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In vitro nuclear egress of herpes simplex virus type 1 capsids

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ABSTRACT

During their life cycles, viruses typically undergo many transport events throughout the cell. These events depend on a variety of both viral and host proteins and are often not fully understood. Such studies are often complicated by asynchronous infections and the concurrent presence of various viral intermediates in the cells, making it difficult to molecularly define each step. In the case of the herpes simplex virus type 1, the etiological agent of cold sores and many other illnesses, the viral particles undergo an intricate series of transport steps during its life cycle. Upon entry by fusion with a cellular membrane, they travel to the host cell nucleus where the virus replicates and assembles new viral particles. These particles then travel across the two nuclear envelopes and transit through the trans-Golgi network before finally being transported to and released at the cell surface. Though viral components and some host proteins modulating these numerous transport events have been identified, the details of these processes remain to be elucidated. To specifically address how the virus escapes the nucleus, we set up an *in vitro* model that reproduces the unconventional route used by herpes simplex type 1 virus to leave nuclei. This has not only allowed us to clarify the route of capsid egress of the virus but is now useful to define it at the molecular level.

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1. Introduction

Herpes simplex virus type 1 (HSV-1) is a member of the herpesvirus family that afflicts many humans. It is indeed estimated that 70–80% of the World population is infected by HSV-1 [1–3]. HSV-1 causes facial mucosal lesions commonly referred to as cold sores [4–6]. HSV-1 is also associated with severe clinical manifestations such as corneal blindness [7,8] and neurological problems [9–12]. Herpes viruses are also characterized by their competence to establish latent infections, with a concomitant reduction of viral protein expression and evasion of the immune system. Despite the existence of efficient treatments against active HSV-1 infections, a drug that can completely eradicate the virus from infected individuals has yet to be discovered. A better understanding of HSV-1 maturation cycle may thus provide novel therapies against this virus and its family members.

The assembly of many viruses with DNA genome (adenovirus, parvovirus, polyomavirus and herpesvirus) and also RNA genome viruses (orthomyxovirus) occurs in the cell nucleus. In the case of HSV-1, the main focus of our research and of the present paper, the release of the virus from the nucleus is poorly understood. It is well accepted that following their entry in the host cell, HSV-1 capsids are targeted to nuclear pores where the viral genome is injected in the nucleus [13–19]. Viral replication, transcription, DNA packaging and assembly of new viral capsids then take place in that compartment [18,20,21]. Newly assembled capsids must subsequently leave the nucleus to pursue their egress to the cell surface. In the past, three eagerly debated models were proposed to depict HSV-1 post-nuclear transport. One of them is the "envelopment/de-envelopment/re-envelopment model" [22]. According to this model nuclear capsids bud into the inner nuclear membrane and accumulate primary enveloped capsids in the perinuclear space. This primary envelope is then rapidly lost following fusion of the viral particles with the outer nuclear membrane. Naked (i.e. unenveloped) capsids are therefore released in the cytoplasm and later on acquire their mature final envelope at the TGN [23–25]. Because the capsids acquire two successive envelopes, the model is also known as the "two envelope model" [19,22,26]. In the second model, the "biosynthetic model" [27], capsids also bud at the inner nuclear membrane and accumulate an enveloped intermediate in the perinuclear space. However, perinuclear enveloped capsids do not interact with the outer nuclear membrane.

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EM, electron microscopy; ER, endosplasmic reticulum; HSV-1, herpes simplex type 1 virus; IRES, internal ribosome entry site; JMEM, Joklik's modified Eagle medium; KDa, kilo Dalton; MOI, multiplicity of infection; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; RNA, ribonucleic acid; RSB, reticulocyte standard buffer; TCA, trichloroacetic acid; TGN, trans-Golgi network; TRITC, tetramethyl rhodamine isothiocyanate.

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Instead, the capsids keep this primary nuclear membrane derived envelope and reach the TGN via the classical ER-Golgi biosynthetic pathway. Since enveloped capsids are transported from the perinuclear space to the lumen of the ER and the Golgi, this model is also referred to as the ''luminal model'' [27]. The third model, the ''nuclear pore enlargement model'', involves an active distension of the nuclear pores induced by the virus [28,29]. This model excludes budding of capsids at the inner nuclear membrane. Though the two membrane model is now favored by many, proving it has been a long and arduous endeavor. To simplify the study of this complex step of the viral life cycle and demystify its regulation, we undertook to isolate this step from the rest of the cell thereby removing all other viral intermediates. Our *in vitro* nuclear egress assay was born!

