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The role of the TGN in the transport of Herpes simplex virus type I capsids

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This thesis called:

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This thesis is dedicated to my 4 years old nephew, Alexandru, currently under the acyclovir treatment for his recently discovered Herpes simplex virus type 1 encephalitis.

Résumé

Le virus Herpès Simplex de type 1 (HSV-1) est un membre de la famille des Herpesviridae et cause une variété de maladies chez les humains et les animaux (Roizman and Knipe, 2001). HSV-1 peut demeurer latent dans les neurones sensoriels et occasionnellement se réactiver et causer une maladie récurrente. Lorsqu'il est réactivé, HSV-1 cause des feux sauvages ainsi que de sérieuses maladies telles que la kératoconjonctivite et l'encéphalite. Les traitements antiviraux tels que les vaccins n'ont toutefois pas réussi à éradiquer HSV-1.

La meilleure méthode cepedant pour empêcher HSV-1 de causer des maladies est d'utiliser des vaccins qui bloquent l'infection initiale. Au niveau cellulaire, une méthode pour bloquer la propagation virale aux cellules voisines serait la plus utile. En vue de trouver une méthode préventive, les détails du cycle viral doivent être explorés, y compris la manière dont le virus entre et infecte les cellules. Nous espérons qu'une meilleure compréhension du transport de HSV-1 dans les cellules infectées nous aidera dans le traitement des maladies causées par HSV-1. Une fois dans la cellule, HSV-1 produit de nouvelles capsides dans le noyau des cellules infectées.

En 2007, parmi de nombreuses autres études, Rémillard-Labrosse et al. suggèrent que les capsides nouvellement assemblées, trop grosses pour sortir par les pores nucléaires, bourgeonnent dans l'espace périnucléaire et fusionnent ensuite avec la membrane nucléaire externe. Par la suite, les capsides cytoplasmiques nues migrent au site de ré-enveloppement, présumé être le TGN ou les endosomes (Turcotte at al., 2005). Plusieurs laboratoires, dont Turcotte et al. en 2005, ont démontré le rôle du TGN dans le cycle viral de HSV-1. Ils ont constaté que, dans les membranes du TGN, les capsides cytoplasmiques acquièrent leur enveloppe finale pour devenir des particules infectieuses dans le milieu extracellulaire. Le TGN est le lieu de triage des protéines avant d'être délivrées à la surface de la cellule et dans diverses organelles; toutefois, le processus par lequel les capsides de HSV-1 quittent ce compartiment n'est pas encore clair.

Dans cette étude, nous suggérons l'implication de la protéine kinase D (PKD) dans le transport du virus du TGN à la membrane plasmique. Dans l'étude du transport intracellulaire des protéines, PKD est présenté comme un important médiateur pour le transport de cargos du TGN à la surface des cellules. Son activité est dépendante du DAG et la réduction de la synthèse de DAG inhibe le transport de molécules du TGN à la membrane plasmique. De plus, une mutation dans le domaine kinase de PKD entraîne la formation de tubules au TGN et la rétention de cargos dans ces tubules. Nos résultats montrent que les virions de HSV-1 sont également pris au piège dans les tubules du TGN formés lors de l'expression de

PKD sous sa forme mutante. Ces résultats proposent l'utilisation par HSV-1 de cette même voie de sécrétion dans son transport à la surface des cellules.

Mots-clés : HSV-1, capsides, TGN, PKD, membrane plasmique.

Summary

Herpes Simplex Virus Type I (HSV-1) is a member of the Herpesviridae, which causes a variety of diseases in humans and animals (Roizman and Knipe, 2001). HSV-1 can remain latent in sensory neurons and occasionally reactivates to cause recurrent disease. When it is reactivated, HSV cause cold sores as well as other serious diseases such as, kerato-conjunctivitis and encephalitis. Anti-viral drugs as well as vaccines have been unsuccessful in eradicating HSV-1.

The best way to prevent HSV-1 from causing diseases, however, is still to utilize vaccines which prevent of the initial infection. At the cellular level a method to stop viral spread to neighboring cells would be most useful. In order to search for a viral prevention method, the details of the virus lifecycle must be explored, including how it enters and infects cells. We hope that a better understanding of the HSV egress from the infected cells will help in the treatment of HSV-1 diseases. Once HSV-1 is in a cell, it produces new capsids within the infected cell nucleus.

Rémillard - Labrosse et al., 2007, among many other studies, suggest that newly HSV-1 assembled capsids which are to big to escape via nuclear pores bud into the lumen of the nuclear envelope and then fuse with the outer nuclear membrane. The subsequent cytoplasmic naked capsids travel to the re-envelopment site, presumed to be the TGN or endosomes (Turcotte at al., 2005). Many laboratories among with Turcotte at al., 2005, demonstrated the TGN role in the HSV-1 life cycle. They found that in TGN membranes, cytoplasmic capsids acquire their mature envelope to become infectious particles within the extracellular medium. TGN represents the station from where the proteins are sorted and delivered to the cell surface and other various organelles; but it is not clear by which pathway HSV-1 capsids leave this compartment.

In this study, we suggest the implication of Protein Kinase D (PKD) in the viral egress from the TGN to the plasma membrane. In intracellular protein transport studies, PKD is presented as an important mediator of cargo transport from the TGN to the cell surface. Its activity is DAG dependent and reduction in DAG synthesis inhibits the transport of molecules from the TGN to the plasma membrane. Also, a mutation in the PKD kinase domain produces TGN tubule formation and cargo retention in these tubules. Our results show that the HSV-1 virions are also trapped in the TGN tubules formed by the expression of PKD mutant. These results propose that HSV-1 utilizes the same pathway as secretory molecules in their transport to the cell surface.

Keywords: HSV-1, capsids, TGN, PKD, plasma membrane.

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LIST OF ABBREVIATION

AP	adaptor protein
ARF	ADP-Ribosylation Factor
bp	base pair
BFA	brefeldin A
CMV	CytoMegaloVirus
DMEM	Dulbecco's modified Eagle's medium
dsDNA	double-stranded deoxyribonucleic acid
E	early
EM	electron microscopy
ER	endoplasmic reticulum
FBS	fetal bovine serum
gB	glycoprotein B
gC	glycoprotein C
gD	glycoprotein D
gE	glycoprotein E
GFP	green fluorescent protein
GGA	Golgi-localizing, γ-adapter ear homology domain ARF- binding protein
FAPPs	four-phosphate adaptor proteins
gH	glycoprotein H
gI	glycoprotein I
gK	glycoprotein K
gL	glycoprotein L

gM	glycoprotein M
gN	glycoprotein N
GST	glutathione-S-transferase
ICP	Infected Cell Protein
HSV-1	Herpes simplex virus type 1
IE	immediate early
L	late
LAT	latency associated transcript
MOI	multiplicity of infection
PA	phosphatidic acid
PBS	phosphate- buffered saline
PKD	protein kinase D
PI4P	phosphatidylinositol-4-phosphate
PI4, 5P2	phosphatidylinositol 4, 5-biphosphate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SM	sphingomyelin
TGN	trans-Golgi network
UL	unique long
US	unique short
VP	Virion Protein
VZV	varicella-zoster virus

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CHAPTER I: Literature Review

1. Introduction

Viruses are the smallest infectious particles, with diameters ranging between 18 and 300 nm. Like all viruses, these particles cannot be seen with a light microscope and are unable to reproduce by themselves because they lack certain functions. The Latin word *virus* means poison. Viruses establish an obligate intracellular parasitism into many different biological organisms in order to produce new virions (infective viral particles).

1.1 Brief history of herpesviruses

Evidences for herpesviruses have been signaled in the 5th century B.C. when the ancient Greek physician and father of medicine Hippocrates mentioned for the first time about skin lesions (Roizman and Whitley 2001, Smith and Cyr 1988). The family name is derived from the Greek word herpein "to creep" which refers to the latent, re-occurring infections typical of this group of viruses. Herpesviridae can cause latent or lytic infections (Wikipedia definition). Herodotus, in the Roman civilization period, described the presence of the fever associated with these lesions, and he named it "herpes febrilis" (Thomas Bateman, 1814). Other references for herpes virus infection are dated from the Shakespeare's era in England, and much later in the king's court of France (Astruc, 1736). The advanced methods of herpesvirus studies began in the late 19th century when researchers started to test scientific hypothesises about how the viruses interact with the host. In the early 20th century, Lowenstein and Gruter demonstrated that human HSV could also produce lesions on the rabbit's cornea (Gruter, 1924). In 1920 and 1930, it was found that many lab animals were susceptible to HSV infections. In 1939, Burnett and Williams published an article describing the nature of latency, noting that HSV seemed to persist for life and could be reactivated under stressful conditions to produce visible lesions (Burnet and Williams, 1939). The work with the cell culture allowed for the discovery of the other human herpesviruses, including cytomegalovirus (CMV) and Varicela zoster virus (VZV) (Craig et al., 1957). Until now, there were about 100 Herpesviruses isolated from many animal species and 9 from humans.

After the 1960s the new technologies such as electron microscopy (EM), DNA sequencing, and DNA cloning made it possible to determine the structure of herpesvirus particles, the sequence of their genomes, the viral gene expression pattern, and the identification of many individual gene products.

Moreover, advanced studies over the last 40 years have resulted in new treatments and vaccines for herpesvirus infections (e.g. VZV) (Epstein, Achong, and Barr, 1964). Recently, herpesviruses have been re-examined for use as viral vectors for certain treatments of human diseases. The modern experimental period has facilitated a better understanding of herpesvirus diseases and made it possible to utilize herpesviruses to potential human health benefit.

1.2 Proprieties and classification of herpesviruses

Herpesviridae find hosts in amphibians, reptiles, fish, birds, and mammals and consist of a wide variety of viruses. The International Committee on the Taxonomy of Viruses defined *Herpesviridae* for the first time as being capable of establishing a latent infection in their natural hosts in a specific set of cells, which varies from one virus to another. There are also other biological properties, such as the length of the reproductive cycle. These were used as the basis of classification, before DNA sequences of the viruses were known. Members of the family *Herpesviridae* were classified by the Herpesvirus Study Group into three subfamilies: the *Alphaherpesvirinae*, the *Betaherpesvirinae*, and the *Gammaherpesvirinae*. This classification is based on host range, length of replication cycle and cell tropism (Roizman, Bartha, and Biggs, 1973; Roizman et al., 1992; Van Regenmortel et al., 2000).

Alphaherpesviruses (α) is represented by Herpes simplex virus type 1 (HSV-1), Herpes simplex virus type 2 (HSV-2), Pseudorabies virus (PRV), VZV and Marek's diseases virus (MDV). They are characterized by a short replication cycle, a rapid multiplication in cell culture, and a large host variety *in vitro* and *in vivo*, and could be latent within neuronal cells (Van Regenmortel et al., 2000).

In contrast, **betaherpesviridae (β)** replicate slower than α; they have a limited host range, and establish latency in numerous tissues, such as secretory glands and lymphoreticular cells. During infection with herpesviruses of this subfamily, host cells frequently become enlarged (cytomegalia) leading to the name of the cytomegaloviruses, the representative members of the β-herpesvirus subfamily (Van Regenmortel et al., 2000).

Although **γ-herpesviruses** have a limited host range similar to the β–herpesviruses, the length of the replication cycle of these viruses varies between species. The γ-herpesviruses infect cells of the lymphatic system, like B or T lymphocytes, and the latent virus is frequently demonstrated in lymphoid

tissue. The Epstein-Barr virus (EBV) is the principal member of this subfamily (Van Regenmortel et al., 2000; Roizman, 1996; Roizman and Sears 1996; Mettenleiter 1994).

There are eight species of human herpesviruses. (HSV-1) and (HSV-2) are etiological agents for oral and genital lesions, keratoconjuctivitis and encephalitis. Varicela zoster virus is the primary cause for chickenpox and shingles, while HCMV causes cytomegalic inclusion disease. EBV causes mononucleosis and tonsillitis. African Burkitt Lymphoma causes B- and T- cell carcinomas. Human Herpes virus 6 (HHV6) and Human Herpes virus 7 (HHV 7) are known to cause T-cell lymphomas. The newly discovered human Herpes virus 8 (HHV 8) is a causative agent of Kaposi's sarcoma and has been renamed as Kaposi Sarcoma Associated Herpes Virus (KSHV).

1.3 The HSV-1 virion structure and genome organization

HSV-1 produces spherical particles that range in size from 120-200 nm (Wildy P. et al., 1963). They contain more than 35 different virally-encoded gene products that assemble into three major structures: the nucleocapsid, tegument and envelope (Mettenleiter, T.C. 2004).

