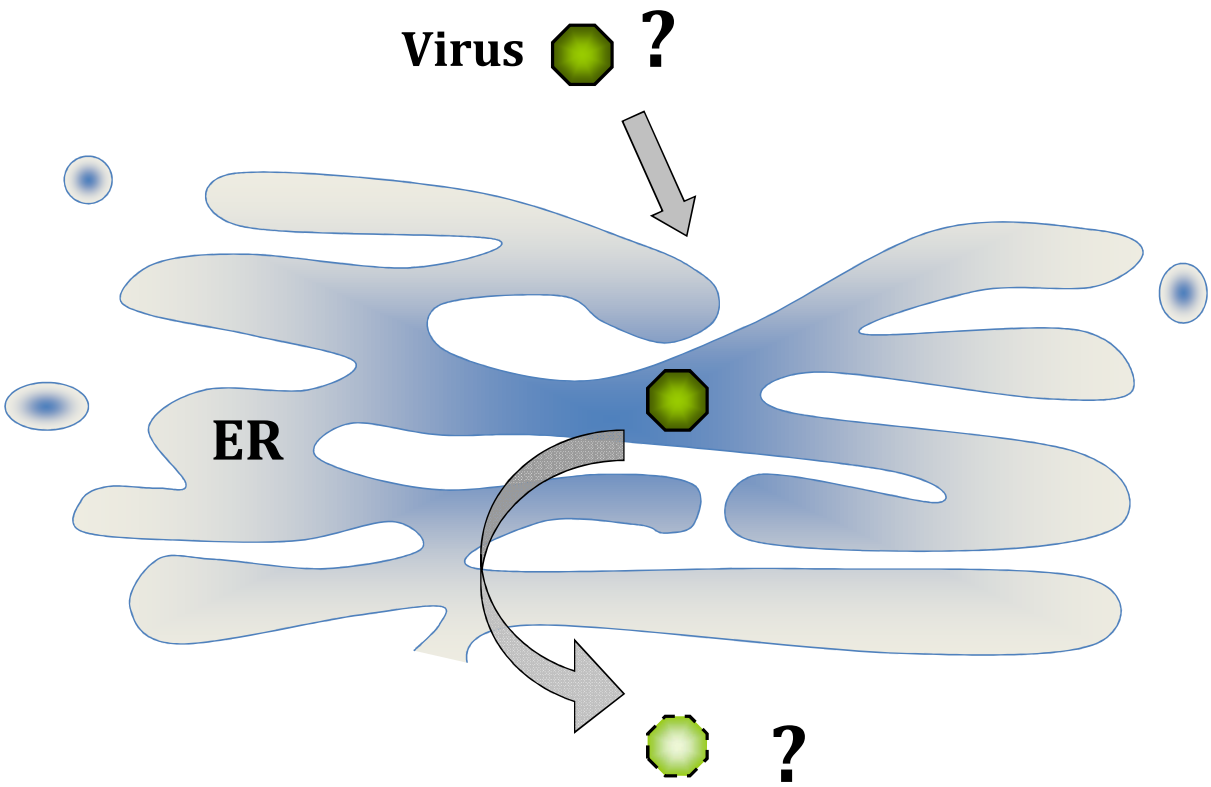
Viruses that abuse ERAD for cell entry



Master Thesis

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Outline

Summary

For millions of years viruses have survived because they take over a cell as intracellular parasites. Within a cell, a virus exploits cellular processes for its replication and reproduction. To gain access to the cells interior, different viruses can have similar but also completely diverse entry mechanisms. Polyomaviruses localize in the endoplasmic reticulum (ER) lumen where they abuses ER associated degradation (ERAD). In this process, specific ER proteins interact with the virus and induce virus uncoating and dislocation to the cytosol. This review describes in more detail how one typical virus uses the degradation mechanisms in the ER. Furthermore we speculate about other viruses that might have similar ERAD hijacking strategies.

Introduction

Viruses are tiny infectious agents that need a host cell for their survival. Packaged into small virus particles, they carry their genomic information (DNA or RNA) and essential accessory proteins. These protected virus particles (virions) mediate the transmission from host to host. A virus relies on the cellular machinery for replication and reproduction.

Before a virus can reproduce within a cell, it proceeds through a stepwise entry process. First, a solid attachment to the cell is required. Every virus has a receptor or attachment molecule what interacts with a cell membrane, this then mediates viral entry. When a virus binds to a receptor it tricks the cell into a (regulated) uptake. Many viruses, but not all, use the cellular internalization pathway which is normally used for instance for receptor recycling. The virus is triggering endocytosis and is then captured in an endosome. From the endosome it fuses with a lysosome, a vesicle that is normally used for destruction of waste material by acidification. For viruses this is the right environment, because within the lysosome the lower pH triggers the uncoating of the virus and induces the release of viral genome and proteins. For replication of the genomic information, some viruses need to enter the nucleus, while others replicate in the cytosol.

To explain the different viral infectivities in more detail, we zoom into four distinct virus types. The infectious routes of Influenza A virus, Herpesvirus, papillomavirus and polyomavirus are different in many aspects, as depicted in figure 1. Influenza A is an enveloped virus that uses the lysosome to uncoat and the ribonucleoproteins (RNPs) are released[1]. Herpes simplex virus (HSV-1) loses its envelope when it fuses at the plasma membrane. The viral capsid moves along the microtubules to the nucleus[2], where it releases its genome while docking on the nucleus. Non enveloped Human Papillomavirus 16 (HPV16) is partly destructed in the lysosome except for the DNA bound to the minor capsid protein[3]. Another non enveloped virus, polyomavirus is captured in a different type of vesicle, a caveosome and localizes with the endoplasmic reticulum for uncoating.

