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Review

Budding and fusion in herpesvirus morphogenesis and transport

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ABSTRACT

Virions of herpesviruses comprise capsid, tegument and envelope with embedded spikes built of glycoproteins. During morphogenesis, capsids are assembled in cell nuclei and filled with viral DNA before being transported to the nuclear periphery. From there, they either bud at the inner nuclear membrane into the perinuclear space acquiring envelope and tegument or else they gain access to the cytosol *via* an impaired nuclear envelope that starts by dilation of nuclear pores. Importantly, we cannot find hard evidence for the much-acclaimed process of "de-envelopment" of enveloped perinuclear virions *via* fusion with the outer nuclear membrane. Moreover, we challenge the logic seemingly supporting this pathway. Instead, we advocate a partially novel herpesvirus exit pathway: Enveloped perinuclear virions, which are protected against premature fusion, are released from the perinuclear space along the luminal continuum between perinuclear space, endoplasmic reticulum and Golgi complex where they are packaged into transport vacuoles. Tegumentation and envelopment occur at different entry sites. Accordingly, the first entry site is by budding at the inner nuclear membrane. Yet, the pathway is also accessible from the cytosolic side by budding of capsids, which escaped the nucleus *via* impaired nuclear envelope, either at the outer nuclear membrane or else further up throughout ER- and Golgi membranes. Alternatively, virions may acquire their envelope and tegument through the more complicated process designated wrapping, whereby capsids bud at Golgi membranes acquiring tegument and envelope simultaneously to enclosing themselves into a transport vacuole. Eventually, vacuoles carry virions to the cell periphery for exocytotic release, whereby vacuolar membranes of transport vacuoles fuse with the plasma membrane releasing virions into the extracellular space.

KEYWORDS: herpesvirus, envelopment, egress, budding, fusion, fission, intraluminal transport, endoplasmic reticulum-Golgi transitions.

ABBREVIATIONS: BoHV-1: bovine herpesvirus 1; HSV-1: herpes simplex virus 1; hpi: hours post inoculation; ER: endoplasmic reticulum; INM: inner nuclear membrane; ONM: outer nuclear membrane; PNS: perinuclear space; PrV: pseudorabies virus.

1. Introduction

Virions of herpesviruses comprise an icosahedral capsid filled with viral DNA and are enclosed by layers of tegument and a membranous envelope with embedded glycoproteins, morphologically seen as spikes. Upon replication, herpesvirus gene expression is regulated in a temporal cascade manner, immediate early, early, and late. Replication of viral DNA occurs during the late stage of gene expression in the cell nucleus and coincides with the synthesis of the majority of structural proteins. Throughout a complex process, various capsid proteins are synthesized at ribosomes in the cytosol before being imported into the nucleus to form a variety of building blocks, which are, in a chaperone- and scaffolding-assisted manner, assembled to empty capsids. Eventually, viral DNA is filled into the

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preformed capsids, which are now ready to leave the nucleus in order to sequentially acquire tegument and envelope on their way to exit as fully infectious virus particles at the cell periphery [1]. While the previous steps may appear complex, they are still pretty well understood and accepted among the scientific community [2]. In contrast, the upcoming steps are still hotly disputed and full of misunderstandings. The purpose of the present communication is to pinpoint and clarify on these issues.

A major reason for the debate comes from the observation of non-enveloped capsids in the cytoplasm, some of which closely interact with the outer nuclear membrane (ONM). Some groups argue that these particles were caught in the process of de-envelopment after having previously acquired a so-called "primary envelope" through budding from the nucleus through the inner nuclear membrane into the perinuclear space (PNS), followed by fusing their primary envelope with the ONM [3-5]. However, other groups explained the same pictures as budding through the ONM into the PNS. They also explained the existence of non-enveloped capsids within the cytoplasm through an alternative exit pathway *via* impaired nuclear membranes [6-8]. Consequently, the groups backing up for the de-envelopment-theory considered perinuclear, enveloped virions as immature and non-infectious raising the dogma that secondary envelopment by Golgi derived membranes is essential for the generation of mature, infectious virus progeny [9]. In contrast, the other groups argued in favour of alternative exit pathways, which included intraluminal transport along ER and Golgi prior to release [7, 10].

Here, we focus on transportation processes at the inner nuclear membrane (INM), outer nuclear membrane (ONM), membranes of the endoplasmic reticulum (ER) and of the Golgi complex primarily of herpes simplex virus-1 (HSV-1), bovine herpesvirus 1 (BoHV-1) and pseudorabies virus (PrV), all members of α-*herpesvirusvirinae*.

2. Methodological constraints

A direct method to study viral membrane interactions during intracellular transportation is by electron microscopy. An indirect method, which is applicable to both light- and electron microscopy, is based on immunolabeling. Importantly, electron microscopy is prone to artifacts including loss of lipids and other substances, and breakdown of cell membranes, especially those organelles that are involved in highly dynamic processes. One of the most delicate organelles is the Golgi complex in endocrine and exocrine secretory cells [11-13] as well as in virus-infected cells in which the Golgi complex is responsible for providing membranes for viral envelopes and for transportation vacuoles both of which require an enormous amount of membranes [14]. The delicacy in preservation of Golgi membranes, the complexity of Golgi functions and the high turnover of membranes are at least in part responsible for the slow improvement in understanding this fascinating organelle [15]. One method to reduce artifacts is cryo-transmission electron microscopy of rapidly frozen isolated particles [16] or cells [13, 17, 18] at temperatures around -120 °C, or high resolution cryo-scanning electron microscopy that achieves much the same resolution as transmission electron microscopy [19]. Another, in many respects, simpler method is examination of cells after rapid freezing followed by freeze-substitution (exchange of cellular water with acetone below the re-crystallization point of water) and embedding in epoxy resin. Employing this technique, loss of lipids is substantially reduced [20, 21], and cell membranes are kept in place even after induced break down [22]. This technology is also highly suitable to study cell membrane-bound processes in the sub-second range, e.g. exocytosis [23]. The crux of the rapid freezing technology is the low thermal conductivity of biological samples, so that the required freezing speed is difficult to achieve in samples thicker than about 10 µm to avoid formation of ice crystals damaging cellular architecture. To overcome this problem a technology was developed that allows freezing of biological samples under high pressure [24, 25]. The technology was postulated to be the base that may lead to precise models of macromolecular assemblies *in situ*, and thus to a better understanding of the function of complex cellular structures [26]. Indeed, studies of vesicle trafficking [27], endocytic compartments [28] and interactions between nematodes and bacteria [12] have led to new horizons in understanding these processes.