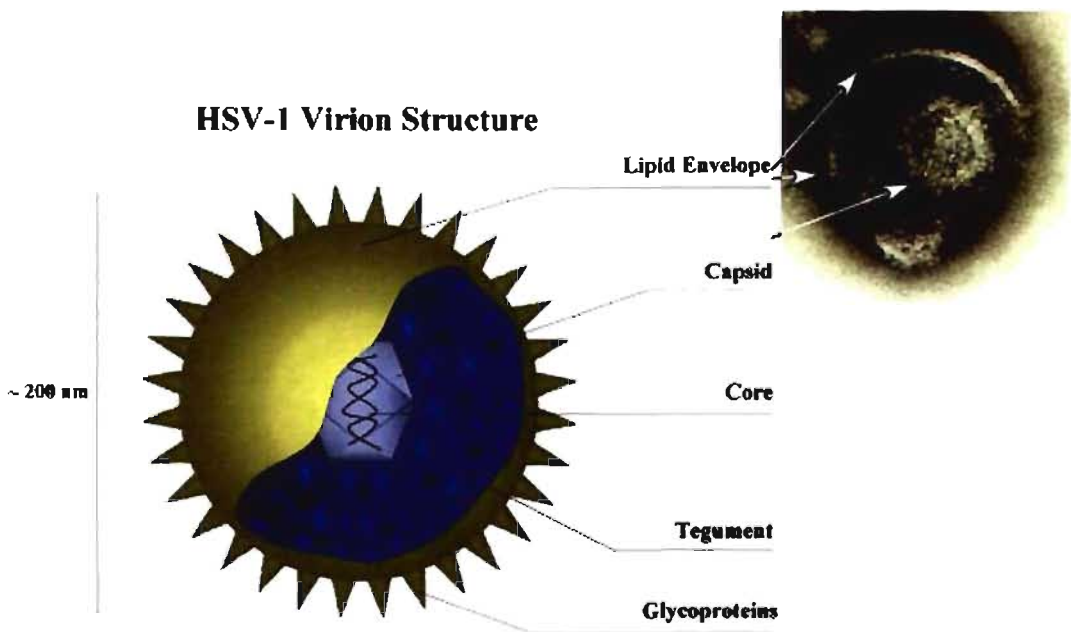

Figure I 1: Herpesvirus virion structure (Roizman and Furlong, 1974).
HSV-1 virion consists of: an electrondense core containing the viral genome, an icosadeltahedral capsid around the core, an amorphous tegument around the capsid, and an envelope derived from cellular membranes containing glycoprotein spikes (Roizman and Furlong, 1974; Travis J. Taylor et al., 2002).

The linear, 152 kbp DNA genome consists of two unique segments (unique long ($\mathbf{U_L}$) and unique short ($\mathbf{U_S}$) flanked by inverted repeats (fig.2) (McGeoch, D.J. et al., 1988, McGeoch, D.J. et al., 1986, McGeoch, D.J., et al., 1985).

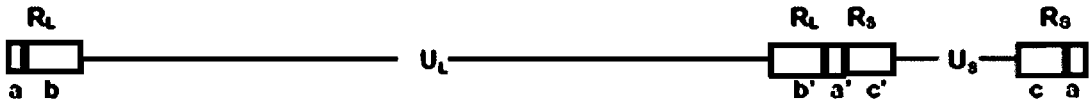

Figure I 2: The HSV-1 virion genome organization.
The HSV genome consists of a long (L) and short (S) component. Each component includes a unique sequence (UL and US) flanked by inverted repeats (RL and RS). The repeat sequence of the long component is designated.

Within infected cells, the "a" repeats that flank both unique segments help to promote the inversion of $\mathbf{U_L}$ and $\mathbf{U_S}$ resulting in the production of four genomic isomers (Hayward G.S. et al., 1975). At present, the viral genome is thought to contain over 80 genes that occasionally overlap with one another and have very few introns (Hardy W.R. et al., 1994, Roizman B and A.E. Sears. 2001).

The icosahedral capsid is composed of four predominant virion proteins (VP5, VP19c, VP23, and VP26) and several less abundant species (Gibson W. and B. Roizman, 1972). The VP5 protein is part of 162 capsomers (150 hexons and 12 pentons) which are linked together by triplex complexes composed of the VP19c and VP23 proteins (Newcomb W.W., et al., 1993, Trus B.L. et al., 1992, Zhou Z.H., et al. 1994). The VP26 protein is bound to the distal tips of each hexon-associated VP5 protein (Booy F. P. et al., 1994, Trus B.L. et al., 1995). The tegument contains more than 20 different virally-encoded proteins, which lie between the nucleocapsid and envelope. This makes the tegument very difficult for study, being the least defined virion substructure (Roizman B and A.E. Sears. 1996). However, recent data suggest that it is a flexible network structure containing extensive protein-protein interactions. The tegument proteins perform several essential functions for the virus, such as host gene expression shut-off, viral gene transactivation and assembly. Surrounding the tegument is the lipid envelope that is derived from host TGN/endosome membranes (Epstein M.A. and S.J. Holt. 1963, Watson D.H. and P. Wildy, 1963, Wildy P. and Watson 1963). The envelope has embedded at least 14 different virally-encoded integral membrane proteins, 12 glycosylated and 2 non-glycosylated with a role in the processes of entry and immune evasion (Campadelli- Fiume G. et al., 2000, Spear P.A. et al., 1970). Cellular components are also present in the

HSV-1 capsids. For example, HSV-1 packages large quantities of polyamines, spermine and spermidine (Gibson W and Roizman 1971). The role of these molecules is to neutralize the negative charges present on the viral DNA, and allow the large genome to fold into preasembeld capsids (Pohjanpelto P. et al., 1988, Raina A. et al. 1988). In addition to cationic molecules, growing evidence indicates that HSV-1 packages both viral and cellular mRNA molecules into virions (Sciortino M.T., et al., 2001). Some hypotheses assume this RNA plays a role in "priming" newly infected cells by delivering transcripts which encode proteins that work early in the replication cycle (Bresnahan W.A. and T. Shenk. 2000). Also, it is possible that packaged RNA serves no function and is only a result of becoming trapped during assembly.

1.4 Pathogenesis

Infections caused by HSV occur worldwide in both developed countries and underdeveloped countries (Black, 1975). Virus transmission, from an infected to a susceptible individual, occurs during close personal contact (Whitley, 2001). Due to HSV infection, more than half of the world's population probably has recurrent HSV infections, enabling the transmission of HSV. The mouth area is the most common location of infection (Whitley, 2001). After primary infection, usually in oral or genital mucosal tissue, the viral replication results in the infection of sensory nerve endings; and the virus is then transported to the dorsal root ganglia (Baringer and Swoveland, 1973; Bastian et al., 1972). In HSV-1 infection, the trigeminal ganglia become the site of the latent virus; whereas in HSV-2 infection, the sacral ganglia is the site of latency (Whitley, 2001). After the establishment of latency, certain stimuli can cause reactivation to occur, and the virus becomes evident at mucocutaneous sites as vesicles or ulcers. Cellular changes, induced by viral infection, include enlargement of infected cells and the appearance of condensed chromatin within the nuclei, followed by degradation of the nuclei. Cells lose intact plasma membranes and form multinucleated giant cells. In infected dermal regions, there is an intense inflammatory response whose intensity decreases substantially with recurrent disease (Whitley, 2001). Primary HSV-1 infection can be either totally asymptomatic or can result in symptoms including fever, sore throat, vesicular or ulcerative lesions. However, asymptomatic infection is generally the rule rather than the exception (Whitley, 2001). Neonatal HSV infections occur at a rate of about 1 in 3000 per year (Nahmias, Keyserling, and Kerrick, 1983; Nahmias, Keyserling, and Lee, 1989), and the highest mortality

rate occurs in babies with disseminated infection (Whitley et al., 1991). Keratoconjunctivitis can also occur in either a single eye or both eyes, and if not treated, causes corneal blindness (Binder, 1977). HSV is one of the most common causes of sporadic, fatal encephalitis (Olson et al., 1967). Some studies estimate a rate as high as 1250 cases per year in the United States (Whitley, 2001). Encephalitis is caused when the virus spreads past the dorsal root ganglia, in which latency is usually established, to the CNS. The mechanisms responsible for this aberrant event in the virus life cycle are unclear. The manifestations of HSV encephalitis include primarily focal encephalitis along with fever, altered behavior, and localized neurological findings. There is usually evidence of localized temporal lobe disease (Whitley et al., 1977; Whitley et al., 1981). In untreated patients, mortality exceeds 70% and only 2.5% of patients return to normal neurological function (Whitley, 2001).

1.5 Treatment

The two methods for control of HSV infections are antiviral therapy and prevention. In theory, any step in the viral cycle, such as attachment, entry, DNA replication, gene expression, virion assembly and egress could be a potential target for antiviral therapy (Coen and Schaffer, 2003). Practically all antiherpesvirus drugs used are nucleoside analogs that target the viral DNA polymerase. Acyclovir, pencyclovir, valacyclovir and famciclovir are members of this class (Wagst ff, Faulds, Goa, 1994). Vaccination would be the ideal method of HSV prevention; however to date no HSV vaccine has been completely successful. Also, patient education can prevent many potential fetus exposures. To date, only one vaccine with 70-90% effectiveness against VZV disease has been accepted for use in humans. The serum was originally isolated from a culture taken in 1970, from a three year old boy named K.Oka in Japan (Asano Y et al., 1977; Takahashi M. 1986; Takahashi M. et al., 1974; Takahashi M. et al., 1985). Moreover, LUPIDON H is an anti-HSV-1 heat inactivated vaccine. Its subcutaneous administration produces cell-mediated immunity in patients (De Maria A., et al., 1995). In order to induce both the cellular and humoral immunity, a disabled infectious single cycle HSV-1 virus (DISC) vaccine was developed. This virus lacks glycoprotein H in the progeny virus and is therefore not infectious (Farrell J.E et al., 1994; McLean C.S, M.Erturk and R. Jennings, 1994).

1.6 HSV-1 LIFE CYCLE

1.6.1 HSV-1 entry

Knowledge about the molecular details of the HSV-1 life cycle has come mostly from the tissue culture systems. The HSV-1 envelope glycoproteins play a central role in virus entry. The initial interaction between the virus and its host begins with the attachment of the viral envelope glycoproteins C and B, to heparin sulphate proteoglycan on the cell surface (Andreas Jacobs et al., 1999; Herold BC et al., 1994; Laquerre S et al., 1998). This binding is followed by the fuzion of the viral envelope glycoproteins gB, gD and the heterodimer H (gH) and L (gL) with their cell surface receptors on the cell membrane, namely Herpes Entry Mediator (HVEM), a member of the tumor necrosis factor receptor family, or nectin-1, a member of the immunoglobulin superfamily, (Montgomery et al., 1996; Nicola et al., 1998 ; Forrester et al., 1992; Sarmiento et al., 1979, Fuller et al., 1989, and Johnson & Ligas, 1988; Manservigi et al., 1977; Roop et al., 1993). Deletion of any one of these glycoproteins results in viruses that are able to bind to cells, but can not penetrate them (Cai W.H. et al., 1988). Also, neutralizing antibodies that target each of these four essential glycoproteins has been isolated, which prove the requirements for each of them in the entry process (Gompels U. and A. Minson 1986). gD binding to its receptor induces a conformational change in gD, that allows gB and the gH:gL heterodimer to complete the fusion process. The gD receptor interaction is extremely important for HSV-1 entry (Montgomery et al., 1996, Whitbeck et al., 1997). It takes between 15 and 30 minutes for HSV-1 to enter into Vero cells (Adi Reske et al., 2007). Originally, HSV was believed to enter into cells by fusion at the cell surface. However, several studies that have been published showed that HSV entry can occur by endocytosis. Nicola et al. demonstrated that HSV entry into CHO and HeLa cells can be inhibited by energy depletion or hypertonic medium, which inhibits endocytosis. Also using lysosomotropic drugs (e.g. bafilomycin A1) the endosome acidification is prevented; thereby the fusion of the virus with endocytic compartment is blocked. These studies suggest that HSV infection of CHO cells occurs through a pH-dependent endocytic pathway by showing that lysosomotropic drugs inhibit productive infection, while entry into Vero cells, in which the original HSV entry pathway studies were conducted, was not affected (Nicola A. et al., 2003, Nicola A. et al., 2004).

1.6.2 Uncoating and genome release

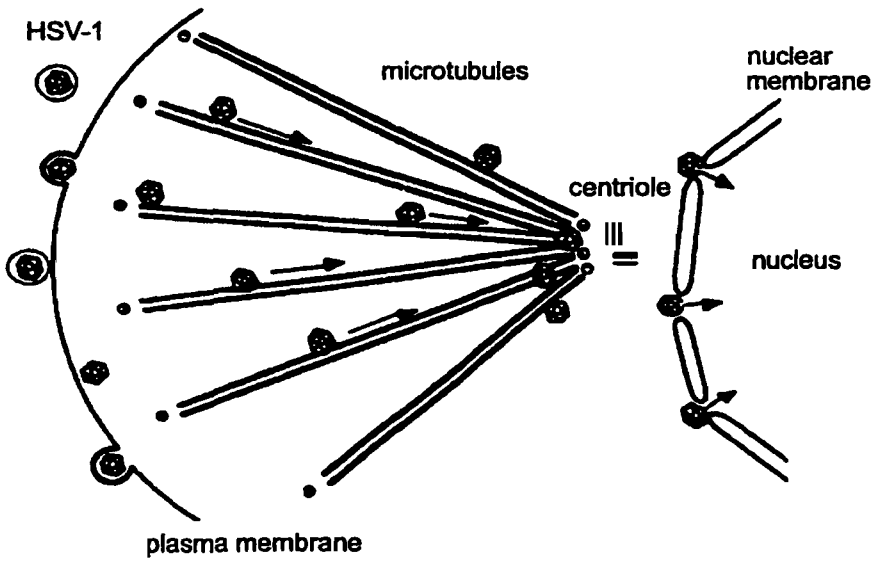

Figure I 3: Transport of the capsid to the nuclear pores with release of the virion DNA into the nucleus (Sodeik et al., 1997).