As for these four viruses (Figure 1), various viral infectious routes are already established. As described above, many viruses use endo- and lysosomes to enter a cell. The previously discussed viruses illustrate that they all use different routes and abuse various cellular mechanisms for entry. For some viruses it is still unknown how they can enter and exit cellular compartments and how their capsid is opened. We think that viruses also use other compartments than endo- and lysosomes for their own purpose, such as the ER. Within the

ER lumen proteins are continuously folded and refolded. Properly folded proteins are transported into the secretory pathway, whereas misfolded proteins are dislocated (retrotranslocated) across the ER membrane for degradation in the cytosol. The latter provides a potential mechanism for viruses to get cellular assistance in crossing the membrane.

The mechanisms and abilities of the ER create an advantageous environment for viruses, which could be used more often than we think. This assumption, together with related papers, is discussed in this review, leading to research proposals and new insights. To understand the relationship between the ER and viruses, we will first focus on the normal activities of the ER, concerning protein folding, quality control and degradation. We will describe polyomaviruses that use the ER as an entry mechanism. Based on polyomavirus mechanisms we try to speculate about more virus types that could use the ER for cell entry.

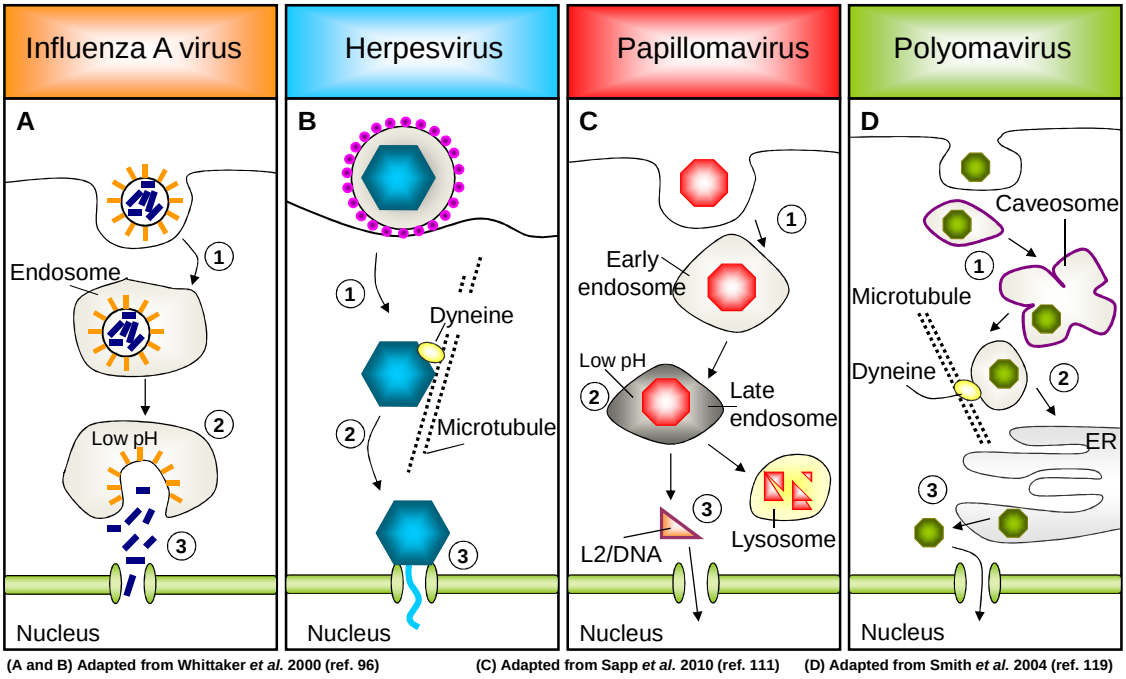


Figure 1. Four different viral entries
(A) Influenza A virus. Influenza A virus binds to the cell surface with spikes on the virus envelope. This triggers the formation of clatherin coated pits and internalization in to endosomes (Step 1). By the low pH in the endo-, lysosome the virus envelope is destructed, what triggers the release of the ribonucleoproteins, these are then transported to the nucleus (Step 2 and 3). **(B)** Herpes Simlplex Virus1 (HSV-1). The envelope of herpesviruses fuses with the cell plasma membrane and release the viral capsid into the cytosol (Step 1). The virus uses dyneine transport on microtubles to travel to the nucleus (Step 2). At the nucleus, it uses its own proteins to attach to the nuclear pore complexes (NPC) and transfers its genome (Step 3). **(C)** Papillomavirus (HPV-16). For HPV-16 it enclosed by the plasma membrane (Step 1). The low pH in the late endosome causes a disruption in the L1 capsid proteins and stimulates the release of DNA associated with minor capsid protein L2 (Step2 and 3). **(D)** Polyomavirus (SV40). The virus is taken up by the cell and the vesicle is surrounded by caveolin-1, called caveosome (Step 1). The virus moves over the microtubules towards the ER (Step 2). Inside the ER the virus is uncoated and the virus is dislocated to the cytosol (Step 3). The capsid releases the genomic information to enter the nucleus.