For microscopic analysis, it has to be borne in mind that the Golgi complex is a not fully understood

complex structure [15] and that it is among the first organelles that rapidly disintegrates during preparation for electron microscopy after improper fixation and processing [11, 13]. Furthermore, the Golgi complex fragments and disperses about 16 hours post inoculation (hpi) with HSV-1 [29] causing additional difficulties to study and understand Golgi function in herpesvirus envelopment.

3. Proposed envelopment and egress pathways

It is well accepted that capsids are assembled in host cell nuclei and translocated to the cytoplasm. At least three general model pathways have been proposed [1], while we advocate here for an additional fourth model pathway (Fig. 1). All four pathways agree on the well documented acquisition of a viral envelope by budding at the INM, although at least one alternative exists. However, we argue that pathway 1, which represents the most cited one, is simultaneously the least documented one, particularly in terms of its second step.

According to pathway 1, virions acquire a primary envelope by budding at the INM but are, in a second step, de-enveloped by fusion of the viral envelope with the ONM or with adjacent ER membranes, thus, releasing non-enveloped particles (capsid and tegument) into the cytosol. Indeed, de-envelopment has never been properly documented. These capsids are thought to be re-enveloped later for a second time by wrapping at membranes of vesicles derived from the *trans* Golgi network (TGN) [9, 30, 31] or endosomes [32-36].

In pathway 2, capsids bud also at the INM but are released from the PNS by formation of vesicles engulfing a single virion [37-40]. Vesicles pass somehow the Golgi complex on their way to the cell periphery, whereby envelope glycoproteins are further processed.

In pathway 3, as an alternative pathway to budding at the INM, capsids are released from the nucleus into the cytosol *via* dilated nuclear pores as has been claimed for Epstein-Barr virus by V. Kushnaryov (personal communication) years ago [41]. Indeed, the nuclear envelope has been shown to break down, starting by dilation of nuclear pores in BoVH-1-infected MDBK cells [42] and HSV-1-infected Vero cells [7]. These capsids are then transported to any sites of the Golgi complex to be enveloped by budding into Golgi cisternae and/or vacuoles, which may enlarge to engulf multiple virions. Alternatively, capsids are enveloped *via* a unique process referred to as wrapping: concomitantly to budding, a small concentric transport vacuole is formed enclosing a single enveloped virion. Additionally, a fraction of naked cytoplasmic capsids was observed to bud at the ONM and ER membranes [7] to get access to the PNS and ER cisternae, respectively.

In pathway 4, after budding at the INM, enveloped perinuclear virions are intraluminally transported into ER cisternae and further *via* ER-to-Golgi transitions or ER-Golgi intermediate compartments (ERGIC) into Golgi cisternae, from where they are transported to the cell periphery *via* vacuoles derived by fission from Golgi membranes, a process designated packaging [43]. However, transportation out of the ER is not fully understood. This transport was first described by Schwartz and Roisman [10]. They proposed that a tunnel is formed, through which virions can be transported from the ER directly into the extracellular space similar as in parietal cells for secretion of hydrochloric acid [44]. The establishment of Golgi-to-ER transitions e.g. in HSV-1-infected cells [45] rather indicates that virions can be transported from the ER into Golgi cisternae for packaging into transport vacuoles. Therefore, we advocate this fourth pathway for all perinuclear, enveloped virus particles, whether they entered the PNS *via* the inner or the outer nuclear membrane or *via* ER membranes.

Finally, vacuoles derived by fission from Golgi membranes transport virions to the cell periphery and release them into the extracellular space *via* exocytosis.

4. Budding

In microbiology, the term “budding” has first been used in the context of yeast to describe smaller yeast daughter cells bulging on the surface, curving thereby the cellular membrane, and pinching, or budding, off from larger mother cells [46]. However, the term has itself established also for other, morphologically similar membrane-associated trafficking processes, for example vesicular trafficking between ER and Golgi complex [47-49], or transmembraneous release of retroviruses [50]. Membrane budding processes range from primarily protein-driven machineries to

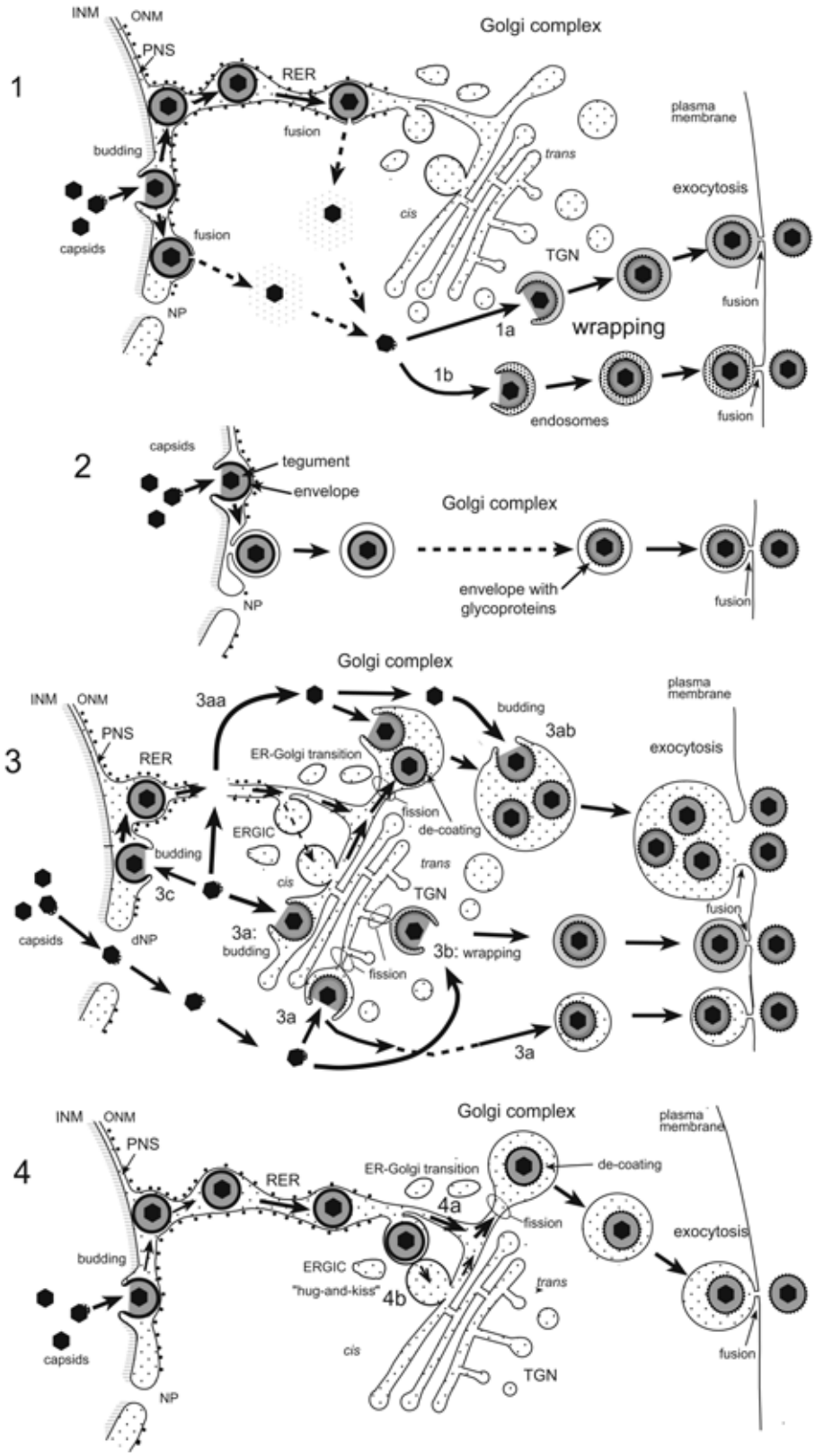
1
INM
ONM
PNS
RER
Golgi complex
budding
fusion
trans
plasma membrane
exocytosis
cis
capsids
fusion
TGN
NP
1a
wrapping
fusion
1b
endosomes
fusion
2
capsids
tegument
envelope
Golgi complex
NP
envelope with glycoproteins
fusion
Golgi complex
3
INM
ONM
3aa
budding
3ab
plasma membrane
PNS
RER
ER-Golgi transition
exocytosis
fission
de-coating
ERGIC
budding
trans
cis
TGN
3c
fusion
capsids
dNP
3a:
budding
3b: wrapping
fission
3a
3a
4
Golgi complex
INM
ONM
plasma membrane
PNS
ER-Golgi transition
de-coating
RER
4a
fission
exocytosis
budding
ERGIC
"hug-and-kiss"
4b
trans
cis
capsids
TGN
fusion
NP