Once the viral and cellular membranes fuse, the capsids still surrounded by tegument enter into the cell. Unenveloped capsids travel inside the cytoplasm to the nucleus along microtubules and dock to the nuclear pore. At this point, the viral DNA is ejected from the capsid and is released into the nucleoplasm (Sodeik et al., 1997). At the same time, the tegument protein vhs (host shutoff) escapes from the naked capsids and degrades the cellular mRNA, which allows ribosomes to preferentially synthesize viral proteins (Read and Frenkel. 1983). In addition, viral tegument proteins down-regulate cellular proteins that interfere with virus detection by the host's immune system (Triezenberg et al., 1988; Baarr & Skulstad, 1994).

1.6.3 Gene expression

Inside the nucleus of infected cells, HSV-1 synthesis involves a synchronized cascade of three phases of gene expression: immediate early (IE or α), early (E or β), and late (L or γ) (Honess and Roizman, 1975).

The cellular RNA polymerase II is responsible for all viral gene transcription. The IE genes are transcribed in the absence of any previous protein synthesis and their products act to mediate the expression of the early and late genes. α genes expression occurs when VP16 (αTIF) is released from the tegument and forms a complex with the two host cell proteins: the POU domain protein, Oct-1 and a host cell factor HCF (Thomas S et al., 1998). This complex activates the TATAGARAT elements and initiates the transcription of viral IE genes into the cell nucleus (Goding and O'Hare, 1988, Hagmann et al., 1995; Preston et al., 1988). Expression of early gene products occurs at 4-5 hours postinfection and they are mostly enzymes necessary for the replication of the viral genome. The L genes expression starts at 6-7 hours postinfection and encodes mostly structural elements, such as capsid, tegument and glycoproteins that will be assembled into the progeny virions (Godowski P.J., and Knipe D. M.1986).

1.6.4 Viral DNA replication

Studies *in vivo* have demonstrated that once β genes have been expressed and translated, there are several proteins that are localized into the nucleus, where they assemble on the parental viral DNA in punctuate "prereplicative sites" near nuclear ND10 structures (Ishov and Maul, 1996; Uprichard and Knipe, 1996). The viral DNA replication initiates on the circular viral DNA, which creates a "theta" structure, and then changes to a rolling circle mechanism producing head-to-tail concatemers of viral DNA (Jacob, Morse, and Roizman, 1979). At this point, replication takes place in "replication compartments" that consist of accumulating DNA molecules and replication complexes (Quinlan, Chen, and Knipe, 1984).

There are seven viral proteins absolutely required for viral DNA replication into cells. These are the viral DNA polymerase (UL30) (Purifoy, Lewis, and Powell, 1977), its accessory protein (UL42) (Conley et al., 1981), an origin-binding protein (UL9), the single stranded DNA binding protein (ICP8), and the helicase-primase complex that consists of three proteins: UL5, UL8, and UL52 (Challberg, 1986). Host cell factors may also be involved in DNA synthesis, and host enzymes that include the DNA polymerase α- primase, DNA ligase, and topoisomerase II are also required. The viral genome contains also the origins of replication, named *oriS*, and *oriL* (Mocarski and Roizman, 1982; Vlazny, Kwong, and Frenkel, 1982; Weller et al., 1985). The basic model for the replication of HSV viral DNA proceeds as follows. First, the parental viral DNA is circularized in the nucleus of the infected cell. After the expression of α and β gene, UL9 binds to either *oriL* or *oriS* and begins to unwind the viral DNA. Then,

UL9 recruits the ssDNA binding protein ICP8 to the unwound portion of the viral DNA. At this point, UL9 and ICP8 recruit the remaining five proteins to the replication forks. The helicase–primase and viral DNA polymerase complexes assemble at each replication fork and initiate the theta replication. Through an unknown mechanism, replication switches from the theta form to the rolling circle form. The rolling circle replication results in long head-to-tail concatamers of viral DNA, which become cleaved into individual units during packaging of viral DNA into empty capsids (Roizman and Knipe, 2001)

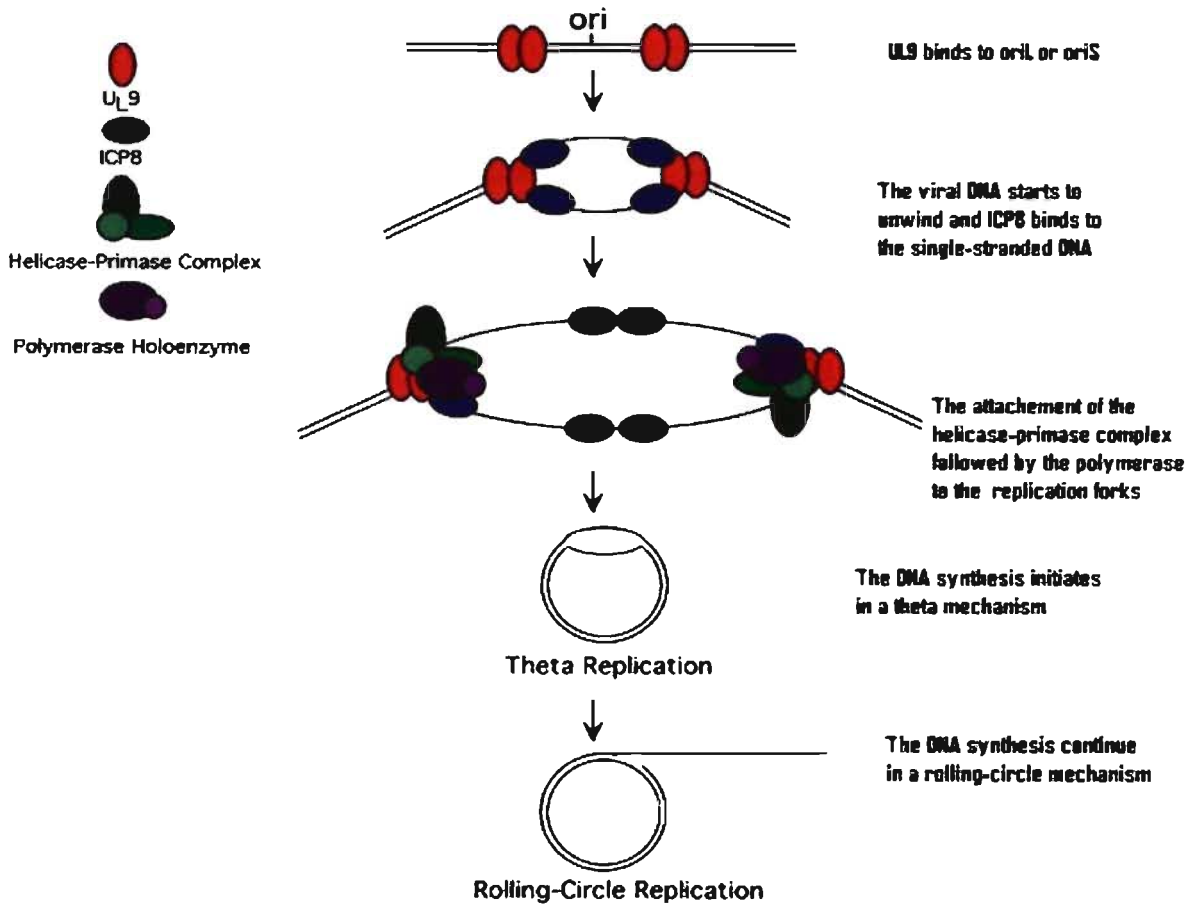

Figure I 4: Mechanism of HSV DNA replication (adapted from Travis J. Taylor et al., 2002)

Also, viral genome is able to stay latently in neuronal cells. Unlike a persistent infection where the virus is constantly replicating, the viral genome is not replicated during the latent infection, with the exception of a subset of HSV genes termed latency associated transcript (LAT) which is abundantly expressed. The role of LAT is still unclear; however recent studies have shown the importance of LAT in limiting viral gene expression, and for the maintenance of the latent state. The major site of HSV latent infection is sensory neurons in ganglion tissue such as trigeminal ganglia for HSV-1 or sacral ganglia for HSV-2. During the latent state, the viral genome remains in the nucleus of the neuron as circular, extra-

chromosomal DNA. After its reactivation by various stimuli, latently infected cells enter into a lytic phase with the production of infectious virus particles (Whitley, 2001). The reactivation from latency occurs upon UV irradiation as a result of excessive sun exposure, stress, fever, damage or perturbation of the ganglia, or menstruation.

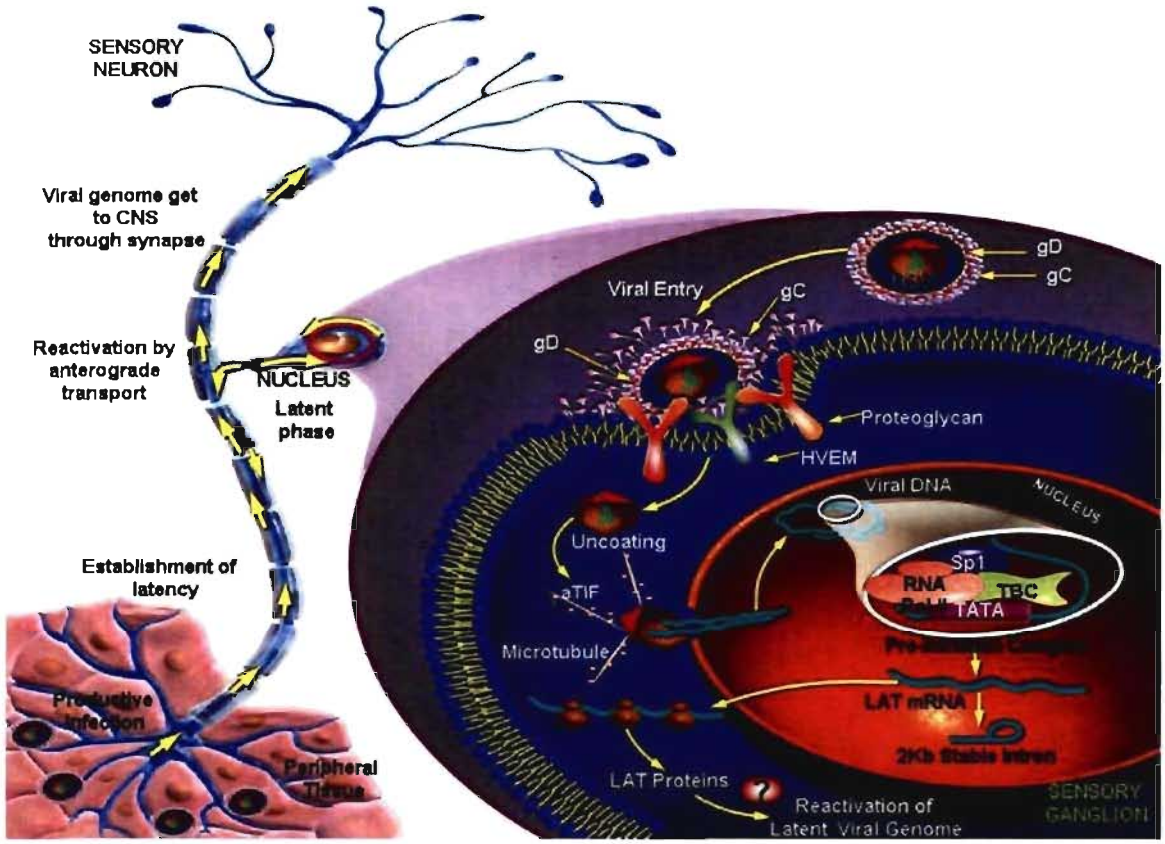

Figure I 5: HSV-1 latent infection (adapted from Protein Lounge Presentation).

1.6.5 Capsid assembly and maturation

As structural proteins accumulate, capsid assembly begins. The viral capsid assembly requires the expression of late genes, the synthesis of the viral structural proteins such as: VP5, VP26, VP23, VP19c and two other polypeptides, UL26 and UL26.5 (ICP35) (Liu and Roizman, 1993). The UL26.5 (ICP35) product is the major scaffold protein for capsid formation, and UL26 encodes a protease (Pra) that cleaves the scaffold. Both products form complexes with VP5 and triplex proteins, consisting of VP23 and VP19. This immature capsid, called procapsid, is characterized as being fragile, thin waled, porous, and easily disassembled at 4°C (Newcomb et al., 1996; Rixon et al., 1996; Trus et al., 1996).