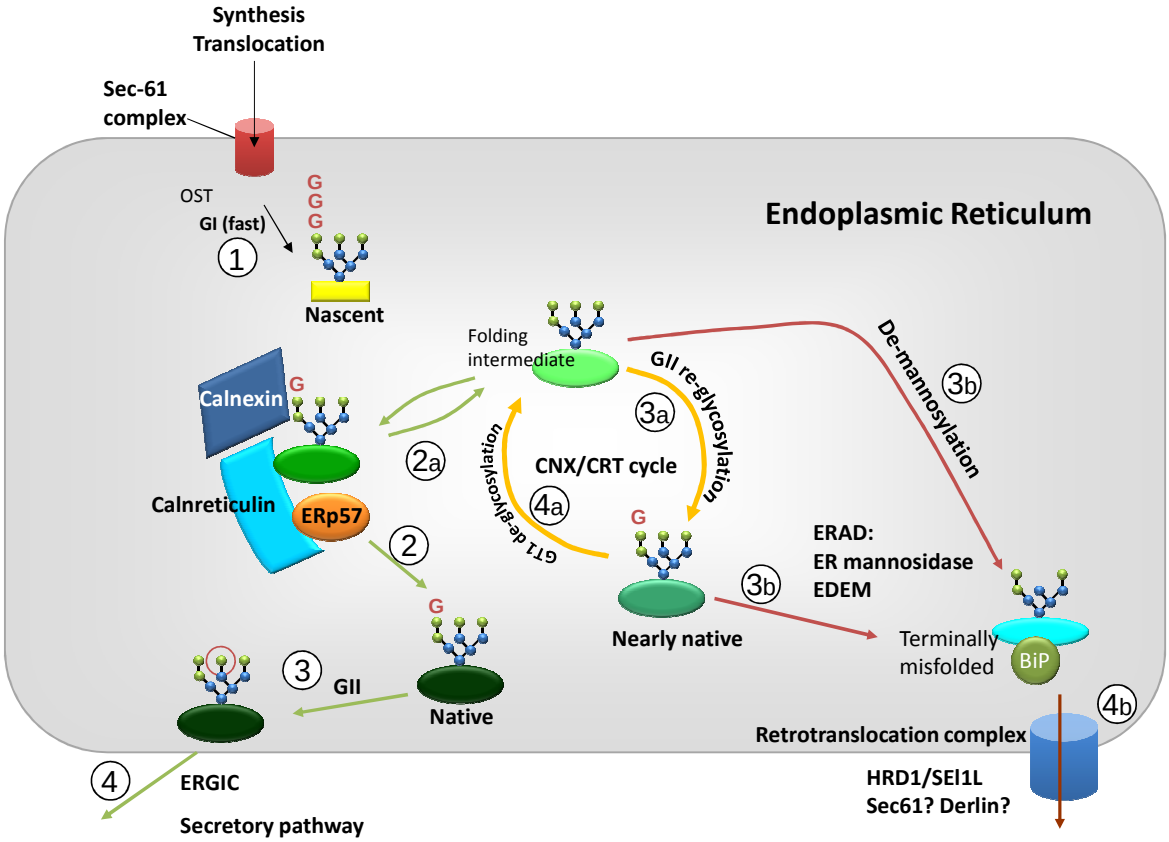
ERAD and Polyomavirus

Protein folding inside the ER

Proteins are the most versatile and structurally complex biological macromolecules[4]. The folding into their native conformation is crucial for function. Therefore the cell is primed to make sure proteins are folded in the correct manner. Approximately one-third of all proteins in a eukaryotic cell are co-translationally translocated via the Sec61 complex into the Endoplasmic Reticulum (ER) lumen or membrane (Figure 2, step 1). Some proteins are immediately glycosylated while they enter. During and after translocation, proteins start to fold. The folding is assisted by a group of chaperones of oxidoreductases. Chaperones calnexin (CNX)[5] and calreticulin (CRT)[6] assist folding of glycoproteins[7]. The free hydrophobic residues of the protein are shielded by BiP and GRP94 to prevent aggregation[8-10]. Oxidoreductases, such as PDI and ERp57 catalyze disulphide bond formation between the free cysteines within the protein (Figure 2, step 2)[11]. Because the process of folding is not perfect, sometimes misfolding can occur. The most lethal genetic disease among Caucasians is Cystic Fibrosis which is caused by protein misfolding. This disease is caused by a single mutation in the chloride transporter, CFTR gene[12]. This mutation results in a folding problem and the transporter is unable to get to the cell surface. The lack of this transporter is causing a defective mucus production what results in severe disease[13, 14]. A different example is the formation of aggregates as result of misfolded proteins, in diseases such as Parkinson and Alzheimer[4, 15].

Endoplasmic Reticulum Quality Control

To avoid the development of diseases caused by misfolded proteins, the ER has a quality control system (QC)[16-18], partially depicted in figure 2. Misfolded proteins are recognized by chaperones with their exposed hydrophobic residues and aggregates[16]. The best characterized QC system in the ER is the so-called glycan code[19]. Most polypeptides entering the ER are modified by preassembled oligosaccharides. The UDP glucose:glycoprotein glucosyltransferase (UGT1) induces glycosylation and deglycosylation of these oligosaccharides[20-22] (Figure 2, step 3a/4a). Thereby misfolded proteins are send back to the CNX/CRT cycle, these chaperones increase the folding efficiency and retain the proteins in the ER long enough to fold correctly[23, 24]. Proteins that cannot be folded, even after several attempts, are terminally misfolded and need to be degraded[25, 26]. However, there is no machinery to degrade proteins in the ER lumen, therefore proteins need to leave the ER to be degraded. (Figure 2, 3b/4b), this process is called Endoplasmic Reticulum Associated Degradation (ERAD).



Adapted from Hebert *et al.* 2007 (ref. 13)

Figure 2. ER modification, Quality Control and ERAD. In this figure three possible routes in the ER are summarized (1-4). (1) When a protein enters the cell, it is directly glycosylated by OST and modified by G1. (2) A protein is folded by chaperones Calnexin and Calreticulin and disulphide oxidoreductase ERp57. (3) The glucose residues are removed by GII and (4) the protein is released into the secretory pathway via the ER Golgi intermediate compartement (ERGIC). (2a) A protein that is not directly folded correctly is recognized (3a) and de-glycosylated. (4a) To let the protein run another cycle of CNX/CRT it is re-glycosylated. A protein that is unable to refold, (3b) is de-mannosylated by ER mannosidase I and recognized by EDEM, this is the direct route for ERAD (4b) the protein is ubiquitinated and dislocated over the ER membrane by the dislocation complex. Inside the cytosol the protein is degraded by a proteasome.