Fig. 1

primarily lipid-driven ones [51, 52]. Protein-driven membrane budding (Fig. 2) is favored in many DNA viruses, including herpesviruses, due to their need to orchestrate these steps in a timely manner. Accordingly, it involves viral proteins to regulate coat assembly, bud formation with negative and positive membrane curvatures, and fission from the donor membrane. Importantly, those protein-membrane complexes forming the budding site are clearly visible as dense curvatures by electron microscopy (Fig. 2). Eventually, the protein coats deform lipid membranes into spherical buds for fission at the neck to become vesicles or a viral envelope [53-55].

5. Fusion

Fusion starts by close apposition of donor membranes to target membranes (Fig. 3). Close apposition is immediately followed by formation of the fusion pore and fusion neck [56-58]. The process is accomplished in the sub-second to second range and, hence, generally demands highly sophisticated methodologies for investigation and visualization. The time scale of vesicle membrane fusion is in the milli-second range [59]. Fusion between influenza virus envelope and liposomal membrane was investigated by quick-freezing [60, 61] and reviewed by Harrison [62]. Intermediate stages of fusion between the envelope of HSV-1 and the plasma membrane were demonstrated applying cryo-electron tomography [18]. Fusion of membranes of secretory granules with the plasma membrane was shown by conventional electron microscopy in lactating mammary cells (Fig. 3C) where fusion can be triggered by gentle mechanical stress immediately prior to fixation [63, 64]. Although it is difficult to visualize fusion by conventional electron microscopy, its appearance can be clearly discriminated from protein-driven membrane budding.

6. Capsid transport across the inner nuclear membrane

Herpesvirus capsids are assembled in replication centers [65] and filled with DNA, before being transported towards the nuclear periphery [66]. However, DNA is not required for capsid transport out of the nucleus. The INM is protected by a complex meshwork of proteins called the nuclear lamina [67-69]. Phosphorylation leading to the disruption of the nuclear lamina is essential and mediated by both viral (pUL13, pUS3) and cellular kinases [70]. The budding machinery at the INM

Legend to Fig. 1. Schematic presentation of proposed herpesvirus envelopment pathways adapted from [45]. **Pathway 1**: Capsids bud at the INM into the PNS acquiring tegument and a "primary" envelope covered with a dense coat. Then, capsids and tegument are released into the cytoplasmic matrix *via* fusion of the viral envelopment with the ONM (de-envelopment). Capsids are then re-enveloped at vesicles derived from the *trans* Golgi network (1a) or at endosomes (1b) by a process-designated wrapping forming a vacuole that carries the engulfed virion to the cell periphery for releasing it into the extracellular space *via* exocytosis. **Pathway 2**: Capsids bud at the INM and are released from the PNS by formation of vesicles which carries the capsid to the cell periphery passing somehow the Golgi complex for maturation of the envelope. **Pathway 3**: Capsids gain direct access to the cytoplasmic matrix *via* dilated/impaired nuclear pores (dNP) and are transported to any site of the Golgi complex where they bud at Golgi membranes. They either bud into small (3a) or large Golgi cisternae (3aa) or vacuoles (3ab) or are enveloped by a process-designated wrapping (3b) that involves budding and concomitant formation of a small transport vacuole engulfing a single virion. They may also be enveloped by wrapping at endosomes (3c) Capsids may also bud at the OM or RER (3d), and the resulting virions are intraluminally transported as in **Pathway 4**: After budding of capsids at the INM into the PNS, virions are intraluminally transported into the RER and further *via* Golgi transitions (4a) or the ERGIC ("hug-and-kiss", 4b) into Golgi cisternae where they are packaged into transport vacuoles, which are detached from Golgi membranes by fission. The dense coat is shed off while vacuoles are transported to the cell periphery for exocytotic release of uncoated virions into the extracellular space. The dense coat, which is derived during the budding process at the INM, probably protects the viral envelope from fusion with membranes the virions are transported along. This coat is shed off (de-coating) from the viral envelope after virions arrive in Golgi cisternae or in transport vacuoles or when virions are released into the extracellular space. During budding at Golgi cisternae and vacuoles, a dense rim of tegument is closely attached to the inner layer of the viral envelope. No dense coat is formed so that spikes (glycoproteins) are readily seen in good electron micrographs.