So as to mature the procapsid into the stable rigid angularized capsid, scaffolds are cleaved by Pra and ejected from procapsid. Subsequent capsid angularization is coupled to packaging of the viral genome. The mature nucleocapsid is known as a C capsid, and is supposed to be capable of becoming an infectious particle. Beside the formation of the C capsid, maturing procapsids may also result in formation of A capsids and B capsids (Gibson and Roizman, 1972; Gibson and Roizman, 1974). The lightest A capsids are composed only of an empty shell, whereas B capsids (intermediate) have some additional internal scaffold proteins Pra and ICP35. Capsid A does not package DNA and is thought to result from an abortive process of DNA packaging (Gibson & Roizman, 1972, Perdue et al., 1975).

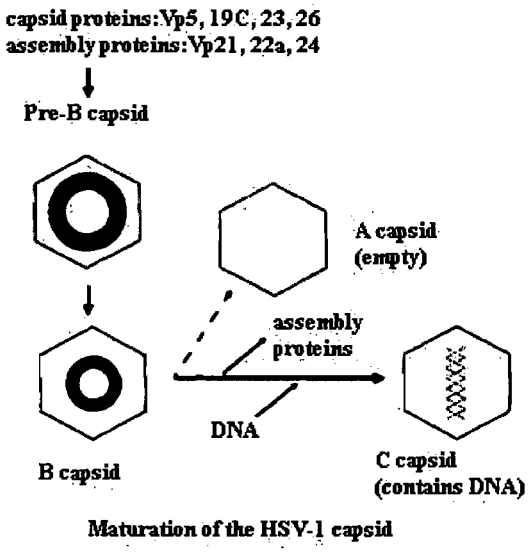

Figure I 6: Maturation of herpes simplex capsids (adapted from D. R. Harper)

Procapsids are observed in cells infected with HSV mutants such as the *ts*1201 (Preston et al. 1983), *ts*Prot A (Gao et al., 1994), or V701 (Register et al., 1996). In these strains, the UL26-encoded Pra has point mutations that inhibit its protease activity in a temperature dependent manner.

The phenotype of these mutant strains has been well used in the study of herpes virus life cycle. Cells infected with these mutants accumulate procapsids in the nucleus at the non-permissive temperature of 39°C (Preston et al., 1983). A significant feature of this mutation is the fact that the mutation is reversible. As the protease function is restored upon return to the permissive temperature of 31°C, subsequent procapsid maturation, angularization and DNA packaging occur in a single synchronized wave (Church and Wilson, 1997). This is a very well used tool in the study of complex biological phenomena as

diverse as protein trafficking through the secretory pathway, endocytosis, control of the cell cycle because of the ability to monitor the accumulation of a protein at a certain stage in its biogenesis, and then to release it as a synchronous wave.

1.6.6 Nuclear egress

After their assembly, the nucleocapsids have to leave the nucleus going into the extracelular space and initiate a new round of infection. The first step is to bud through the inner nuclear membrane. How this is achieved is not well understood, and the literature proposes three concurrent models of egress (Enquist et al., 1998; Mettenleiter 2002, Johnson and Juber, 2002).

The luminal model, also known as the single envelopment model, was described by Johnson and Spear in 1982. This model involves virus envelopment at the inner nuclear membrane, which already contains viral glycoproteins. The enveloped capsids leave the perinuclear space and enter into the ER and Golgi vesicles. Following the exocytic partway, the viral envelope interacts with the membrane of the surrounding vesicle where the final maturation of viral glycoproteins can take place, and the viral particle is released from the cell.

The second model of egress is named de-envelopment re-envelopment (Skepper et al., 2001). The model proposes that the primary envelopment of HSV capsids at the inner nuclear membrane is followed by a fusion with the outer nuclear membrane, resulting in its de-envelopment, and release of naked capsids into the cytoplasm. The nucleocapsid surrounded by some tegument proteins, further accumulates other tegument proteins, buds into the TGN/endosome membranes where it undergoes a secondary envelopment (re-envelopment), and is released into the extracellular environment (Cheung et al., 1991). This model is supported by studies in which the cellular retention signals of viral glycoproteins were mutated. For example, the construction of a mutant gH protein with an ER retention signal failed to package gH into the virus (Browne et al., 1996), and a gD mutant constructed with an ER localization signal produced significantly less of the virus, than a mutant with a Golgi retention signal (Whiteley et al., 1999). In addition, the extracellular viral envelope is more similar to plasma membrane composition than to that of the nuclear membrane (vanGenderen et al., 1994).

The third model of egress, proposed by Wild et al. 2005, and Leuzinger et al, 2005 implies a disassembly and dilatation of the nucleare pores, resulting in a direct passage of the capsid from the nucleus to the cytoplasm.

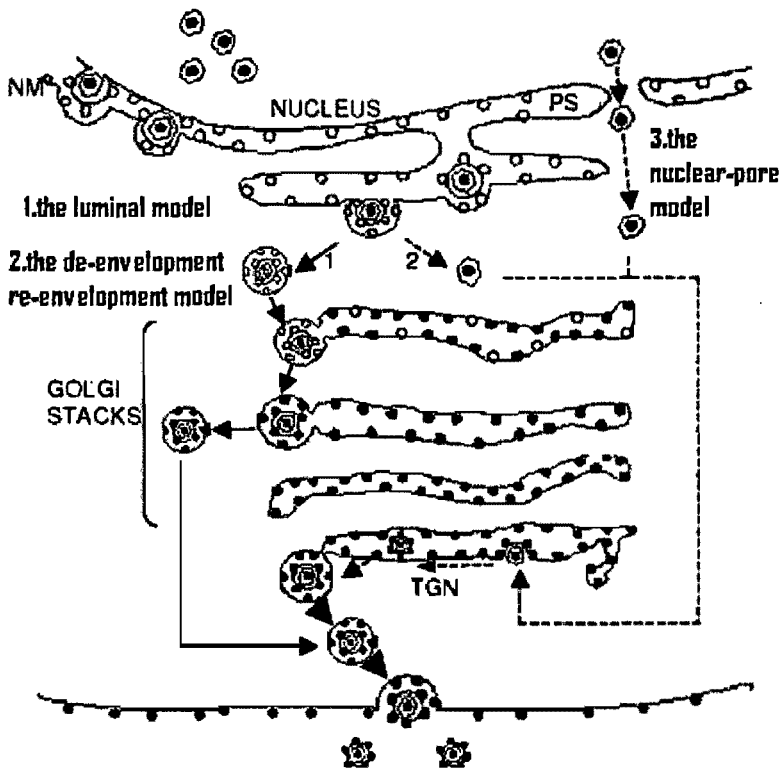

Figure I 7: Models of herpes simpex virus egress.
1- the luminal model ; 2- the de-envelopment re-envelopment model; 3- the nuclear pore exit model , NM- the nuclear membrane; TGN - trans-Golgi network.; PS, perinuclear space (Campadelli-Fiume G. and Gianni T. 2006).

The correct pathway of HSV-1 nuclear egress is still controversial and much examination is still needed to elucidate it. There are molecular studies that involve a crucial role of two virally encoded proteins, U_L31 and U_L34 in primary envelopment (Chang et al., 1997; Roller et al., 2000). U_L31 and U_L34 interact with each other, co-localizing at the nuclear membrane in infected cells (Bjerke et al., 2003; Fuchs et al., 2002; Reynolds et al., 2001). In the absence of either protein, primary envelopment is inhibited and capsids accumulate in the nucleus (Chang et al., 1997; Fuchs et al., 2002; Roller et al., 2000; Klupp et all., 2000). Both the U_L 31 and U_L 34 proteins have been found associated with newly enveloped virions in the perinuclear space, but not with cytoplasmic or extracellular virions (Reynolds et al., 2001; Reynolds et al., 2002). U_L34 is a substrate for the U_S3 who is itself phosphorylated by U_L13. The interaction among these proteins has been demonstrated to depolymerize the nuclear lamina through the cellular protein kinase C (PKC) pathway (Bjerke, S. L., and R. J. Roller. 2006). This local lamina depolymerization (Reynolds et

al., 2004; Simpson Holley et al., 2004) presumably allows the capsids to reach the inner nuclear membrane (Muranyi et al., 2002, Reynolds et al., 2004).

Some evidence which supports the de-envelopment re-envelopment model considers that naked capsids are necessarily present in the infected cell cytoplasm. Others evidence considers them a result of the enveloped virus's disassembly found in a non-productive state (Roizman and Sears, 1993). In addition, glycoproteins with an ER retention motif are not incorporated into extracellular virions (Browne et al., 1996; Whiteley et al., 1999). For example, Browne and coworkers have constructed a recombinant HSV in which the glycoprotein gH has been modified to contain a KKXX endoplasmic reticulum (ER) retention motif. When cells are infected with this recombinant, the amount of viruses released into the medium is the same as that in cells infected with the wild-type virus. However, these viruses are completely devoid of gH and have a 100-fold-lower infectivity than cells infected with the wild-type virus. This result suggests that the ER nuclear membrane is not a donor of viral envelope, and virions acquire their final envelope in a post-ER compartment, from which the modified gH is absent because of the ER retention motif. The work of van Gederen and coworkers have shown that the lipid composition of isolated HSV envelopes is very different from that of nuclear membranes. This means the viral envelope formed at the nuclear membrane is lost and the naked capsids in the cytoplasm will acquire a different lipid bilayer from the other intracellular compartment, which is post ER (van Genderen et al., 1994).

The validity of the two models of envelopment was also studied using a fungal metabolite known to block the anterograde transport of cargo through the secretory pathway, named brefeldin A (BFA) (Klausner et al., 1992; Lippincott-Schwartz et al., 1989). In the case of single envelopment model, the virus in cells treated with BFA accumulates inside the perinuclear space and does not pass through the secretory pathway because of the block of export from ER by BFA (Anindya Dasgupta and Duncan W. Wilson, 2001). In contrast, in the de-envelopment re-envelopment model, the enveloped virus in the perinuclear space fuses with the external nuclear membrane and leads to an accumulation of naked capsids in the cytoplasm. BFA blocked its traffic through Golgi and naked capsids accumulated in the cytoplasm. These experiments support the second model where the Golgi complex, the most sensitive BFA-organelle, is the major envelopment site of HSV-1 nucleocapsids leading to the formation of the infectious progeny virus (Chatterjee and Sarkar, 1992; Cheung et al. 1991; Anindya Dasgupta and Duncan W. Wilson, 2001; Koyama and Uchida, 1994).

1.6.7 Cytoplasmic capsid assembly and secondary envelopment

The mechanism of secondary envelopment is not well understood. The final envelopment requires a combination of capsid, tegument, and the final envelope at the site of re-envelopment, i.e. the TGN or endosomes. After nuclear egress, intracytoplasmic capsids were found to contain pU_L36 and pU_L37 proteins which mediate transport of nucleocapsids to the envelopment site. That indicates that U_L36 is necesary for the attachment of tegument components to the capsids (Desai, 2000). Its gene deletion results in accumulation of unenveloped capsids in the cytoplasm of infected cells. The same effect was observed by deletion of the U_L37 gene, but in this case nucleocapsids were shown to accumulate also in the nucleus, which suggests U_L37 protein may be involved in both stages of viral egress, at the nucleus and in the cytoplasm (Desai et al., 2001). In principle, all components of the mature viral envelope need to be present in the correct compartment for the virion incorporation. From 11 glycoproteins encoded by HSV-1, most of them have been reported to be present at the TGN including gB, gD, gE/gI, gK, gM, and gH. gB and gL do not completely colocalise with the TGN as the other cited glycoproteins that implies they play another role in other compartiments (Turcotte et al., 2005). However, other envelope glycoproteins such as the gH/L complex and gD do not contain any TGN signal and it is thought they localize to the plasma membrane (Hutchinson et al., 1992; McMillan & Johnson, 2001) even that Turcotte et al., 2005 found a partial presence of gL in the TGN at 20°C. The mechanisms by which the envelope glycoproteins could be targeted to the final envelopment compartment are unclear. Many studies have shown that gM forms a complex with gN and together have important roles in viral assembly and egress. gM/N colocalize with the TGN marker TGN46, and cause a relocalization of several membrane proteins from the plasma membrane such as gD and gH/L to the TGN (Crump et al., 2004). The ability of gM/N to cause localization of the herpesvirus envelope proteins gD and gH/L to the TGN, could be part of the mechanism by which herpes viruses maintain sufficient concentrations of envelope proteins in the secondary envelopment compartment, thus allowing efficient assembly and viral egress. Even if the entire process still remains poorly understood, these results provide clues about how HSV-1 virion components are driven to the site of envelopment.

1.6.8 TGN to plasma membrane viral egress

Another step in HSV-1 envelopment that is poorly understood is the process by which virion initiate budding at TGN membranes. The budding process comprises an aggregation of virion proteins on the inner surface of a membrane followed by induction of membrane curvature and subsequent "pinching off" of the particle. Unlike for the cellular proteins, the HSV-1 machinery to initiate the membrane curvature and the pinching-off has not been identified. The discovery of this machinery is important because it provides an indication about how the virion components are selectively packaged during assembly. For example, which proteins are packaged by forming interactions with components of this machinery and which are packaged in the correct compartment during envelopment?