Endoplasmic Reticulum Associated Degradation (ERAD)

Misfolded glycosylated proteins are recognized by the lectin (sugar binding) ER mannosidase I[27,28] which cleaves off one of the mannoses of the protein's glycan. By this demannosylation, the protein becomes a substrate for EDEM1[29,30](Figure 2, step 4b). EDEM prevents aggregation and cleaves a second mannose. In concert with EDEM, a reductase called ERdj5 reduces the incorrect formed disulphide bonds[31]. The processes of demannosylation and reduction make the target recognizable for a next lectin, OS-9[32]. OS-9 has a mannose 6-phosphate receptor domain which recognizes the terminal mannose which is necessary for ERAD targeting[33, 34]. OS-9, together with more ER chaperones (BiP, XTP-3B and PDI), interact with the misfolded proteins. Together they deliver the reduced and unfolded substrate at the ER membrane for dislocation back into the cytosol[33, 35].

At the ER membrane a complex of proteins forms a scaffold to connect important proteins which facilitate the ER associated protein degradation[34, 36, 37]. This complex is visualized in figure 3. Dislocation is a process that assists the transport of ER proteins across the ER membrane back into the cytosol[38, 39]. For proteins to exit the ER lumen, a channel is needed. It is still uncertain which protein forms this portal for dislocation. Many researchers search for the actual dislocation channel, what resulted in a lot of debate[13, 40, 41].

One candidate for the dislocation channel is found in the dislocation complex. This protein is called Derlin and spans the ER membrane six times[42, 43] (Figure 3). The proposed channel, Derlin-1, has two more isoforms, Derlin-2 and -3 [41,44]. For Derlin-2 it was discovered it acts in concert with Derlin-1 to assist ERAD substrate dislocation. It appears that these Derlins work together to successfully dislocate proteins[43, 45].

In contrast to the finding that Derlin serve as dislocation channel, another possible dislocation channel is the Sec61 complex[46, 47]. This complex, known for its ability to import proteins into the ER, seems also important for the export of ER proteins. ERAD substrates associate with the Sec61 complex[48, 49], both in co-immunoprecipitation experiments[46] and genetic analyses[47, 50]. ERAD of tail anchored proteins was however found independent of Sec61. Loss of function mutations of Sec61 neither reduces the turnover of these proteins, nor causes an accumulation in the ER[51]. Also, the by Human cytomegalovirus (HCMV) induced degradation of the host MHC is not mediated by Sec61, instead the dislocation is associated with Derlin-1[41]. Taken together, the exact dislocation channel remains to be defined. At the moment both channels Sec61 and Derlin-1 are considerable candidates. It could be that, depending on the substrate, either the Sec61 or Derlin channel is used[49].

Next to the dislocation channel, the E3 ligase HRD1/SEL1L is spanning the ER membrane. The HRD1/SEL1L complex is important for ERAD[41, 52, 53] and the pairing of the proteins HRD1 and SEL1L is crucial for ERAD function[53]. During dislocation the E3 ligase HRD-1 adds ubiquitin chains to the misfolded proteins as it crosses the ER membrane (Figure 3)[54]. At the cytosolic site the dislocation is completed by the AAA ATPase p97 (also called VCP or, in yeast, Cdc48)[55, 56]. In the study of Ye *et al.*[44], a p97 binding protein was found, which links p97 to the dislocation channel in the ER membrane. This binding protein, VCP interacting membrane protein (VIMP), forms the receptor for p97[44]. At the cytosolic site, VIMP interacts with p97 and its two co factors recognize the polyubiquitinated substrate derived from the degradation process (Figure 3)[43, 57]. With the use of ATP, p97 has the ability to create a mechanical force which eventually pulls a substrate across the ER membrane into the cytosol[35, 44, 57] (Figure 3).

When the proteins is fully dislocated and arrives in the cytosol the polyubiquitination makes the protein a target for a 26S proteasome. Within the proteasome the protein is cleaved in small peptides and is no longer functional (Figure 3). With this type of degradation, the cell is protected from misfolded proteins being released from the ER.

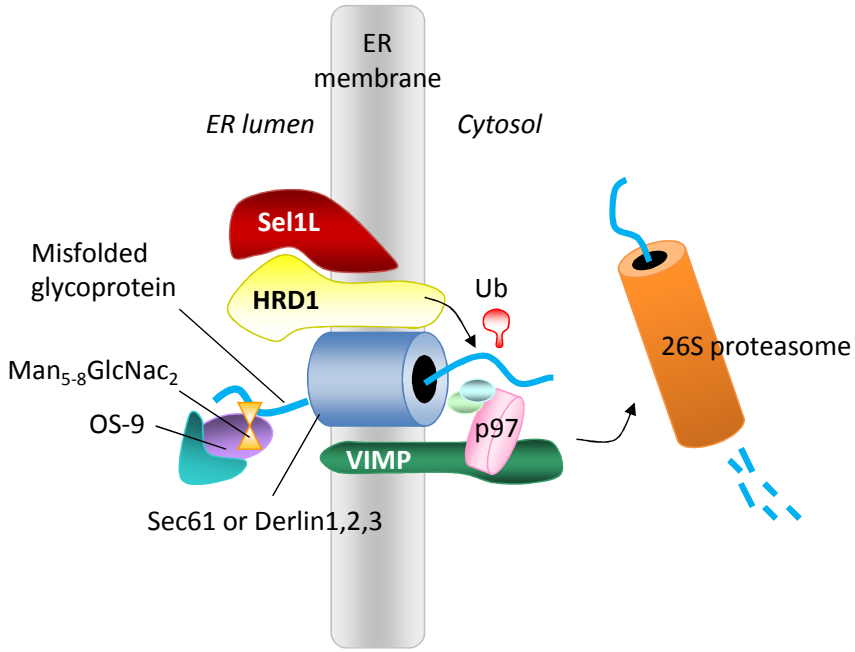


Figure 3. Dislocation complex. Generalized and simplied overview of the dislocation complex at the ER membrane. A misfolded glycoprotein is trimmed by ER mannosidase and EDEM. The $Man_{5-8}GlcNac_2$ is recognized by OS-9 and XTP3B and guided to the ER membrane. The glycoprotein is translocated over the ER membrane by Sec61 or Derlin1,2 or 3. The glycoprotein is ubiquitinated by E3 ligase HRD1, which is binding to SEl1L. The misfolded protein is pulled to the cytosol by the mechanical force of ATPase p97 which binds to VIMP. Once the polyubi-quitinated protein has crossed the membrane it is degraded by a 26S proteasome.