2. Purpose and potential of the *in vitro* HSV-1 nuclear egress assay

Until recently, there has been limited ways to quantify HSV-1 nuclear egress. These include microscopic approaches (EM and fluorescence microscopy) as well as plaque assays (e.g. the quantification of intracellular infectious particles). These approaches have often been coupled to immuno-labeling to study particular proteins implicated in HSV-1 nuclear egress and/or viral deletion mutants. While these approaches have proven very useful, quantitative EM is fairly tedious. On the other hand, plaque assays do not specifically evaluate nuclear egress since they monitor total viral infectious particles found in the cell irrespective of their origin (i.e. coming directly from the nucleus or a downstream step). The development of a quantitative and specific assay to monitor and characterize HSV-1 nuclear egress at the molecular level was therefore fitting. *In vitro* assays have proven instrumental to decipher numerous intracellular transport events and for the study of various viral life cycles [30–34]. As discussed above an *in vitro* assay was set up to specifically analyze the nuclear egress of HSV-1 [30]. The assay basically consists in the isolation of nuclei from infected cells and their incubation in conditions that allow HSV-1 capsid release. The main advantage of this assay is its focus on only one step of the virus maturation, i.e. nuclear egress, since the assay is devoid of other organelles. Furthermore, because isolated nuclei are used instead of whole cells, viral particles in the system are unequivocally coming from the nucleus. In addition, most reagents can be tested in this assay, contrary to intact cells where the plasma membrane acts as a barrier to many substances. The assay is also flexible, fast, reproducible and quantitative, allowing multiple parameters to be tested in parallel in a single experiment. Finally, the capsids released by the nuclei can readily be characterized biochemically, morphologically and by systemic approaches to ultimately define the various factors implicated in HSV-1 nuclear egress. Thus far, this assay allowed us to confirm the two envelope model [30] and is now being used to define the complex and sequential acquisition of the tegument, an amalgam of viral and host proteins found between the viral capsid and its envelope (Rémillard-Labrosse et al., in preparation). Finally, the assay is particularly helpful to probe nuclear egress at the molecular level.

3. Reagents preparation

3.1. Isolation of infected nuclei

The first steps of the *in vitro* assay are to prepare all the ingredients for the assay, nuclei being the most important component. To efficiently accumulate large amounts of material, we opted for HeLa cells adapted for growth in suspension (HeLa S3 cells were originally obtained from ATCC #CCL-2.2) [35]. The assay may be adapted to other cell types and growth systems but great care should be taken to re-optimize each step. The following steps indicate how to make stocks of HSV-1 infected nuclei. First, grow HeLa S3 cells in Joklik's modified Eagle's medium (JMEM) + 5% fetal calf serum (Sigma–Aldrich and Medicorp respectively) in magnetic spinner flasks with agitation between 50 and 100 rpm (*i.e.* without tearing the cells and creating lather but rapid enough to prevent cells from adhering to bottle surface or sediment). Cells should be grown to obtain a density around $1–2 \times 10^6$ cells/ml just before the infection. Prior to infection, count and centrifuge the cells at 300*g* for 10 min then discard the supernatant and gently resuspend the cells in one tenth of the original volume in JMEM + 0,1% bovine serum albumin. Then, add HSV-1 virus to a multiplicity of infection (MOI) of 3 and let adsorb for an hour at 37 °C with agitation in a small Erlenmeyer with a baffled bottom to avoid clustering of the cells. Subsequently, transfer infected cells to a magnetic spinner flask and top up the medium to its initial volume with culture media (JMEM + 5% fetal calf serum) before incubating the infected cells for an additional 6–8 h at 37 °C with continuous agitation. For the next steps of this protocol, always work in the cold room (4 °C) with ice-cold media and buffers and keep cells and nuclei suspensions on ice to preserve cellular structures. After the infection period, count and centrifuge the cells at 300*g* for 10 min, discard the supernatant and gently wash the cells in phosphate buffered saline (PBS) supplemented with 5 mM $MgCl_2$ (13.7 mM NaCl + 0.3 mM KCl + 0.2 mM KH_2PO_4 + 1 mM Na_2HPO_4 anhydrous + 5 mM $MgCl_2$). Then, re-centrifuge the cells at 300*g* for 10 min and resuspend them in RSB buffer (10 mM NaCl + 10 mM Tris–HCl pH 7.4 + 5 mM $MgCl_2$). Ten minutes later, the cell suspension is mechanically broken by cavitation (we use a customized cell homogenizer) and visually inspected by microscopy to insure the cells are ruptured but the nuclei are intact. Typically, two passages in the cell homogenizer are sufficient. Quickly top up the cells with PBS + 5 mM $MgCl_2$ to re-equilibrate osmolarity. After that, deposit the cell lysate on a 40% iodixanol (Optiprep Axis-Shield) cushion and centrifuge at 700*g* for 15 °C at 4 °C. The interface is then collected and the residual volume of the 40% iodixanol cushion measured to adjust the cell lysate to 25% of iodixanol. Afterward, add the 25% iodixanol cell lysate on a discontinuous gradient consisting of 35%, 29% and 27% iodixanol layers. The gradient is centrifuged at 10 000*g* for 30 min at 4 °C in a SW28 Beckman rotor, then the 29%/35% interface, where the nuclei are found, is harvested with a needle. For storage, the nuclei preparation is supplemented with 50% glycerol + 10 mM dithiothreitol. Smoothly mix the nuclei to ensure a homogenous preparation but avoid vortexing. Snap freeze the nuclei in liquid nitrogen and keep at −80 °C.

3.2. Nuclear integrity

The integrity of isolated nuclei should be tested, particularly for new users, to fine tune their preparation and insure the correct results. Note that these assessments can also be performed to monitor nuclear integrity following their incubation in the *in vitro* nuclear egress assay.

3.2.1. Morphological assessment

The nuclei can be observed by electron microscopy (EM) to evaluate the overall integrity of the nuclei, the presence of both nuclear envelopes and the absence of other contaminating organelles. Various protocols abound in the literature and are equally satisfactory. In our laboratory, the nuclei preparations are treated as follows. Fix the nuclei for 1 h with 2.5% glutaraldehyde (Canemco and Marivac) in sodium cacodylate buffer (0.1 M, pH 7.2–7.4) then postfixed an additional hour in 1% osmium tetroxide + 0.1 M sodium cacodylate (Mecalab). The samples are then contrasted with 2% aqueous uranyl-acetate (Canemco and Marivac) for 1 h at 4 °C,

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