1.6.9 Host transport from the TGN to the plasma membrane

Various intracellular transport studies have revealed the complex formation of vesicles at the TGN membranes and they consider the TGN as a major protein exit station towards various destinations inside the cell. After their maturation in the Golgi, proteins are sorted in the TGN, and transported to different locations inside the cell, with respect to biochemical sorting signals that are found on the individual proteins (Balch W. E., and B. A. Bernard. 1999). In this sense, the TGN is considered a critical gateway for protein transit. In the lumen of TGN, proteins interact with specific receptor molecules. It is considered that after proteins find their specific receptors, they accumulate within TGN subdomains, then bud off in order to form diverse secretory vesicles (Mark A. McNiven and Heather M. Thompson. 2006). In addition, some proteins are retained within the TGN due to the presence of a phosphosorting acidic cluster motif adaptor (McNiven M. A. and Thompson H. M. 2006). The decision of sorting and transporting for a given protein from the TGN, is taken in association with resident adaptor molecules, such as ARF and Golgi-localized gamma-ear which contains ADP ribosylation factor (ARF)-binding proteins (GGAs) (Boman A. L. 2001).

Some proteins exit inside COPI-coated vesicles in the retrograde direction back to the ER (Fernandez-Ulibarri *D.*V. I. et al., 2007). Finally, TGN vesicles that travel to endosomes, or plasma membrane, depend of the presence of GGAs and/or PKD (Kirchhausen, 2000; Brodsky *et al.*, 2001; Robinson and Bonifacino, 2001).

Coat proteins induce the membrane curvature or regulate the association of motor proteins with membranes. This prevents the vesicles detachment from the organelle before the protein sorting has been completed (Kirchhausen 2000; Boehm and Bonifacino 2001; Bonifacino and Lippincott-Schwartz 2003). Clathrin coats found on the cytosolic face of the membranes mediate the releasing of transmembrane proteins at the plasma membrane, TGN or endosomes (Kirchhausen, 2000; Brodsky *et al.*, 2001). At each location, clathrin coats contain adaptor protein (AP) complexes that mediate both the attachment of the clathrin to membranes and the concentration of specific transmembrane proteins. While AP-2 complex functions at the plasma membrane, AP-1 complex is present at the TGN and/or endosomes (Kirchhausen, 2000; Brodsky *et al.*, 2001; Robinson and Bonifacino, 2001). A protein family, named, GGAs (GGA1, GGA2, and GGA3 in humans) was presented to promote the recruitment of clathrin and the releasing of transmembrane proteins at the TGN (Robinson and Bonifacino, 2001; Boman, 2001). The ARF family (for ADP-Ribosylation Factor), of small GTPases plays an important role in vesicle formation. They recruit the Golgi-associated adaptor AP-1 and GGAs to the Golgi membranes, and interfere in the releasing of proteins in endocytic pathways (Taylor et al., 1994). De Matteis and collegues further reported a new family of coat proteins, called FAPPs (the four-phosphate adaptor proteins), that function at the TGN. Like clathrin, FAPPs are coat proteins that bind to the vesicles or tubules which extend from the TGN and contain cargo. FAPP1 and 2 are recruited in TGN by phosphatidylinositol-4-phosphate (PI, /PI4P), and they are considered as mediator of the apical protein transport (Godi et al. 2004, Vieira et al. 2005). Overexpression of a dominant-negative of FAPPs or depleting FAPP1 levels with small-interfering RNAs inhibits protein transport from the TGN to the plasma membrane (Godi et al. 2004). Although FAPPs control the formation of transport carriers at the TGN, they are not present on transport intermediates that detach from the TGN, which means they disassemble from transport vesicles after budding.

While GGA proteins play a role in regulating the TGN vesicular egress to endosomes, PKD has been identified as a molecule which regulates TGN vesicular transport to the plasma membrane (Liljedahl et al., 2001). PKD is a family of serine/threonine protein kinases that belongs to the Ca2+/calmodulin-dependent kinase superfamily. Three members of the PKD family have been identified so far in humans: PKD1, PKD2, and PKD3. They share homology in their catalytic domain and distinct sequences located between the conserved motifs in the regulatory region confer isoform specific functions (Rykxa A., et al. 2003).

The three PKD isoforms are implicated in basolateral protein transport from TGN to the plasma membrane (Yeaman et al. 2004). They bind to DAG from the TGN membranes via their cysteine rich (C1) domain (Maeda et al. 2001). DAG depletion inhibits their binding to the TGN and consequently, their activation (Baron and Malhotra 2002). The kinase-inactive form of PKD, the lysine to asparagine PKD-K618N mutant, has been shown to accumulate at the TGN and cause tubulation of the TGN. PKD-K618N tubules contain cargo, but do not detach from the TGN. The mutant also produces an inhibition of cargo transport between the TGN and plasma membrane. In contrast, PKD overactivation by ilimaquinone induces fragmentation of the Golgi apparatus (Keller et al. 2001, Liljedahl et al. 2001, and Polishchuk et al. 2003.

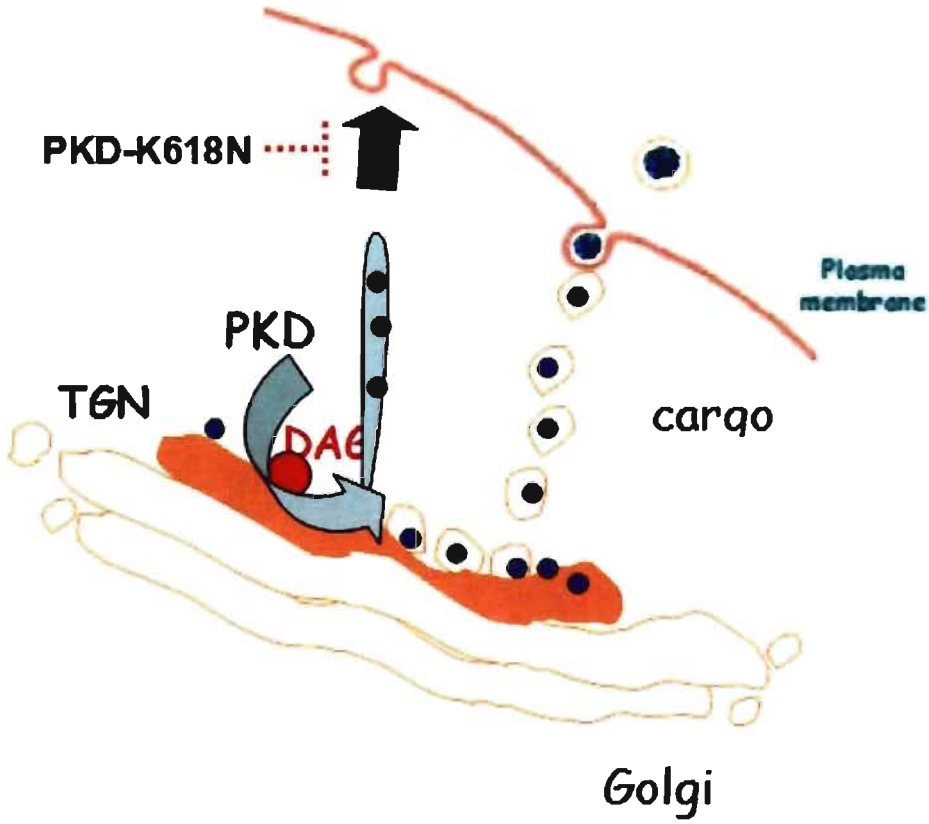

Figure: I 8. Kinase dead PKD block HSV-1 transport from the TGN to the plasma membrane.

The activity of PKD depends on its recruitment from the cytosol to the TGN membranes by DAG (Prestle *et al.*, 1996; Liljedahl *et al.*, 2001; Baron and Malhotra, 2002). At the same time, DAG

production in the Golgi depends on the conversion of phosphatidic acid (PA) mediated by phosphatidic acid phosphohydrolases (PAPs). Finally, PA could be a result of phosphatidylcholine (PC)-specific phospholipase D enzymes (PLD) activity (Siddhanta and Shields, 1998). Therefore, PLD and PAP are important in DAG production in Golgi membranes. Another source of DAG is represented by sphingomyelin synthase (SMS) activity, which generates sphingomyelin (SM) and DAG (Ichikawa and Hirabayashi, 1998). Moreover, phosphoinositides such as (PI, PI4P) or phosphatidylinositol 4, 5-biphosphate (PI4, 5P2) are converted to DAG and inositol bis- or tris-phosphate through phosphoinositide-specific phospholipase C (PI-PLC) (Claro *et al.,* 1993; Rhee, 2001).

Intracellular VSV-G post-Golgi transport has been studied in the presence of some drugs, such as fumonisin B1 (FB-1), l-cycloserin (L-CS) and propranolol (Liebisch G. Schmitz G. Hoekstra D. 2004). Those drugs all cause an inhibition in the TGN-derived transport carriers as a result of Golgi-associated DAG level reduction (Baron and Malhotra, 2002; Brindley & Waggoner 1998, Pyne et al. 2004). These results show the DAG is a lipid involved in neck formation of the Golgi-derived vesicles or tubules. Consequently, DAG creates membrane insertion sites that allow peripheral membrane proteins to gain access to the hydrophobic portion of the bilayer, where they induce the generation of membrane curvature (Nie and Randazzo, 2006). Moreover, a reduction in DAG levels of Golgi membranes cause the inactivation of the molecular machinery necessary to induce membrane fission (Bard and Malhotra, 2006).

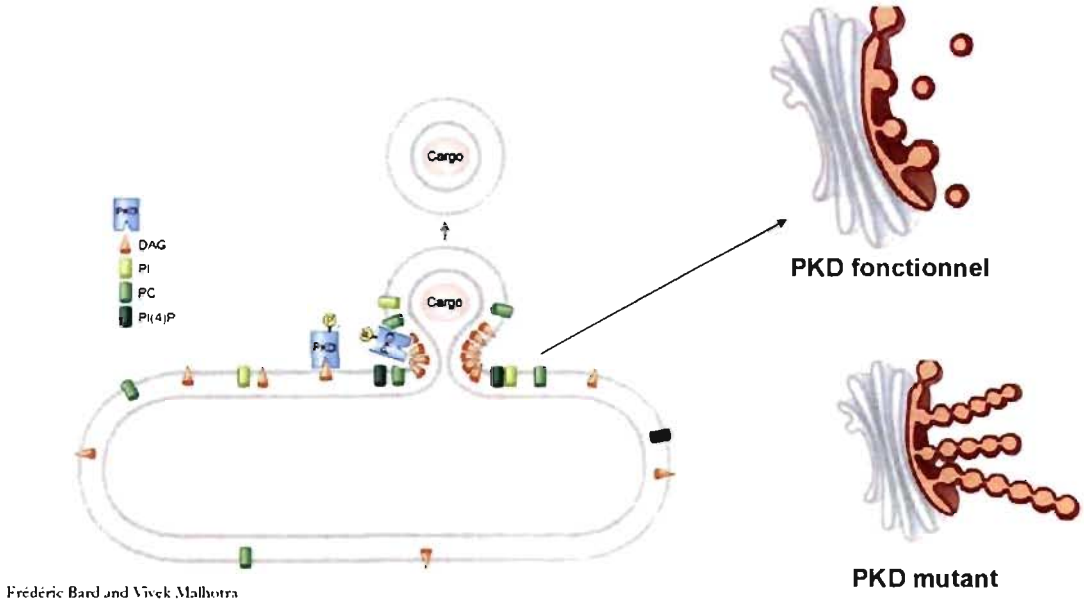

Figure I 9: Recruitment and activation of protein kinase D (PKD) at the TGN (adopted from V. Malhotra and modified by CM).

CHAPTER II: Objectives of research

Unlike the cellular proteins, nothing is known about the machinery that HSV-1 virions utilize to initiate the TGN to plasma membrane egress. The present work tries to identify whether HSV-1 virions utilise the same machinery as the cellular proteins in their exit from TGN to the plasma membrane. In the cellular biosynthetic pathway it is known that the serine-threonine protein kinase D (PKD) plays a central role in vesicle formation at the TGN. As discussed above, PKD function itself depends on the pool of DAG in the TGN membranes. We have investigated the role of PKD in virus cargo exit from TGN. To verify this hypothesis we made use of the synchronized infection with HSV-1 termosensitive mutant virus V701 previously used (Turcotte et al. 2005). Since pharmacological inhibitors could acts at multiple stages of the viral life cycle, a mutant form of PKD to and RNA interference were also used to determine the real implication of this machinery in the HSV-1 virion egress.

CHAPTER III: Article

Summited to the Cell Biology Journal

Protein kinase D dependent trafficking of the large HSV-1 capsids from the TGN to plasma membrane

by

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Running title: PKD regulates large cargo transport from the TGN

Character counts: 32087

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3.1 Abstract

The conventional biosynthetic pathway has been extensively studied for small cargo. Interestingly, large particles such as procollagen, chylomicron and various virions reach the TGN by alternative routes. Given this dichotomy, we probed which machinery large cargo uses downstream of the TGN. Using Herpes simplex virus type 1 virions as a model, a collection of specific inhibitors and a synchronized infection protocol, the data surprisingly revealed a role in HSV-1 egress for the cellular serine-threonine protein kinase D. This established mediator of TGN to cell surface transport for small cargo unexpectedly highlights the trafficking of entities as large as virions by the common machinery used by small cargo. This substantially alters the range of cargo that the conventional biosynthetic pathway can accommodate. Given the apical release of HSV-1 in neurons, it also raises the possibility that PKD might regulate basolateral sorting. Lastly, it addresses for the time the molecular basis of egress of any viral particle transiting at the TGN.