ERAD and pathogens

The ERAD process is a highly regulated process what makes it possible for proteins to cross a membrane. Some pathogens have thought of a way to make use of this process for their own survival. Viruses use the retrograde transport as they move from the plasma membrane to vesicles and eventually pathogens can reach the ER[58]. For cholera toxins it is well established that the toxin enters the ER, where it unfolds and releases the enzymatic A1 chain, accommodated by chaperones and PDI in the cell's ERAD process[59]. Summarized by Hazes *et al.*[60] it was found that at least six bacterial toxins are using ERAD to release their toxin in the cytosol, to eventually intoxicate the cell. It seems that not just toxins but more pathogens are using the ERAD pathway for their own function. Polyomaviruses were found to accumulate in the ER, exploiting the ERAD process for their own purpose.

Polyomavirus

A family of viruses that is known to depend on the ERAD process is the polyomavirus family. Polyomaviruses (or papovaviruses) are able to induce tumor development in many different tissues[61]. Two members of the family were first identified in animals. In 1953 the mouse polyomavirus (mPy) was described to cause contagious leukemia[62] in mice and a few years later Simian Virus 40 (SV40) was isolated from rhesus monkey kidney cells[63]. Later, the human homologues of these viruses were found and are known as BKV[64] and JCV[65], named after the first patients carrying the virus. More recently human polyomaviruses were isolated[66], known as KI [67] WU[68] Merkel cell virus (MCV)[69] and Trichodysplasia spinulosa virus (TSV)[70]. In a study by Kean *et al*.[71] it was found that for human polyomavirus types such as BKV, more than 80% of the population is carrying the virus[71]. This indicates that polyomaviruses are widely spread and mostly asymptomatic.

The family of *polyomaviridae* can be classified as group 1 in the Baltimore scheme, since they contain double stranded DNA[72]. The DNA is circular and is wrapped around 20-22 histones[73, 74]. Furthermore the virus is non-enveloped and the icosahedral capsid consists of homopentamers of the major capsid protein VP1, associated with minor structural capsid proteins VP2 and VP3[75].

Viral entry and endocytosis

How does a polyomavirus infect a cell? Therefore we first look at the attachment of the virus to the host. mPy, BKV and JCV all seem to attach to the host cell via sialic acid[72]. By adding neuraminidase, the sialic acid bonds are broken down, and binding to a host cell could be prevented. For SV40 this was not the case, SV40 has a narrow specificity for GM1 gangliosides, specifically to the sialic acid residues in GM1[76]. Upon binding of SV40 to GM1 gangliosides, Caveolin-1 proteins are attracted and start to form polymers, this event is probably stabilized by the cholesterol that is attracted to this site[77]. SV40 binding to the plasma membrane causes lipid rafts to get in a closer proximity[78] and eventually form a cluster, inducing invagination and uptake of SV40[77].

Viral trafficking to the ER

When studying the internalization of SV40, the virus particles eventually start to accumulate in the ER[86]. During the internalization, the virus passes through an endosome like organelle, surrounded by Caveolin-1, hence named caveosome[77, 79] (Figure 4). Given that endosomes are normally not directed towards the ER, Pelkmans *et al.*[80] suggested that caveosomes mediate the transport of virus particles to the ER[80, 81]. The study of Damm *et al.*[78] showed that SV40 is also able to enter a cell in a caveolin independent, cholesterol dependent manner, triggered by tyrosine kinases. SV40 infection was however partly blocked by specific caveolin inhibiting drug[82].

A study on the intracellular trafficking of SV40 showed that the virus uses a two step approach to reach the ER[80]. First the virus is enclosed in a caveolin-1 surrounded vesicle forming a caveosome. The second step was revealed by live cell imaging, the virus containing vesicles become tubular and move fast in a linear direction towards the ER, completely bypassing the endosomal-lysosomal route[83]. This implied that the virus exploits carrier vesicles that make use of the host cell cytoskeleton, more specifically the microtubules[79] (Figure 4). The vesicles eventually fuse with the ER and deliver the virus particles in the ER lumen (Figure 4). This route of trafficking is similar to that of many other viruses but instead of pH mediated uncoating in the lysosomes, the intact virus is delivered to the ER. This implies another interesting topic for research, since the destination ER is highly unusual for endocytosis. How the virus is then admitted into the ER is still unclear, the Golgi apparatus is considered as and intermediate step[84]. Even though the trafficking towards the ER is unknown, Norkin *et al.*[83] showed that the destination is apparent, because SV40 is largely depending on the ER. Treatment with the drug Brefeldin A, which interferes with ER trafficking, resulted in a complete blocking of SV40 infection[58, 85].

Polyomavirus inside the ER, abuse of ERAD

Like more DNA viruses, polyomaviruses depend on the nuclear replication machinery; therefore the small viral genome has to enter the nucleus. For a virus to reproduce it has to release its genomic information, therefore polyomaviruses use the ER to disassemble[83, 86]. As previously stated the SV40 capsid consists of VP1 pentamers and smaller capsid proteins VP2 and VP3. The VP1 pentamers are connected with cysteine residues disulphide bonds, the capsid is further stabilized by Ca^{2+} ions[74, 87]. Especially the essential bonds between cysteines at position C9-C9 and C104-C104 are forming the backbone for the capsid coat networks of SV40[74, 75, 88]. For mPy the essential bonds are formed between C19 and C114[89-91]. The breakdown and rearrangement of these bonds is important for the uncoating of the virus.