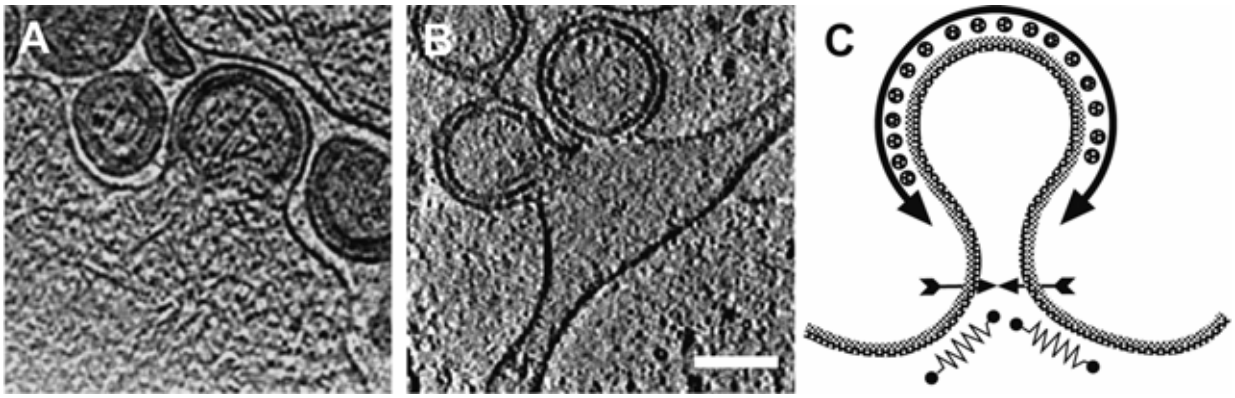

Fig. 2. A, B) Budding of HIV analyzed by cryo-electron tomography, reproduced from copyright: © 2010, Carlson, L. A., de Marco, A., Oberwinkler, H., Habermann, A., Briggs, J. A., Krausslich, H. G. and Grünewald, K. PLoSPathog, 6(11), e100117. https://doi.org/10.1371/journal.ppat.1001173 [133]. C) Scheme of budding adapted from Antonny, B. 2006, Cur. Opin. Cell Biol., 18, 386 [53], indicating the positive and negative curvatures, the budding neck, and the forces involved in budding.

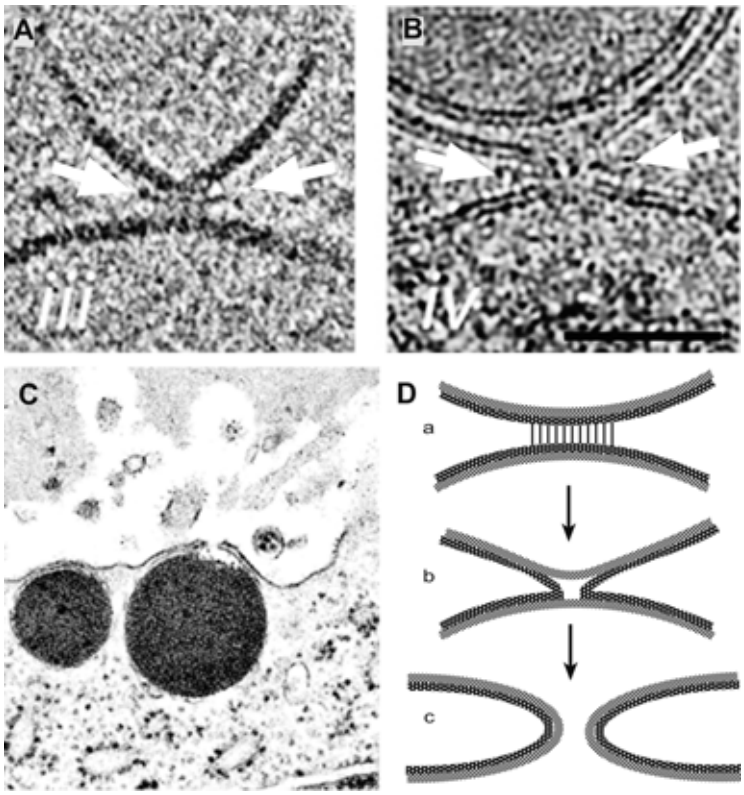

Fig. 3. A, B) Fusion of influenza virus with liposomal membranes as revealed by cryo-electron microscopy showing membrane contact and formation of fusion pore. Reprinted by permission from Calder, L. J. and Rosenthal, P. B. 2016, Nat. Struct. Mol. Biol., 23, 853. copyright © 2016, with permission from Nature Publishing Group. C) Close apposition of a secretory granule with the plasma membrane and fusion pore in a lactating mammary cell. Reproduced from copyright © 2008 Wild, P. Introduction to electron microscopy for biologists; methods in cell biology, Allan, D. (Ed.), Elsevier, San Diego, 497 with permission from Elsevier. D) Scheme of fusion adapted from [133] indicating a) initiation of fusion, b) hemifusion, and c) fusion pore.

involves the proteins pUL31/pUL34 designated as the nuclear egress complex (NEC), reviewed by Johnson *et al*. and Mettenleiter *et. al.* [71, 72]. The NEC was reported to be responsible and sufficient for budding without requirement of cellular factors [73-76]. In the absence of pUs3, the pUL31/UL34-proteins are not phosphorylated, which was reported to obliterate budding into the PNS [77, 78]. Nevertheless, packets of viral particles were formed, which were wrapped with one or more layers of nuclear membrane [77, 78]. The glycoprotein K (gK) was shown to be located on nuclear membranes [79] as well as on perinuclear virions [80]. The significance of gK in envelopment at the nuclear level is not well understood, not least because its function was mainly investigated considering the envelopment-de-re-envelopment theory that obviously hindered open minded interpretation of data. Thus, gK of PrV was shown to be involved in viral egress probably by preventing immediate re-infection [81]. gK was postulated to be a fusion inhibitor and involved in capsid envelopment and transportation of virions from the cytoplasm to the extracellular space [82], whereas gK is not involved in de-envelopment [32] by fusion of the viral envelope with the ONM. In Vero cells infected with a HSV-1 gK deletion mutant, capsids aggregated in front of the INM in large clusters as has been impressively shown by Jayachandra *et al*. [82] clearly indicating that capsids do not bud into the PNS in the absence of gK suggesting that gK is rather involved in budding than in fusion. gK was also proposed to contain domains in its amino-terminal portion that promotes aberrant nucleocapsid envelopment and/or membrane fusion between different virion envelopes [83]. Quantitative electron microscopic analysis revealed that budding at nuclear membranes is inhibited in cells infected with a HSV-1 gK deletion mutant [84] implying that gK is involved in budding of capsid also at nuclear membranes rather than only at cytoplasmic membranes (see section 9) [85]. Yet, with all components in place, the capsids approach the INM for the actual stage of budding (Fig. 4A) forming during the process a dense proteinaceous coat covering the rising viral envelope [7, 42]. Tegument proteins are deposited around the capsid starting at the budding front while the capsid pushes the INM towards the ONM. At the back side of the rising envelope, the INM is pulled behind the budding capsid for fission from the INM to give rise to an enveloped virion covered by a dense coat. The PNS is normally 20 to 40 nm wide so that the intruding viral envelope comes in close apposition to the ONM even before budding is completed. Although close apposition is the initial step for membrane fusion [86, 87], fusion of the viral envelope with the ONM at this stage has never even been proposed. Instead, enveloped and tegumented virions measuring 200 nm in diameter can be observed in the PNS. The envelope-embedded glycoproteins are, at this stage, hidden by a dense coat. In analogy to the clathrin-coated vesicles, which need to be uncoated to enable fusion [88], we consider it likely that this dense coat on the viral envelope prevents fusion with the ONM – and possibly – with the INM.