3.2 Introduction

The biosynthetic pathway is a well studied route by which membrane bound and secreted proteins sequentially travel in the ER, the Golgi apparatus and the TGN before being sorted to their final destination. Along this route, the serine-threonine protein kinase D (PKD) is a key player at the TGN, since it regulates the fission of cargo filled carriers destined for the cell surface (Liljedahl et al., 2001). Three PKD isoforms exist in humans and differentially modulate cargo transport to the basolateral surface (Sanchez- Ruiloba et al., 2006; Yeaman et al., 2004). Interestingly, recruitment of PKD from cytosol requires its interaction with diacyl glycerol (DAG), a lipid critical to PKD function and whose presence at the TGN is determining (Baron and Malhotra, 2002). Procollagen, chylomicron and virions are large particles reaching several hundred nanometers (Canty and Kadler, 2005; Fromme and Schekman, 2005). Their substantial size poses major challenges for their intracellular transport (Fromme and Schekman, 2005; Mettenleiter, 2004). For instance, it has been reported that both procollagen and chylomicron bypass the classical COPII coated vesicles to escape the ER (Siddiqi et al., 2003; Starkuviene and Pepperkok, 2007; Stephens and Pepperkok, 2002). Similarly, Herpes simplex virus type I (HSV-1) is a large particle assembled in the nucleus that also reaches the TGN by an alternative route. Too large to leave the nucleus via the pores, the 125 nm capsids sequentially bud across the inner nuclear membrane and fuse with the second nuclear envelope to be released in the cytoplasm (Mettenleiter, 2004; Remillard- Labrosse et al., 2006). They then bypass the Golgi apparatus and acquire an envelope from the TGN to form mature 200-300 nm virions (Harley et al., 2001; Turcotte et al., 2005). The virions finally leave this last compartment by a completely unknown mechanism.

Given the unconventional transport pathways employed by large cargo to arrive at the TGN, we sought to examine if they use the conventional transport machinery further downstream. Using HSV-1 as a model and a collection of inhibitors, the egress of virions from the TGN to the plasma membrane was monitored using a recently developed protocol (Turcotte et al., 2005). The data surprisingly revealed a critical contribution of PKD in HSV-1 virion egress, a unique finding for any of the known viruses transiting at the TGN. These results clearly indicate that large particles can share the same route as small cargo to escape the TGN, in sharp contrast to earlier steps of transport. Given the exceptional size of HSV-1 virions, this also substantially broadens the range of cargo the classical transport machinery can accommodate. Finally, given the apical release of HSV- 1 in neurons, it raises the possibility that PKD may not uniquely be devoted to basolateral sorting.

3.3 Results

Synchronization of HSV-1 intracellular transport from the TGN to the cell surface.

There is little information regarding the mechanism by which HSV-1 leaves the TGN. Unfortunately, the rapid life cycle of HSV-1 makes it difficult to characterize this viral transport step. Nonetheless, others and we have showed that viral egress can be synchronized using mutants of the viral protease UL26, a protein required for encapsidation of the herpes DNA and capsid maturation (Church and Wilson, 1997).

Hence, thermosensitive UL26 viral mutants such as ts1201 (Preston et al., 1983), tsProt.A (Gao et al., 1994) and V701 (Register and Shafer, 1996) accumulate immature viral capsids in the nucleus at the non permissive temperature of 39.5°C but release mature extracellular virus at 31°C. To ensure a tight wave of viral egress, cycloheximide – an inhibitor of protein synthesis - is typically added after the 39.5°C incubation to prevent the assembly of new capsids (Church and Wilson, 1997; Turcotte et al., 2005). Viral egress can further be dissected as HSV-1 capsid transport is reversibly arrested at the TGN at 20°C, much like host proteins along the biosynthetic pathway (Turcotte et al., 2005). This reversible 20°C block represents an ideal mean to define the molecular requirements of HSV-1 transport from the TGN to the plasma membrane. It is also optimal to probe the potential role of PKD in the transport of cargo as massive as fully assembled virus. To first confirm the efficacy of this block, 143B cells were infected with the thermosensitive HSV-1 stain V701 and the virus was monitored at 39.5°C, 20°C and 31°C (fig. 1). To detect the virus, the samples were stained with an ICP5 antiserum, which recognizes the major viral capsid protein. As previously reported, the virus was retained in the nucleus at 39.5°C, was efficiently retained in the TGN at 20°C and could reach the cell surface at 31°C (fig. 1; Turcotte et al., 2005). Most important, the 20°C block was reversible as the virus could escape the TGN when followed by a chase at 31°C. It was therefore possible to synchronize the transport of the large HSV-1 virus from the TGN to the cell surface.

PKD inhibitors arrest TGN to plasma membrane transport.

Recruitment of cytosolic PKD to the TGN is essential for cargo release from that compartment. This requires an interaction between PKD and the TGN bound pool of DAG (Baron and Malhotra, 2002). Fumonisin B1 (FB-1) and L-cycloserine (L-CS) block DAG production by preventing at two distinct steps the synthesis of ceramide, which is ultimately converted into DAG and sphingomyelin (Baron and Malhotra, 2002; van Ooij et al., 2000). In contrast, propranonol blocks DAG synthesis by inhibiting the

conversion of phosphatidic acid into DAG. Importantly, all three inhibitors strongly perturb PKD mediated transport of cargo from the TGN to the surface in HeLa cells (Baron and Malhotra, 2002; van Ooij et al., 2000). To insure that the inhibitors also worked in the 143B cell line used in this study and examine the role of PKD on HSV-1 transport from the TGN to the plasma membrane, their impact of VSV G ts045 transport was evaluated. This protein is a long established thermosensitive marker of the biosynthetic pathway relying on PKD for its transport to the cell surface (Griffiths et al., 1985; Liljedahl et al., 2001). It accumulates at the ER at 39°C, travels to the TGN at 20°C and is exported to the cell surface at 31oC. 143B cells were thus transfected with GFP labelled VSV G ts045 and incubated at various temperatures. As expected, VSV G accumulated in the TGN of 143B at 20°C and was chased to the cell surface when the temperature was switched to 31°C (fig. 2). In contrast, VSV G only occasionally reached the cell surface and strongly remained TGN associated in the presence of 25 μg/ml FB-1, 1.5 mM L-CS or 50 μM propranolol added during the 20°oC incubation and the subsequent 31°C chase. Manual counting of transfected cells positive for VSV G at their plasma membrane indicated that the inhibition was 72%, 70% and 67% for the three drugs respectively and complete at 20°C, i.e. with 100% inhibition (n=118 to 260). Note that all transfected cells were positive for VSV G at the plasma membrane when incubated at 31°C in the absence of drug. Albeit not absolute, the relatively efficient block of transport confirmed the ability of the drugs to hamper TGN to plasma membrane transport in 143B cells, as observed for other cell lines (Baron and Malhotra, 2002).

Block of viral egress by PKD inhibitors.

Having confirmed that inhibitors of DAG synthesis function as expected, their impact on HSV-1 egress was examined. The virus released in the extracellular medium was first quantified by plaque assay. As above, the infection was first synchronized at the nucleus with a 7 hour pre-incubation at 39.5°C, followed with a chase at 31°C in the presence of cycloheximide. Control infections without the drugs typically yielded 1-2 x 10^5 total plaque forming units (pfu) when chased for 30 hours at 31°C (fig. 3). In contrast, the extracellular viral yield was reduced by 99% at 20°C, consistent with figure 1 and previous results (Turcotte et al., 2005). This 20°C block was reversible and rescued to 60-65% of normal levels when followed by a chase of 24 hours at 31°C. Importantly, when 25 μg/ml FB-1, 1.5 mM L-CS or 50 μM propranolol was added during the 20°C block and the subsequent 24 hour 31°C chase, hardly any virus escaped the cells (fig. 3; 5.7% for FB-1, 8.0% for L-CS and 8.2% for propranolol). Collectively, the

inhibition of HSV-1 egress by the three independent inhibitors of DAG synthesis pointed at a role for PKD during HSV-1 egress.

PKD inhibitors act downstream of nuclear egress.

PKD has multiple roles in cells and is found in various compartments, including the nucleus, cytoplasm, plasma membrane, mitochondria and TGN (Rozengurt et al., 2005). It was thus possible that FB-1, L-CS and propranolol blocked viral transport at any moment during egress. To examine at which step the drugs acted, the transport to the cytoplasm of capsids newly assembled in the nucleus was first evaluated. To this end, a recently established *in vitro* nuclear egress assay was used to quantify viral transport (Remillard-Labrosse et al., 2006). The assay reconstitutes in the test tube the exit of capsids from the nucleus into the cytoplasm. It is based on the isolation of nuclei from infected cells and their incubation with buffer, energy and cytosol. The specific release of capsids by the nuclei is then quantified by scintillation counting, as the viral genome is preloaded with 3H thymidine during the initial infection (Remillard-Labrosse et al., 2006). Using this assay, viral egress from the nucleus to the cytoplasm was evaluated. Controls included nuclei incubated with energy and cytosol (basal level of egress normalized to 100% for easier comparison) and nuclei incubated without energy and cytosol (negative control). Figure 4 shows that none of the PKD inhibitors had any impact on viral egress from the nucleus, indicating that the block of viral egress occurred further downstream.

PKD inhibitors trap HSV-1 in the TGN.

Given the reduced viral yield in the extracellular medium and the normal transport of HSV-1 across the two nuclear membranes in the presence of the PKD inhibitors (fig. 3-4), the cells were examined by immunofluorescence to determine where the virus might be trapped. 143B cells were infected with V701, the infection synchronized at the TGN and the virus released at 31°C in the presence or absence of the PKD inhibitors. As before, HSV-1 strongly associated with the TGN at 20°C and reached the cell surface at 31°C (fig. 5). Once again, the block of transport at 20°C was fully reversible when followed by a chase at 31°C. In the presence of FB-1, L-CS or propranolol during the 31°C chase, the virus could not escape the TGN (fig. 5). Thus HSV-1 seemingly leaves the TGN by a PKD regulated host transport route.

Kinase dead PKD hampers HSV-1 transport from the TGN to the plasma membrane.

The inhibition of HSV-1 egress by three independent inhibitors of PKD argued in favour of its involvement in the transport of the virus. To ensure this was the case, cells were transfected with PKD or the PKD K618N inactive form of the kinase. This mutant causes cargo retention in the TGN and blocks transport to the cell surface, as the inactive PKD fails to fission the transport carriers (Liljedahl et al., 2001). Consequently, long TGN46 positive tubules are formed at the TGN. Malhotra and colleagues have shown that these PKD induced tubules tend to resorb at higher temperatures and are more easily observed below 25°C (Liljedahl et al., 2001). 143B cells were therefore initially transfected for 24 hours with either GST tagged wild type or mutant PKD and incubated 6 hours at 20°C. The samples were then fixed and stained for the TGN or PKD using a polyclonal antibody against GST. PKD labelling was clearly specific as no signal was detected in the absence of transfected plasmid (fig. 6A, panels a-c). Furthermore, a strong recruitment of wild type PKD to the TGN was observed (fig. 6A, panels d-f). As anticipated, long tubules labelled with TGN46 were seen in the presence of mutant PKD (fig. 6A, panels gi).

To examine whether HSV-1 co-localized with such structures, cells were transfected for 24 hours with wild type or PKD K618N and subsequently infected with HSV-1. Following 7 hour incubation at 39.5°C to synchronize the infection, the cells were chased 6 hours at 20°C in the presence of cycloheximide and stained for PKD, TGN and capsids. To ensure that fully mature capsids were observed, the conformation dependent 8F5 antibody known to solely detect such capsids was used (Trus et al., 1992). The results indicate that tubules were absent in wild type PKD transfected cells and control cells only treated with transfection reagent (fig. 6B, panels a-h). In contrast, long PKD and TGN46 positive tubules were once again found in K618N transfected cells (fig. 6B, panels i-l), indicating the virus didn't alter the K618N phenotype. Under those conditions, the virus strongly co-localized with the PKD K618 / TGN positive tubules (fig. 6B, panels i-l). Oddly, in cells transfected with wild type PKD, the virus occasionally reached the cell surface, suggesting that over expression of PKD could rescue the 20°C block (fig. 6B, panels e-h). Since the virus cannot normally escape the TGN at 20°C, it was difficult to determine the true impact of PKD on viral egress. The experiment was therefore repeated with a 9 hour chase at 31°C instead of 6 hours at 20°C as above. Despite the leaky nature of the PKD induced tubules at that temperature (Liljedahl et al., 2001), the virus co-localized to a large extent with PKD K618N induced tubules (fig. 6B, panels q-t) and reached the cell surface in wt PKD transfected cells (fig. 6B, panels m-p), suggesting PKD indeed modulated viral egress at the TGN .