By entering the ER, the virus is uncoated by an interplay between viral cysteine residues and host reductases, isomerases, and chaperones that all act differently on Py virus to promote infection. Walczak *et al.* identified PDI and three other thiol-disulphide oxidoreductases (ERp57, ERp29, ERp72) as important molecules for polyomavirus infection. Studies in SV40 demonstrate that ERp57 in concert with PDI and ERp72 breaks the disulphide bonds between the C9-C9 bonds and links the C9 to a C104[74]. With this process the virus particle is partly uncoated. *In vitro* the chaperones and oxidoreductases seem to work in a close proximity because they are pulled down in a precipitation. Therefore Walczak *et al.*[91] suggest that they are binding together in a so-called PDI network[91].

Schelhaas *et al.* found that depletion of several PDI family members resulted in reduced infectivity of SV40[74]. Knocking down these proteins in mPy infection resulted in similar observations as found in SV40 infection and showed a reduced infection up to 40%[91]. Down regulation of PDI showed that virus particles move into the ER and accumulate, because they fail to exit the cell compartment. Silencing of PDI has multiple effects, ERAD becomes defective and membrane crossing is impossible, consequently nothing can leave the ER[85].

In mPy infection the oxidoreductase ERp29 seems important for the conformational change within the VP1 C-terminal arm. This conformational change increases the binding efficiency of the viral capsid to the ER membrane for export[91, 92]. The binding to the ER membrane by SV40, was recently described by Geiger *et al.*[93]. This binding appears different from mPy and involves proteins BiP and BAP31[93]. Taken together, the virus appears to use a specific subset of ERAD components. The essential ER chaperones calnexin and calreticulin for protein folding seem uninvolved, because silencing of these proteins did not have an effect on SV40 infectivity[91].

These experiments showed that polyomaviruses exploit the ER QC system to disassemble. But these viruses must have a mechanism to exit the ER, to eventually reach the nucleus for replication.

Polyomavirus ER dislocation

If polyomaviruses are completely exploiting the ERAD pathway, these viruses may use the dislocation channel to cross the ER membrane. The silencing of the dislocation proteins Derlin-1 and SeL1L revealed their importance for SV40 infection[40, 74]. For the virus to leave the ER, all polyomaviruses make use of ERAD, for each virus the channel for the dislocation seems to be different.

In a study conducted by Lilley *et al*, it was apparent that specifically Derlin-2 is involved in the ER membrane crossing during mouse polyomavirus infection[45]. In contrast to mPy, SV40 makes primarily use of Derlin-1 for translocation to the cytosol[72, 74]. For human BKV, the significance of a dislocation channel was demonstrated when a double negative form of Derlin-1 resulted a tremendously reduced BKV infection[94]. The capsid protein VP1 of BKV even showed interaction with Derlin-1 in a pull down assay indicating the association of BKV and Derlin-1[94].

When the virus is successfully dislocated across the ER membrane the uncoating of the viral capsid continues. In the ER lumen the viral capsid is partly disassembled, but is held together by the calcium ions that are stabilizing the VP1 pentamers. When the virus enters the cytosol the low calcium levels induce the further uncoating, due to the loss of the stabilizing calcium ions[87]. This process disassembles the virus particles and the capsid proteins promote nuclear targeting of the viral genome[95]. The genomic information can enter the nucleus through the nuclear pore complexes[96, 97]. Within the nucleus, the virus is able to replicate and induce DNA transformations of the host's genome. When compared to other well studied viruses such as Adenovirus, the cellular trafficking of polyomaviruses seems unusual (The infectious route of SV40 is summarized in figure 4). However, we believe that the family of polyomaviridae is not unique in the usage of the ERAD components. Therefore we speculate about more virus candidates that could move to the ER to use the ER resident proteins and ERAD regulators.