7. Capsid transport across the outer nuclear membrane

It is undisputed that non-enveloped virus particles in the cytosol can easily be detected. However, the mystery how naked capsids get there has caused heated debates and controversies. As non-enveloped capsids are often found in close neighborhood to the ONM, it is tempting to speculate that these naked capsids emerged as a consequence of enveloped perinuclear viral particles shedding their "primary" membrane through fusion with the ONM, releasing tegument and capsids into the cytosol [5]. This hypothetical process, designated "de-envelopment", was widely accepted, yet, firm proof was never presented and it ignored two basic reports [6, 10] as well as the fundamentals of membrane-bound transport. Indeed, Darlington and Moss showed that capsids were intraluminally transported following budding from the cytosolic side into the PNS, whereas Schwartz and Roizman showed that virions were intraluminally transported out of the PNS into the ER and further into cisternae bound by ribosome-free membranes. As the two NEC-forming proteins pUL31 and pUL34 are present at both the INM and the ONM (Reynolds 2002), the transport into the PNS from the cytosolic side may be quite similar to the budding process *via* the INM (Fig. 4), showing positive and negative curvatures of membranes becoming densely coated, and deposition of tegument proteins at the budding front [6, 8, 75, 89]. Although clearly visible by electron microscopy, budding from the cytosolic

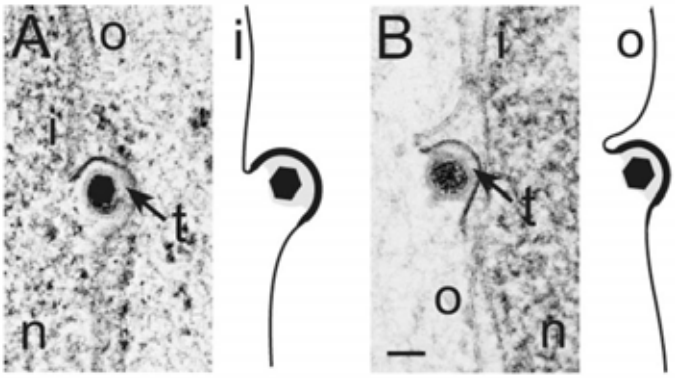

Fig. 4. Capsid transport (A) across the INM (i) and (B) across the ONM (o); nucleus (n), tegument (t). Both the INM and the ONM form positive and negative curvatures typical for budding. A dense coat is formed at the budding front, and tegument deposited between capsid and rising envelope. Reproduced from copyright © 2005 American Society of Microbiology, Leuzinger, H., Ziegler, U., Schraner, E. M., Fraefel, C., Glauser, D. L., Heid, I., Ackermann, M., Müller, M., and Wild, P. 2005, J. Virol., 79, 13047 [7] and copyright © 2005 American Society of Microbiology, Wild, P., Engels, M., Senn, C., Tobler, K., Ziegler, U., Schraner, E. M., Loepefe, E., Mueller, M. and Walther, P. 2005, J. Virol. 79, 1071 [42]. Bar 100 nm.

side into the PNS was not taken seriously or, worse, even misused to "document" the de-envelopment process. Thus, close neighborhood of capsids to the ONM at the cytoplasmic face remained as one important indication that capsids are released from the PNS by de-envelopment e.g. [90].

In the absence of direct evidence, e.g. of convincing images of the fusion process between the "primary" envelope and the ONM, indirect arguments were used to support the "de-envelopment theory". These included apparent differences in tegumentation between intracellular and extracellular HSV-1- [91] and PrV-virions [92] reviewed by Mettenleiter [30] as well as the various roles of viral envelope glycoprotein functions [4].

Concerning tegumentation in PrV, [92] observed formation of cytoplasmic capsid aggregates upon deletion of the UL47 homologue, which encodes for the viral tegument protein VP13/14. According to a one-step growth analysis, the viral titers obtained with this mutant were 10-fold reduced in comparison to wild-type virus and the plaques were smaller. Without showing evidence and although they detected the UL47 protein in the nucleus, the authors claimed that VP13/14 was not incorporated in perinuclear virions, whereas it was abundantly detected in cytoplasmic and extracellular viral particles. Eventually, they used this circumstantial evidence in support of the "de-envelopment" theory, claiming that deletion of UL47, which encodes VP13/14, did neither affect "primary envelopment" nor the consecutive "de-envelopment" but impaired to a certain extent "secondary envelopment". Similarly, Naldinho-Souto *et al.* [91] did not detect VP13/14 of HSV-1 in perinuclear enveloped virions, while they showed its presence in individual extracellular virions by immune-gold-labeling. At the same time, these authors detected VP16, the product of UL48, in both perinuclear and extracellular particles. Based on this, they also counted their findings to supporting the "de-envelopment" theory.

The envelope of alpha herpesviruses contains numerous glycoproteins, some of which are essential for receptor binding and entry into the cell, i.e. gB, gD, gH and gL [93, 94]. In the context of egress, deletion of gB in PrV [95] or one of the other glycoproteins in HSV-1 had no impact on capsid transport across the ONM whereas simultaneous deletion of the two glycoproteins gB/gH led to accumulation of virions in the PNS [96]. This phenotype was also taken as evidence for the "de-envelopment" theory and gB/gH were held responsible for it. However, as shown in Fig. 5 reprinted from [96], more than a dozen of capsids were caught in the process of being transported across the ONM. The phenotypes of virus interaction with the ONM show all characteristics