To ensure that the co-localisation between the virus and PKD induced tubules was meaningful and not merely fortuitous; the impact of the kinase dead PKD mutant on the extracellular release of the virus was evaluated. Unfortunately, it is not possible to reliably monitor viral release in transiently transfected cells, as a mix of transfected and untransfected cells co-exist. However, the stable cell line HeLa GF17 constitutively expresses PKD K618N (Liljedahl et al., 2001). Thus to confirm the role of PKD in HSV- 1 egress, control HeLa and HeLa GF17 cells were infected with V701 under similar conditions used for 143B cells and viral released was quantified by plaque assay. Hence, the cells were incubated 7 hours at 39.5°C, then 6 hours at 20°C to accumulate the virus at the TGN and finally 4 hours at 31°C to release the virus. As control, the cells were incubated 7 hours at 39.5°C, then 10 hours at 31°C. Surprisingly, there was no significant difference in viral output between HeLa and HeLa GF17 cells under these conditions (data not shown). Since PKD K618N is somewhat leaky at higher temperatures (Liljedahl et al., 2001), it was possible the virus had sufficient time to escape even if slowed down by the inactive PKD. To ascertain this, the experiment was repeated with shorter kinetics.

HeLa GF17 and control HeLa cells were then infected with thermosensitive HSV-1 and incubated 6 hours at 39.5°C. The virus was subsequently chased for 2 hours at 20°C to the TGN and released for 2 hours at 31°C. Figure 7 reiterates the efficiency of the 20°C block to hamper viral egress. As previously reported for 143B cells (Turcotte et al., 2005), viral egress was fully restored in HeLa cells when subsequently incubated at 31°C. In HeLa GF17 cells, some viruses did manage to reach the extracellular milieu under those conditions, but viral output was strongly reduced (by 60.6% + 2.7). These results confirm the role of PKD in HSV-1 transport from the TGN.

PKD specific siRNA inhibit viral egress.

To independently confirm the role of PKD in HSV-1 egress and evaluate whether any of the three known human isoforms (Hausser et al., 2005; Yeaman et al., 2004) specifically regulate capsid transport, PKD expression was first verified at both the mRNA and protein levels. As positive controls, 143B cells were transfected for 24 hours with GST-PKD1, 2 or 3 constructs prior to analysis. To distinguish the various PKD isoforms, we resorted to commercial PKD antibodies that readily detected the exogenous PKD by Western blotting (fig. 8A) and immunofluorescence (fig. 8B). Interestingly, analysis of endogenous PKD only revealed PKD3 expression by both Western blotting and immunofluorescence, with no detectable levels of PKD1 or PKD2. To determine if the other isoforms were transcribed and perhaps expressed at levels too low to be detected, a RT-PCR was performed using both total (data not

shown) and mRNA extracts (fig. 8C). The data corroborated the above results with a positive band for PKD3 but no signal for PKD1 and PKD2, despite up to 50 cycles of amplification with isoform specific oligos. Thus, 143B cells solely express PKD3.

The unique expression of PKD3 in 143B cells greatly simplified further analysis, as no functional redundancy between the PKD isoforms was possible in these cells. siRNA targeting PKD3 were thus tested for their ability to block PKD3 expression. It is noteworthy that transfection experiments revealed a strong penetrance of a control fluorescent siGLO siRNA (85.7% of cells positive by immunofluorescence). Similarly, siRNA duplexes against PKD1, PKD2 or PKD3 were highly efficient to down regulate their respective exogenous GST-PKD expression (fig. 9). Most important, while PKD3 siRNA very efficiently inhibited endogenous PKD3 protein expression as early as 24 hours post transfection, siRNA against PKD1, PKD2 or the commercial "On-Target" control (Dharmacon) had no major impact on the endogenous level of PKD3. The occasional On-Target or PKD1 siRNA partial reduction of PKD3 expression was not reproducible (compare panels A and B) and never reached the extent seen with PKD3 siRNA. It was thus possible to efficiently and specifically block PKD3 expression with siRNA.

To finally evaluate if inhibition of PKD3 protein expression halted HSV-1 transport from the TGN to the cell surface, 143B cells were transfected for 24 hours with the above siRNA and subsequently infected with V701 for 7 hours at the non permissive temperature of 39.5°C to accumulate the virus in the nucleus. The virus was then chased at 31°C for 9 hours and viral egress monitored by immunofluorescence using the capsid dependent 8F5 antibody. Staining of PKD3 with isoform specific antibodies confirmed its down regulation (fig. 10, panels A, B). In the absence of siRNA, the virus readily reached the cell surface (fig. 10, panel D). Similarly, siRNA against PKD1 or PKD2 as well as the On-Target control did not prevent viral egress (fig. 10, panels E-G). In sharp contrast, PKD3 specific RNAi duplexes strongly inhibited HSV-1 egress (fig. 10, panel H).

Though some of the virus remained associated with the nucleus, much of it co-localized with the TGN marker, indicating the virus was trapped in that compartment as expected. The diffuse TGN in some experiments is caused by the infection despite the use of 143B cells, which are more resilient to TGN disruption by the virus (Turcotte et al., 2005). Altogether, these results were fully consistent with our previous observations and confirmed the implication of PKD3 in viral egress.

3.4 Discussion

PKD plays a key role in the transport of cargo from the TGN to the cell surface (Liljedahl et al., 2001). Given that the large HSV-1 capsids transit through the TGN during their egress, it was of interest to examine if PKD could mediate the transport of such large cargo to the plasma membrane or whether the virus employs an alternative transport pathway. To address this issue, we used a synchronized infection protocol that reversibly accumulates the virus at the TGN ((Turcotte et al., 2005); fig. 1). The results show that HSV-1 indeed relies on PKD for its release from the TGN. This was initially demonstrated with three independent inhibitors of DAG synthesis, each of which efficiently blocked the transport of VSV G from the TGN to the plasma membrane (fig. 2). Thus, FB-1, L-CS and propranolol all strongly prevented the release of HSV-1 to the extracellular milieu (fig. 3, 5). This block occurred downstream of the nucleus, since the chemical inhibitors had no impact on the release of capsids from the nucleus into the cytoplasm (fig. 4). Instead, the PKD inhibitors clearly trapped nearly all the virions at the TGN (fig. 5).

While the results with DAG inhibitors only indirectly pointed out PKD, several pieces of evidence confirmed it. First, the transfection of 143B cells with kinase dead PKD trapped the virus at the TGN, whereas HSV-1 readily travelled to the cell surface in the presence of wild type PKD (fig. 6). Second, upon infection of HeLa GF17 cells, which constitutively express PKD K618N, a strongly reduced viral yield was measured compared to control HeLa cells (fig. 7). Third, the virus co-localized with PKD K618N induced tubules at the TGN (fig. 6). Finally, siRNA against PKD3, the only isoform expressed in 143B cells (fig. 8), strongly down regulated HSV-1 egress and retained the virus at the TGN (fig. 10). The involvement of PKD in HSV-1 egress in at least two independent cell types is worth noting, indicating that the phenotype is not cell line specific. Moreover, the residual viral egress observed in HeLa GF17 cells is expected, as these cells only moderately express PKD K618N (Liljedahl et al., 2001) and data not shown) and the PKD induced tubules tend to resorb at higher temperature (Liljedahl et al., 2001). In fact, a high dose of inactive PKD would presumably be fatal, as transport of several proteins to the cell surface would be hampered. Though 143B cells don't express PKD1, the block of capsid transport in these cells by the PKD1 kinase dead mutant most likely reflects a functional redundancy between the different PKD isoforms (Hausser et al., 2005; Maier et al., 2007). Taken together, the results show compelling evidence that HSV-1 egress from the TGN to the cell surface is PKD dependent.

The requirement for PKD during HSV-1 egress has a number of implications. The first obvious one is that HSV-1 uses the host transport machinery during that part of its life cycle. This was not

necessarily obvious, as many viruses including HSV-1 typically shut down host protein synthesis. Furthermore, some cargo can leave the TGN by unconventional means (Kinseth et al., 2007). This is also in sharp contrast with the non classical route used by HSV-1 during its first steps of egress (see introduction). So, as it is often the case with viruses, HSV-1 is clearly ambivalent, using host transport machinery when possible and elaborating its own transport machinery when necessary. Importantly, this defines for the first time the molecular basis of egress from the TGN for any of the several viruses transiting in that compartment, including the Herpesvirus, Rotavirus, Coronavirus, Bunyavirus and Poxvirus families (Griffiths and Rottier, 1992).

Given this novel involvement of PKD in HSV-1 egress, it would be of interest to determine whether PKD also regulates the transport of these other viruses. The second implication concerns to the ability of PKD to regulate the transport of various proteins from the TGN to the plasma membrane. HSV-1 virions are large 200- 300 nm wide enveloped complexes composed of over three thousands protein subunits for a total mass in the 100 million Dalton range (Baines and Duffy, 2006). It is clear from the present data that PKD not only promotes the transport of individual proteins but also of large structures as massive as HSV-1 virions. This is the first report on the trafficking of a large particle, a virion, which requires the same machinery from the TGN as shown thus far for small molecules. This is of particular relevance to other large host cargos such as the 300-400 nm procollagen and chylomicrons, which may use a similar pathway (Canty and Kadler, 2005; Fromme and Schekman, 2005). For instance, while procollagen accumulates in PKD and VSV G positive tubular carriers (Polishchuk et al., 2003), the chylomicrons transit through the Golgi before being secreted (Sabesin and Frase, 1977). However, a direct requirement for PKD for the surface transport of these molecules remains to be established. Conceptually, the incorporation of large cargos in PKD transport carriers may be possible owing to the heterogeneity and large size of the tubules (Polishchuk et al., 2000), in comparison with the small 60-70 nm COPII vesicles leaving the ER that cannot accommodate such large particles (Barlowe et al., 1994; Fromme and Schekman, 2005). This point to the TGN as an important station where conventional and unconventional pathways may meet. It will be of interest to examine more dynamically HSV-1 transport out of the TGN to determine if it travels within the same structures as other cargos or if the virus monopolizes the transport machinery to travel in solo. The TGN is an important sorting station. While cargo incorporated in clathrin coated vesicles are typically destined for endosomes (Robinson, 1994), COPI coated vesicles deliver their content back to the Golgi stacks and ER (Duden, 2003). In contrast, PKD regulates transport from the TGN to the basolateral surface (Yeaman et al., 2004). This preferential transport of cargo to the

basolateral membrane by PKD is in apparent contradiction with the release of HSV-1 along the axons of neuronal cells, which are believed to be the counterpart of the apical membrane (Dotti et al., 1991). Unless this neuronal asymmetry is not preserved among different neurons, our results suggest that PKD may mediate apical membrane targeting of some cargo. This raises several key questions. The first one is whether PKD regulates axonal transport of HSV-1. Does PKD only regulate the release of HSV-1 at the cell body of neuronal cells, i.e. at their basolateral membrane? Do the three known PKD isoforms (Hausser et al., 2005; Yeaman et al., 2004) participate in HSV-1 transport in neurons? Finally, could one of these isoforms regulate apical transport of certain cargos? Experiments to address these issues are now under way.

In conclusion, we have shown by complementary means that PKD mediates the transport of HSV-1 from the TGN to the cell surface. This indicates that PKD regulated transport carriers can carry immense cargos as large as HSV-1 virions and that both small and big cargo likely compete for the same transport machinery at the TGN.

3.5 Materials and Methods

Cells and viruses

HeLa, HeLa GF17 constitutively expressing PKD K618N (kindly provided by Vivek Malhotra), BHK, 143B TK-, and Vero cells were grown at 37°C in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Medicorp), 2 mM L-glutamine (Invitrogen), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Except when infected, 143B cells were further supplemented with 15 µg/ml of 5-bromo-2'-deoxyuridine (BUdR, Sigma) and HeLa GF17 cells were grown in the presence of 500 µg/ml G418 (Invitrogen),. The HSV- 1 V701 *ts*80-1C2 mutant (strain 17) encodes a thermosensitive UL26 protease and was provided by Bruce Register and Jules A. Shafer. It was propagated on BHK cells and titrated on Vero cells.

Infections

143B cells cultured without BUdR were grown on glass coverslips in 24-well plates for 24 hours. The cells were then mock treated or infected with HSV-1 V701 at a multiplicity of infection of 5 for 1 hour at 37°C. The infection was then synchronized at 39.5°C for 7 hours to arrest the virus in the nucleus (Church and Wilson, 1997; Turcotte et al., 2005). Cells were shifted to 20°C for 6 hours in the presence of 20µg/ml cycloheximide (Sigma) to accumulate the virus at the TGN (Turcotte et al., 2005). To reverse the 20°C block, the samples were finally shifted to 31°C for 6 hours, in the continuous presence of cycloheximide. When indicated the 31°C chase was performed in the presence of 25µg/ml FB-1 (Sigma Aldrich), 1.5mM L-CS (Sigma Aldrich) or 50µM propranolol (Calbiochem). The samples were treated for immunofluorescence or analyzed by plaque assays as indicated below.