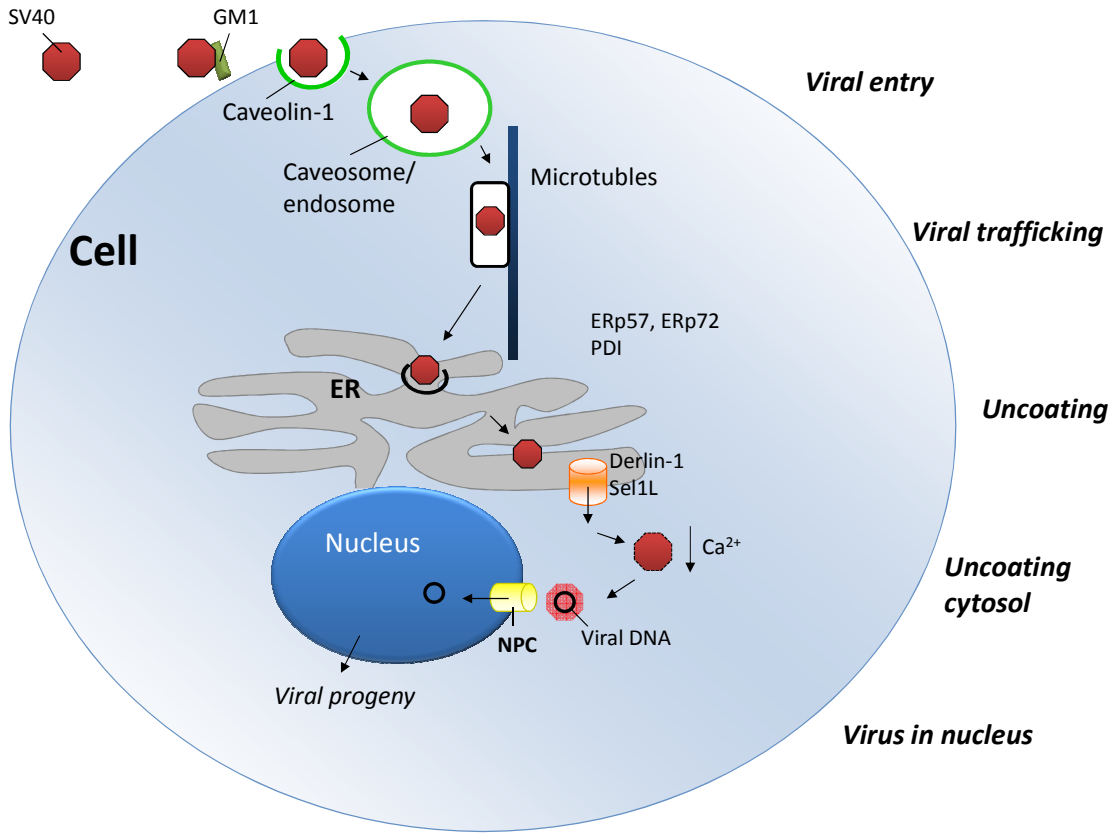


Figure 4. Schematic overview of SV40 infection. SV40 binds to the GM1 ganglioside on the cell surface. As a result of GM1 binding a lipid raft starts to form an attracts caveolin, this is a caveolin dependent endocytosis. Inside the cell the virus is enclosed by a caveolin-1 coated organelle. The virus travels fast in tubular formed vesicles across the microtubules toward the ER. The virus enter the ER and is partly uncoated by PDI and ERp57. The virus exploits the dislocation channels to exit the ER and further uncoat in the cytosol. Uncoating in the cytosol is achieved by the low calcium levels. The virus enters the nucleus via nuclear pore complexes.

Discussion

The past decade showed a remarkable increase in the understanding of the infectious route of many viruses. As we have seen, the polyomaviruses exploit ERAD, the ER resident proteins uncoat the virus and release them to travel to the nucleus[74, 91, 98]. We suggest that the viral entry mechanism of polyomaviruses is used by other viruses as well. Based on viral properties, we can speculate which viruses also abuse ERAD.

Viruses that are not depended on pH

To search for viruses that could also use ERAD, we first looked at viruses that do not depend on a low pH for uncoating. It is well established that most viruses move to the lysosome, the low pH in the lysosome than causes a disruption in the envelope or capsid structure what leads to viral uncoating. Still, there are many viruses that were found independent on a low pH in the lysosome. These viruses must have found a different mechanism to uncoat. They exit from the endosome and somehow uncoat in the cytosol, because the actual mechanism is unknown. In table 1 the majority of the pH independent viruses are listed[80]. We speculate that a few of these viruses for example papillomaviruses also use the ER, similar to polyomaviruses.

Whereas Human papillomavirus-16 (HPV-16) seems to depend on a low pH for uncoating, [99] Bovine papillomavirus-1 (BPV-1) uses a different unknown mechanism. BPV-1 enters the cell in a clatherin-dependent endocytosis, and then switches to a caveolin surrounded vesicle, similar to polyomaviruses. This could imply that the virus particles move to the ER. The virus capsid protein L2 of BPV-1 carries conserved residues which bind to an ER localized receptor called syntaxin 18. Mutating the syntaxin 18 resulted in a significantly decreased BPV infection[100]. Probably the virus uses this receptor to reach the ER. Nevertheless a real co localisation with the ER was not described so far. The localization in the caveosome, together with capsid binding to an ER receptor may suggest that BPV-1 enters the ER where it could abuse ERAD.

Association of viruses with oxidoreductases

When looking at the known polyomavirus strategies for ERAD abuse, we could search for similar mechanisms in other viruses. For example, we have seen polyomaviruses depend on oxidoreductases for capsid disassembly inside the ER. Looking into viruses that also depend on oxidoreductases, could imply that ERAD is involved.

For retroviruses such as HIV-1, a low pH is not required for the uncoating of the virus[80, 101]. The viral envelope fuses with the plasma membrane by the envelope glycoprotein Env[102, 103]. HIV-1 was found to be dependent on oxidoreductases, especially to the disulphide oxidoreductase PDI. PDI disrupts the HIV-1 envelope disulphide bonds in the GP120 protein[98]. Inhibition of PDI also inhibited HIV-1 infection[98]. These PDI induced reductions seem to take place at the cell surface to release the inner HIV-1 capsid into the cell[104]. Despite the assumption that HIV-1 is interacting with PDI at the cell surface, we believe that the HIV-1 is (also) interacting with PDI intracelullarly. PDI is an ER resident protein and therefore HIV-1 could move to the ER instead of PDI to the cell surface. A study that supports ER localization, found that in the molecular structure of the HIV-1 capsid, disulphide bonds are stabilizing CA pentamers[105]. As we have seen in for polyomaviruses disulphide bonds are broken inside the ER, perhaps the same occurs for the HIV-1 capsid. Based on the found interaction of HIV-1 with PDI and the disulphide bonds in the capsid, we suggest that HIV-1 could use ERAD.

Usage of ERAD regulators outside the ER?

Unlike HIV-1, SARS coronavirus (SARS-CoV) does not depend on oxidoreductases[98], though this virus is associated with ERAD regulators OS-9 and EDEM. The ERAD components are present in small vesicle derived from the ER. In an uninfected cell these vesicles exist for ERAD tuning. This is the regulated break down of ER Mannosidase I, EDEM and OS-9 in an endo/lysosome compartment[38]. These double membrane vesicles (DMV) contain endogenous EDEM and are therefore called EDEMosomes[38, 106].

In coronavirus infected cells, these vesicles accumulate. Virus particles co localize with the EDEMosomes and use the vesicles for replication and reproduction[106]. SARS CoV seems to abuse ERAD, it hijacks vesicles that contain ERAD regulators, derived from the ER.