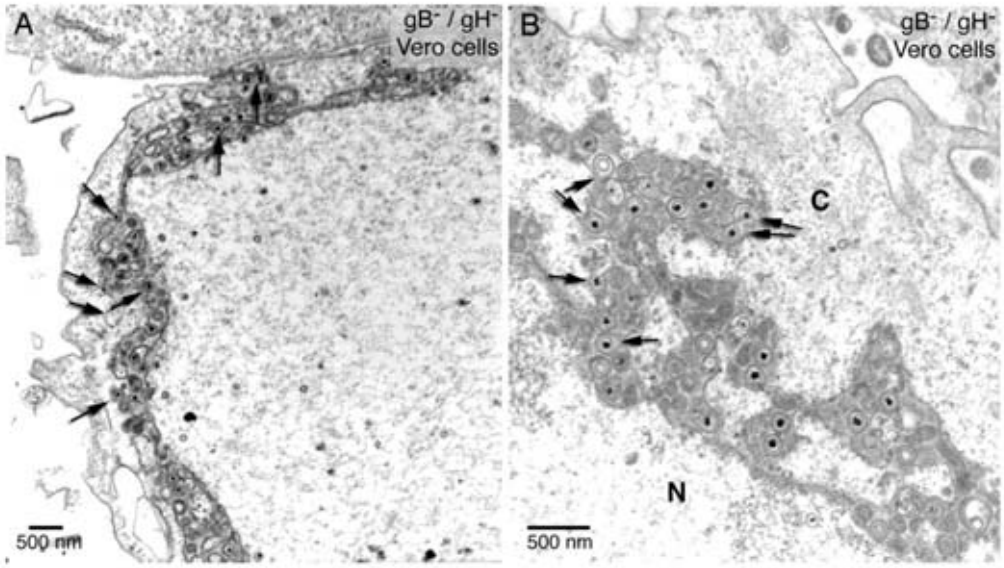

Fig. 5. Virus transport across the ONM in the absence of gB/GH. gB/gH null virions accumulate in the PNS-ER compartment. The arrows (inserted by PW) point to interactions of virus particles with the ONM and ER membranes showing the characteristics of budding. The fusion proteins gB/gH are missing. Therefore, these virus membrane interactions represent various stages of budding – not of fusion. Reproduced from Fig. 2 of Farnsworth, A., Wisner, T. W., Webb, M., Roller, R., Cohen, G., Eisenberg, R. and Johnson, D. C. 2007, Proc. Natl. Acad. Sci. USA, 104, 10187, copyright © 2007 with permission from National Academy of Sciences, USA.

of budding as first published by Darlington and Moss [6]. Thus, naked capsids in the cytosol remained unexplained, while proof for budding through the ONM was provided. As budding through the INM was not seen at all in this picture, it seemed as if the transport from the cytosol to the PNS had been enhanced, with the result that virions accumulated in the PNS.

8. Capsid transport into the cytosol

In the absence of hard evidence for "de-envelopment" at the ONM, the hypothesis arose that naked capsids gained access to the cytosol *via* impaired nuclear envelope, probably starting at dilated nuclear pores. To our knowledge, an impaired nuclear envelope had first been shown in cells infected with simian agent 8 [8] which was confirmed later by others and with other herpesviruses [42, 97-101]. The pathway of nuclear membrane breakdown is apparently similar to that in dividing cells, where nuclear pore complexes disintegrate and pores dilate, subsequently leading to breakdown of the nuclear envelope during mitosis [102, 103]. Transmission and scanning electron microscopy at high resolution of HSV-1- and BoHV-1-infected cells clearly revealed that nuclear pores dilated and that capsids escaped *via* dilated pores. Furthermore, the curvature connecting the INM with the ONM remained intact, even in severely impaired nuclear envelopes implying that breakdown had started at a nuclear pore [7, 42, 98].

9. Capsid transport across cytoplasmic membranes

Nuclear membranes extend directly into the ER. Therefore, it is not surprising that capsids have been reported to bud at ER membranes including ONM [7]. Budding capsids at the ONM and ER membranes have been also shown in cells infected with a gB/gH deletion mutant (Fig. 5). The question, hence, is not whether capsid can bud at the ONM and ER membranes, rather the question is how frequently they bud, which is difficult to assess considering the entire time span from infection to cell death. Quantitative analysis of budding capsids in cells harvested at different time points after inoculation my give a rough idea [7]. By examination of 20 sections through HSV-1-infected cells 7 capsids were found to bud at the INM, 16

at the ONM, yet only 1 at an ER membrane. The probability to find budding capsids at both nuclear and ER membranes in ultrathin sections (≈80 nm) at a given time is extremely low. For example, in the same study only 1 budding capsid was found at the INM by 12 hpi. However, cryo-scanning electron microscopy revealed close to 2 budding capsids per μm^2 nuclear surface at 10 hpi, yet only 0.1 per μm^2 at 15 hpi [99] giving ≈1000 and ≈50 capsids per the entire nuclear surface at 12 and 15 hpi, respectively.

The main target of capsids are Golgi membranes. For understanding the mechanisms of budding at Golgi membranes, two different ways need to be distinguished – wrapping and "simple" budding – because wrapping is a more complicated process requiring different or additional machineries than "simple" budding. During wrapping, capsids bud at Golgi membranes pushing the membrane towards the opposite membrane so that an envelope and concomitantly a vacuole is formed (Figs. 6 and 7). The result is a virion - comprising capsid, tegument and envelope - in a concentric vacuole, which is detached by fission from its donor membrane. For wrapping, capsids bud at any Golgi membranes [7, 42] or, as postulated at vesicles derived from the TGN [30, 31] or at endosomes [36]. To successfully complete this process, first, membranes must be provided to form the envelope and its surrounding vacuole. Therefore, wrapping at vesicles is unlikely to be accomplished unless the vesicle is of the size to provide the required membranes for formation of both the viral envelope and the vacuolar membrane, or, alternatively, membrane constituents are provided by vesicular trafficking from e.g. Golgi membranes to the rising envelope-vacuole unity, a process never considered so far. Second, the space between envelope and vacuolar membrane becomes filled with substances with the staining properties of proteins, which is forced from the cisternal or vacuolar lumen between the rising envelope and vacuolar membrane. However, these substances become largely extracted during processing by conventional procedures e.g. [96, 104]. They might serve as a space holder and protect the viral envelope from fusion with the vacuolar membrane, which likely would come in close contact with the rising envelope during the process of wrapping.

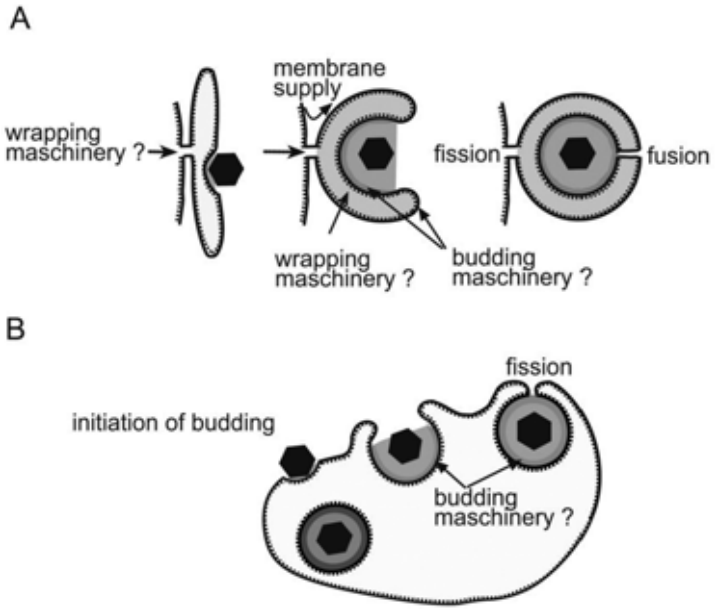

Fig. 6. A) Wrapping of capsids by Golgi membranes. Wrapping by Golgi membranes or endosomal membranes requires additional machineries to budding for bending the rising vacuolar membrane, preventing fusion of the envelope with the vacuolar membrane, and for fission of the vacuolar membrane. B) Budding of capsids into Golgi cisternae or vacuoles. The budding machinery differs from that at nuclear membranes and is located at the rising envelope and possibly underneath the envelope.