Echovirus and caveolin

Pelkmans *et al*. suggested that viruses with similar properties as SV40 may also use ERAD. One family of such viruses is the family of picornaviridae. These virions are, like polyomaviruses, relatively small and non-enveloped. An example of these viruses is the Enteric Cytopathogenic Human Orphan (Echo) viruses. It is often described that Echoviruses, similar to polyomaviruses, are able to enter a cell in a caveolin-dependent endocytosis[107]. As stated, viruses which use the caveolin-endocytosis and caveosomes may have the ER as their destination instead of lysosomes[86,108]. Therefore the Echovirus is a possibly also abusing ERAD. There are studies which support this hypothesis, because the virus particles were found to accumulate in a membrane surrounded compartment close to the nucleus[109]. This could indicate the virus localizes in the ER, similar to polyomavirus. On the contrary, a study by Pietiäinen *et al.*[110] indicated that Echovirus uses the caveosome, but does not enter the ER, Golgi or endosome[110]. Instead, it is suggested the virus replicates in the cytosol[110].

The finding that Echoviruses are moving to caveosomes led to the assumption that they also enter the ER. It was argued by Engel *et al*. that SV40 also moves to endosomes instead of the mentioned caveosome[84]. This result indicates that even if viruses enter an endosome, they are still able to move to the ER.

Conclusion and suggestions for research

For a whole range of viruses the exact viral entry is unknown or incomplete. As discussed, most viruses depend on a low pH in the lysosome to uncoat. Because for many viruses their release from the endosome and uncoating is unclear we predict that the ER is a possible intermediate step. Based on published data we would select the viruses indicated green in table 1 as viruses that could abuse ERAD. Especially for viruses such as BPV-1, HIV-1 and SARS CoV, Echovirus we have gathered supportive information what should motivate in depth research on viruses and ER associated degradation. Therefore we propose experiments to test the ERAD abuse of more viruses.

For the suggested viruses, the localization of the virus in the ER can be tested with electronic and fluorescence microscopy. Co-localization in the ER could already indicate ERAD abuse. Second, the requirement for ERAD components should be tested. To conduct these experiments accurately, the specific components involved in polyomavirus can be used as reference. To test ER involvement, general ERAD associated processes can be inhibited. Schelhaas *et al.*[74] perturbed ER processes by adding inhibitors for proteasomal degradation, ER Ca2+ homeostasis and disulfide bond formation (DTT). These inhibitors all blocked SV40

infection[74]. If virus infectivity is reduced by these inhibitors, their dependence on specific ER proteins, PDI and more oxidoreductases (ERp57, ERp72 and ERp29) should be tested. Specific siRNA known downs of these reductases will imply the localization in the ER and reveal the exact involved reductases.

If viruses enter the ER, do they use the ERAD dislocation complex to get out? To explore the involvement of the ERAD dislocation complex, the requirement for important proteins Derlin-1, -2, -3 and Sec61 can be tested. In the study done by Lilley *et al.*[45] the involvement of Derlin in mPy dislocation was revealed. Therefore dominant negative forms of Derlins were made with a GFP tag. The non-functional Derlin-2 inhibited the mPy infection, what indicated that this Derlin is involved in mPy dislocation[45]. If viral infectivity is reduced by the inhibition of ERAD components, we can conclude that a virus depends on the ER and/or abuses ERAD

Based on the scientific publications so far, we propose a whole subset of viruses to test for their ERAD hijack abilities. With an overview of candidate viruses, we have created a whole new attractive subject for research. With our suggestions and implications for research we hope more ERAD dependent viruses can be defined. With exact knowledge as to how viruses are exploiting a cell, we may specifically stop certain virus infections.

Overview of viruses considered for ERAD abuse

Virus family	Virus example	Intracellular trafficking	Uncoating in ER/ERAD?
Polyomaviridae	SV40 BKV JCV KI WU MCV TSV	Caveosome / endosome	Yes, virus is dependent on ERAD components
Paramyxoviridae	Measles virus	-	No, fusion with membrane → release virion[101]
Herpesviridae	Herpes simplex virus 1	-	No, fusion with membrane → release viral genome[101].
Papillomaviridae *	HPV-16 BPV-1	Clatherin and caveolin independent Caveosome	No, HPV-16 depends on low pH in lysosome ? Possibly moving to ER[111, 112] (Sapp) ER receptor binding[100].
Poxviridae	Vaccinia virus	Macropinosome	? Uncoating in cytosol[113] Capsid disulphide bonds [114]
Reoviridae	Human rotavirus Infectious subviral particles	Endosome / Lipid rafts Endosome	? Depends on calcium levels[115] No, Indication of acidification
Hepadnaviridae	Hepatitis B virus	Early endosome	No, Intact virion in nucleus, nuclear basket[116]
Retroviridae *	HIV-1	Membrane fusion	? Fusion with membrane[102] Redox reactions[98, 104] Capsid disulphide bonds[105]
Picornaviridae *	Human rhinovirus Poliovirus Echovirus	Endosome Endosome Caveosome	No, genome is released through membrane channel ? Echovirus also caveole[110, 117]
Coronaviridae *	SARS-CoV	Endosomes EDEMosome[106]	? EDEMosome, ERAD components[106, 118]

Table adapted from Pelkmans *et al*. 2003 (ref. 80)

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Table 1. Overview of viruses considered for ERAD abuse. Viruses that do not use the low pH in the lysosome to uncoat, are considered as ER associated candidates. Viruses marked in red are viruses which are unlikely to be involved with the ER. These viruses do not have any properties or characteristics like polyomaviruses or have a well defined viral entry mechanism. Viruses indicated in green are possible ERAD abusers; these viruses have properties similar to polyomaviruses. With respect to their capsid disulphide bonds, caveolae-dependent endocytosis, ER co-localization, association with ERAD regulators and/or oxidoreductases requirements. The question marks indicate the unknown mechanism and the found publications on the viral entry and/or uncoating. Virus families indicated with * are discussed in the main text.

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