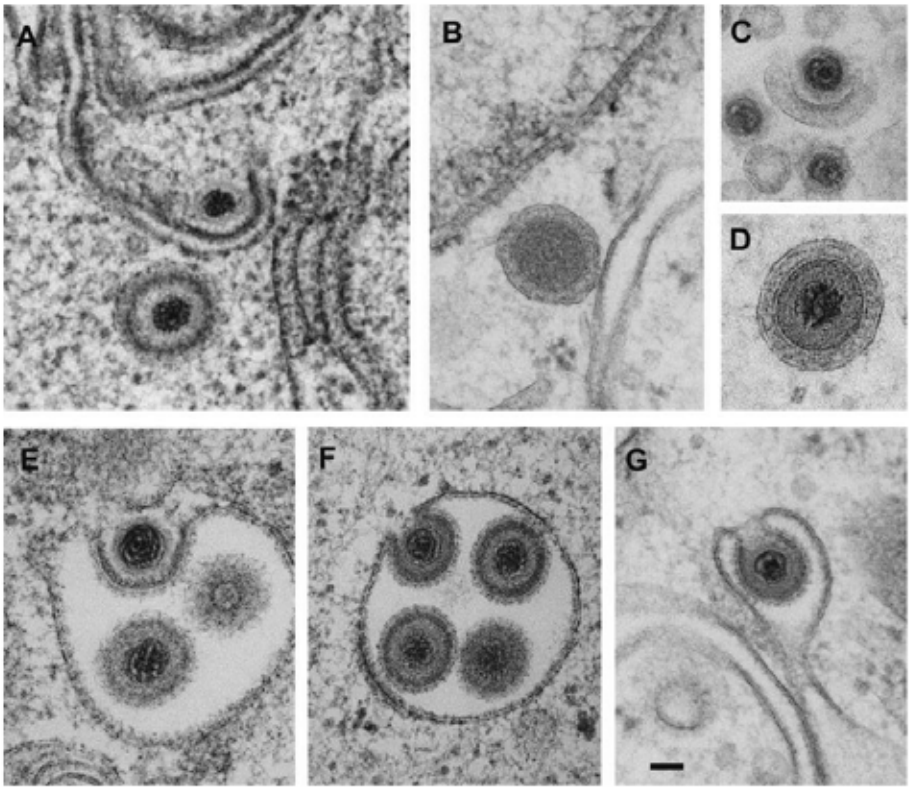

Fig. 7. A) Early phase of wrapping by Golgi membranes, B) wrapping vacuole immediately prior to scission from Golgi membranes (tangential section), C) initial stage of wrapping at a vesicle. Note: for completion of wrapping, membranes need to be supplied. D) Concentric vacuole derived by wrapping engulfing one virion. E-G) Capsids may "simply" bud at Golgi membranes. Early (E) and late phase (F) of a budding capsid into a vacuole or Golgi cistern already containing virions. G) Budding of a capsid into a Golgi cistern immediately prior to completion by fusion of the Golgi membranes and scission of the viral envelope, respectively. Panel B and G are reproduced from copyright © 2005 American Society of Microbiology Wild, P., Engels, M., Senn, C., Tobler, K., Ziegler, U., Schraner, E. M., Loepefe, E., Mueller, M. and Walther, P. 2005, J. Virol. 79, 1071 [42]. Bar 100 nm

Alternatively to wrapping, capsids can also "simply" bud into vacuoles or Golgi cisternae (Figs. 6 and 7) as has been shown 5 decades ago [6], and later by Leuzinger *et al*. and Homman-Loudiyi *et al*. [7, 105]. The result is one, or two or numerous virions in vacuoles or Golgi cisternae of various sizes. Vacuoles and/or Golgi cisternae engulfing one or two or plenty of virions were repeatedly shown e.g. [6, 7, 10, 14, 29, 92, 95, 105-110]. In three-dimensional thinking, a vacuole of almost any size in a thin section might be or might be not connected somewhere to Golgi membranes so that structures appearing as vacuoles could be Golgi cisternae. Vacuoles are formed by fission of the vacuolar membrane from Golgi membrane.

In addition to enveloped virus particles, Golgi cisternae and vacuoles also contain proteins, which are largely lost during conventional preparation procedures e.g. [110]. They are difficult to recognize in well frozen cells (Fig. 7) but easily seen in poorly frozen samples that leads to segregation [7]. These proteins might hinder virions to come in close contact with the cisternal or vacuolar membrane that most likely would initiate immediate fusion. Whether or not these proteins are identical to those covering the viral envelope or even derive from them (see section 10) remains to be investigated.

The phenotype of budding at Golgi membranes with or without concomitant vacuole formation (wrapping) differs from budding at nuclear membranes insofar as no dense coat covering the rising envelope is formed. Budding at Golgi membranes depends on the cytoplasmic ESCRT

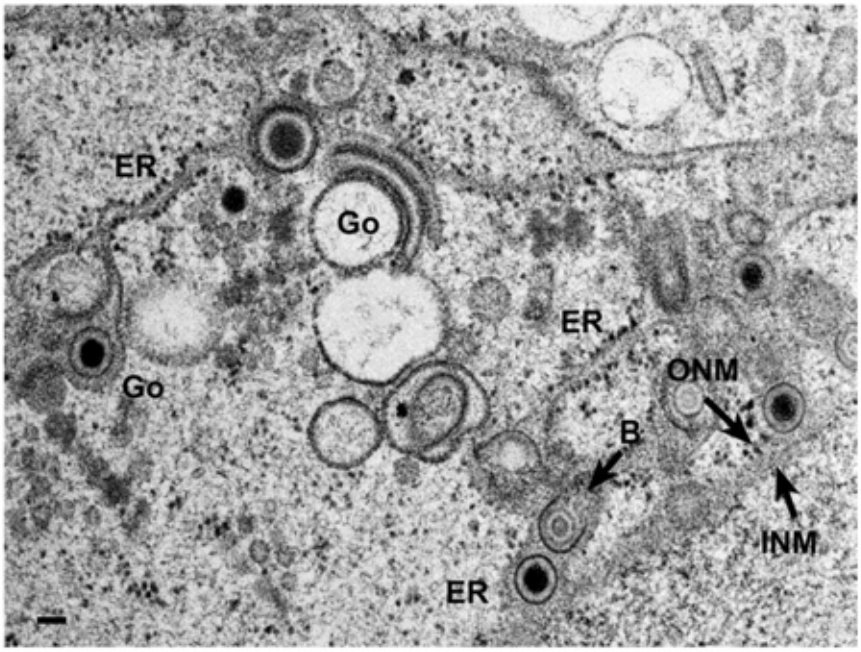

Fig. 8. The PNS-ER-Golgi entity. PNS delineated by the INM and ONM connect to the ER that continues into Golgi cisternae so that virions can be intraluminally transported from the PNS into Golgi cisternae (Go). Note the virions in the PNS, ER and Golgi cisternae with electron dense envelope. A capsid devoid of DNA buds (B) into the ER. Bar 100 nm

machinery [34, 111]. Other factors are gK, and the membrane-associated pUL20 protein [112] interacting with gK [113]. In the absence of pUL20, virions accumulated in the PNS-ER compartment [112] indicating that pUL20 plays an essential role in maintenance of Golgi function in HSV-1 envelopment. Besides gK and pUL20, three tegument proteins encoded by UL36 [114], UL37 [115] and UL48 [116], are involved in HSV-1 envelopment at the Golgi complex. Deletion of UL36, UL37 or UL48 resulted in accumulation of capsids in the cytosol indicating that envelopment by Golgi membranes is impaired. Clathrin was claimed to be involved in envelopment of human herpesvirus-6 capsids at Golgi membranes together with viral proteins [117].

10. Virus transport from the perinuclear space to the Golgi complex

While no hard evidence for fusion of the viral envelope with the ONM has been presented yet, virions have been repeatedly shown within ER cisternae [3, 7, 10, 14, 106, 108, 118, 119]. Of course, they may enter ER cisternae by budding from the cytosol as described in section 9. Alternatively, they may intraluminally transported from the PNS into the ER. However, the viral envelope must be protected at this stage from fusion with the INM, the ONM and ER membranes that form *per se* an entity. In fact, a dense coat has been observed to cover the viral envelope not only in the PNS but also in the ER (Fig. 8), suggesting that it protects the viral envelope from fusion with ER membranes (including nuclear membranes). Images showing "queuing" virions in adjacent ER cisternae to the PNS [14, 108] strongly suggest that virions are transported from the PNS into the ER, indeed. Other indications for transportation of virions from the PNS into the ER are the accumulation of virions in the PNS and adjacent ER cisternae in cells infected with Us3 deletion mutants, or with HSV-1 and subsequent exposure to brefeldin A [104] similar as proteins [120, 121]. The question thus remains how virions are transported out of the ER and how they are translocated into the Golgi complex. In the secretory pathway, proteins are transported to the Golgi complex *via* vesicles that derive from ER exit sites [122]. Proteins may

also be transported through an ER-Golgi intermediate compartment (ERGIC) [123, 124]. Whether or not the ERGIC is a stable structure is under debate [125]. Furthermore, membranes of the *cis*-Golgi may approach the ER and contact ER exit sites to capture proteins for transportation to the Golgi complex, a process described as 'hug-and-kiss' behavior [126]. There are also transitional elements connecting Golgi membranes to ER membranes [44, 127, 128] possibly enabling direct transportation of proteins from ER cisternae into Golgi cisternae. As shown by Schwartz and Roisman [10], virions move into cisternae bound by membranes devoid of ribosomes, most likely Golgi membranes. They hypothesized that these membranes tunnel virions directly to the extracellular space. More likely, virions are transported from the ER into Golgi cisternae *via* ER-to-Golgi transitions [45]. The ONM continues into ER membranes, which in turn merge with Golgi membranes forming an entity (Fig. 8) as has been shown in BoHV-1 [106] and HSV-1 [45]-infected cells. Therefore, virions can be easily transported form the PNS into Golgi cisternae, which is supported by the fact that about 80 HSV-1 virions per mean cell volume were within the PNS-ER compartment at 12 and 16 hpi, respectively, but close to 300 by 24 hpi [107] suggesting that virus transportation out of the ER is inhibited after Golgi fragmentation [29]. Despite hard evidence for both budding of capsids at the ONM and virus transport within the ER, the theory of capsid release *via* fusion of the viral envelope with the ONM was [129] and still is heavily disputed [130]. Intraluminal transport of virions from the PNS into Golgi cisternae implies two diverse populations of virions in Golgi cisternae, one derived by intraluminal transport covered by a dense coat, the other derived by budding.

11. Virus transport from the Golgi complex to the extracellular space

The goal of packaging of secretory products into granules is to produce a carrier to transport the content to the cell periphery for exocytotic release [43]. There, the membrane of the carrier fuses with the plasma membrane releasing the content into the extracellular space. Similarly, virus particles are transported to the cell periphery for exocytosis [131] in vacuoles derived by wrapping or by packaging. The two types of vacuoles can be distinguished at any time from formation until fusion of the vacuolar membrane with the plasma membranes [42, 106]. Vacuoles are directed to virally induced, specialized egress sites of the plasma membrane [132]. During exocytosis, the vacuolar membrane fuses with the plasma membrane releasing virions into the extracellular space. The vacuolar membrane including spikes (glycoproteins) is inserted into the plasma membrane [130]. Consequently, glycoproteins are transported from the Golgi complex to the plasma membrane. Endocytic activity is significantly reduced but not ceased in HSV-1-infected cells [14] so that membranes including glycoproteins are transported back *via* endosomes that is proposed to be the main source of membranes for HSV-1 envelopment [35].

12. Conclusion

Budding at nuclear membranes is driven by the viral proteins pUL31/pUL34 involving dense coat formation that is not required for budding at Golgi membranes. Virions derived by budding at the INM into the PNS are infectious. Apparently, these virions are intraluminally transported into the ER implying that the viral envelope is protected from fusion with the ONM or ER membranes. ER-to-Golgi transitions strongly suggest an intraluminal transportation route from the PNS into the Golgi complex for packaging into transport vacuoles. Capsids, which bud into Golgi cisternae and vacuoles or are wrapped by Golgi membranes need to gain access to the cytosol *via* another route than by de-envelopment, i.e. *via* impaired nuclear envelope that starts by dilation of nuclear pores. Herpesvirus egress will not be understood as long as the fundamentals of membrane-bound transportation at the two nuclear membranes are not strictly considered, as long as the two ways of vacuole formation is not conceived, and as long as release of capsids *via* impaired nuclear membrane is negotiated.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interests.

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