Inhibition of VSV G transport

143B cells were plated on glass coverslips in DMEM with 10% FBS and antibiotics but without BUdR until 60 to 70% confluence was reached. Twenty four hours later, they were transfected with a GFP tagged thermosensitive vesicular stomatitis virus G glycoprotein mutant (GFP VSV G ts045; obtained from Patrick Keller) using Lipofectamine 2000 as per manufacturer instructions (Invitrogen). This construct accumulates VSV G in the ER at the non permissive temperature (39.5°C), the TGN at 20°C and the plasma membrane at 31°C (Griffiths et al., 1985). Following an initial incubation of 4 hours in

transfection medium (i.e. without serum or antibiotics), the cells were incubated for a further 20 hours at 39.5°C in medium containing 10% FBS, 2 mM L-glutamine, and 10 mM sodium butyrate (Research Chemicals, Ltd.). The cells were then switched to 20°C for 2 h in CO2-independent medium and 20μg/ml cycloheximide to allow the egress of VSV G *ts*O45 to the TGN (Turcotte et al., 2005). VSV G was then chased for 6h to the cell surface at 31°C in the presence or absence of 25μg/ml FB-1, 1.5mM L-CS or 50μM propranolol. Cells were then fixed, permeabilized and analyzed by immunofluorescence (see below). To quantify VSV G transport, the cells were examined by immunofluorescence and phase contrast. Cells positive for VSV G at the plasma membrane were manually counted and compared to all transfected cells irrespective of VSV G localization (n varies between 118 and 260).

Transfection with GST-tagged PKD constructs

143B cells plated one day earlier on glass coverslips were transfected with 5 μg/well (24-well plate) of pME-Py-GST PKD1 wt, pME-Py-GST PKD1 K618N, pME-Py-GST PKD2 or pME-Py-GST PKD3 (all generously provided by Vivek Malhotra) using Lipofectamine 2000. Cells were cultured 24 h at 37°C. When indicated, they were then mock treated or infected with V701 as above and incubated an additional 7 hours at 39.5°C to accumulate the virus in the nucleus. The cells were fixed and permeabilized immediately or after an additional incubation of 8 hours at 31°C or 6 hours at 20°C. The samples were finally examined by immunofluorescence as detailed below.

Immunofluorescence

Samples were fixed in 3% paraformaldehyde for 30 min at 4°C, washed with PBS, and neutralized with 50 mM NH4Cl in PBS for 30 min at room temperature (RT). After a permeabilization of 4 minutes with 0.1% TritonX-100, they were blocked 30 min at RT with 10% FBS in PBS and further incubated for 30 min at RT with primary antibodies diluted in 10% FBS–PBS. The coverslips were washed, incubated with secondary antibodies for 20 min, washed again and mounted on glass slides in Mowiol (Calbiochem). TGN46 antibody (Serotec) was used to label the TGN, while two distinct VP5 antibodies were used to label the viral capsids. The first one (ICP5; Virusys Corporation) detects total VP5 (both mature and immature capsids), while the second (MAb 8F5; kindly provided by Jay Brown) is specific for VP5 present in mature capsids (Trus et al., 1992). In experiments involving pME-Py-GST tagged PKD constructs, staining was performed with a goat polyclonal against GST (Amersham) or PKD specific antibodies for PKD1 (Santa Cruz), PKD2 (Upstate Cell Signalling) and PKD3 (Bethyl Laboratories).

Alexa 350, 488, or 568 secondary antibodies were used as appropriate (Molecular Probes). When indicated, the cell surface was labelled by incubating live cells (i.e. unfixed and not permeabilized) with the Sulfo-NHS-LC-Biotin reagent (Pierce) and streptavidine conjugated Alexa 568 (Molecular Probes). Finally, 0.1μg/ml Hoechst 33342 (Sigma) was used to stain nuclei. Fluorescence microscopy was performed with an Axiophot wide-field fluorescence microscope (Zeiss) equipped with filters and a Retiga 1300 Camera (Q Imaging). The images were acquired and analyzed with Northern Eclipse imaging software (Empix Imaging). They were processed and assembled with Photoshop 6.0 (Adobe).

Nuclear egress assay

The impact of DAG synthesis inhibitors on HSV-1 egress was measured with a recently developed nuclear egress assay (Remillard-Labrosse et al., 2006). Succinctly, HeLa cells grown in suspension were infected with HSV-1 17+ at 37°C for 8 hours at a multiplicity of infection of 3 and radiolabelled with 25 μCi/ml of 3H thymidine (PerkinElmer). Cells were then harvested and the nuclei isolated. These HSV-1 containing nuclei were incubated 6 hours at 37°C in duplicates with cytosol, an energy regenerating system and nuclear buffer (20 mM Tris-Cl pH 7.4, 5 mM MgCl2, 100 mM KCl, and 1 mM dithiothreitol; Remillard-Labrosse et al., 2006). The negative control consisted of the same reaction without energy and cytosol. When indicated, 25 μg/ml of FB-1, 1.5 mM L-CS or 50 μM propranolol were directly added to the assay. At the end of the incubation period, the capsids released by the nuclei were quantified by liquid scintillation on an LKB Beta rack 1211 counter (Remillard-Labrosse et al., 2006).

Extracellular release of HSV-1

143B, HeLa or HeLa GF17 cells grown in 60-mm dishes were infected with V701 at a MOI of 5 and incubated at 39.5°C for 6-7 hours to accumulate the capsids in the nucleus. When indicated, they were incubated 0, 2 or 6 hours at 20°C and chased at 31°C up to 30 hours (see figure legends). The extracellular medium was collected and centrifuged for 1 hour at 39,000*g*. The viral pellet was resuspended in MNT (30 mM morpholine ethanesulfonic acid, 100 mM NaCl and 20 mM Tris pH 7.4) before being titrated on Vero cells. For experiments involving siRNA, see additional details below.

Reverse transcriptase PCR (RT-PCR)

To evaluate PKD expression, untransfected or 143B cells transfected with the GST-PKD 1, 2 or 3 constructs (see above) were grown in 6-well plates and the mRNA extracted using a PolyATtract System 1000 kit as per manufacturer's instructions (Promega). In some experiments, total RNA extracted with a SV Total RNA kit (Promega) was used with identical results. Reverse transcription and PCR amplification was performed with 50 to 100 ng of RNA, PKD isoform specific primers and an Access Quick RT-PCR kit as per manufacturer's instructions (Promega). Primers were GATGTGGCCAGGATGTGGGAG (forward) and GGGGGTACCCACCACTGACCT (reverse) for PKD1, TCATTGACAAACTGCGCTTC (forward) and GCGTTCTGGATCTGGTCATT (reverse) for PKD2, CAGAGCTGGGAAAAAGCA (forward) and TGCCACTGAGGCTCACATA (reverse) for PKD3. Half the PCR reaction was analyzed on a 2% agarose gel. To insure the survival of the polymerase when long cycling was used (up to 50 cycles), fresh TAQ was added at the 30th cycle. Note that the PKD →primers were specific for their respective isoform (data not shown). Furthermore, each PCR yielded a distinct amplification product which could readily be distinguished based on its size (see fig. 8).

siRNA transfection

All siRNA reagents were purchased from Dharmacon, including PKD1, PKD2 and PKD3 siRNA. Dharmacon's "On-Target" non-targeting siRNA and siGLO Green transfection indicator were used as transfection controls. siRNA transfection for all of the experiments was performed as suggested by the manufacturer.

Briefly, 143B cells in 24-well plates (immunofluorescence) or 6-well plates (Western blotting) were grown to 60-75% confluence and transfected with 20 nM siGLO or 100nM siRNA and Lipofectamine 2000 (Invitrogen) in serum free medium. Four to five hours later at 37°C, complete medium was added to each well. Twenty-four hours posttransfection, the cells were either harvested immediately, mock infected or infected with HSV-1 V701 at a MOI of 5 for 7h at 39.5°C followed by a chase of 9h at 31°C. The same kinetics was used for the immunofluorescence and to evaluate the extracellular release of HSV-1 by plaque assay in presence or absence of siRNA. When indicated, the cells were harvested after 24h, 48h and 72h post-transfection prior to Western blot analysis.

Western Blottin

To verify which PKD isoform was expressed in 143B cells, they were mock transfected or transfected with 5 µg/well (6-well plate) of pME-Py-GST PKD1, pME-Py-GST PKD2 or pME-Py-GST PKD3. The cells were harvested 24 hours later, washed in PBS, counted and subjected to gel electrophoresis. The same number of cells was loaded in a 8% gel, corresponding to approximately 40 µg of proteins. The samples were transferred to a PVDF membrane (Millipore), blocked with 5% non-fat milk in PBS and probed with 1:500 dilution of rabbit polyclonal antibody against PKD1 (Santa Cruz Biotechnology), PKD2 (Upstate) and PKD3 (Bethyl Laboratories). Their detection was with a 1:5000 dilution of peroxidase-labeled anti-rabbit immunoglobulin G (Jackson Immuno-Research). An anti gamma actin antibody was used as loading control (Chemicon).

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Abbreviation list

BUdr: 5-bromo-2'-deoxyuridine

DAG: Diacyl glycerol

FB-1: Fumonisin B-1

HSV-1: Herpes simplex virus type 1

L-CS: L cycloserine

Pfu: Plaque forming units

PKD: Protein kinase D

VSV: Vesicular stomatitis virus

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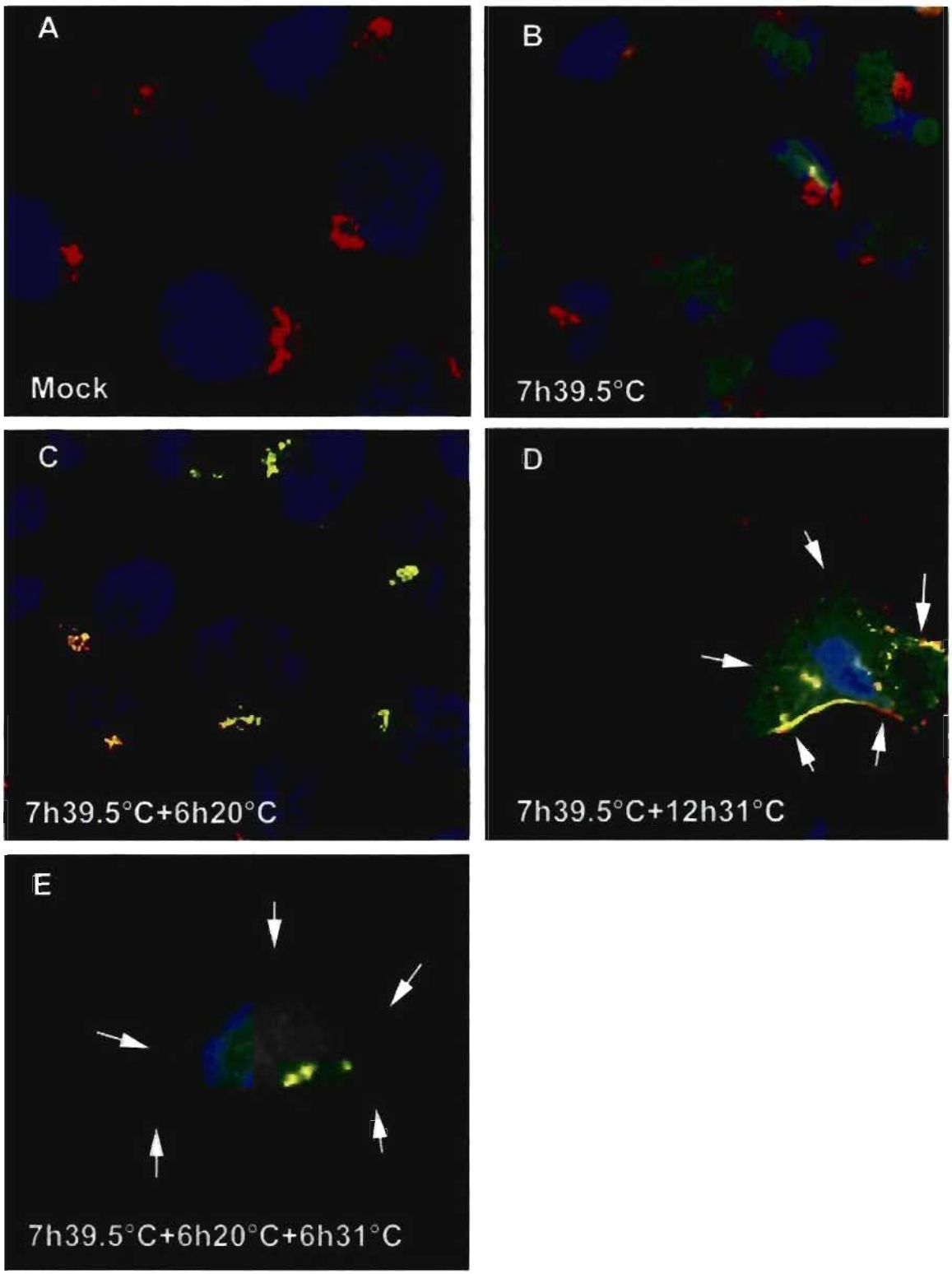
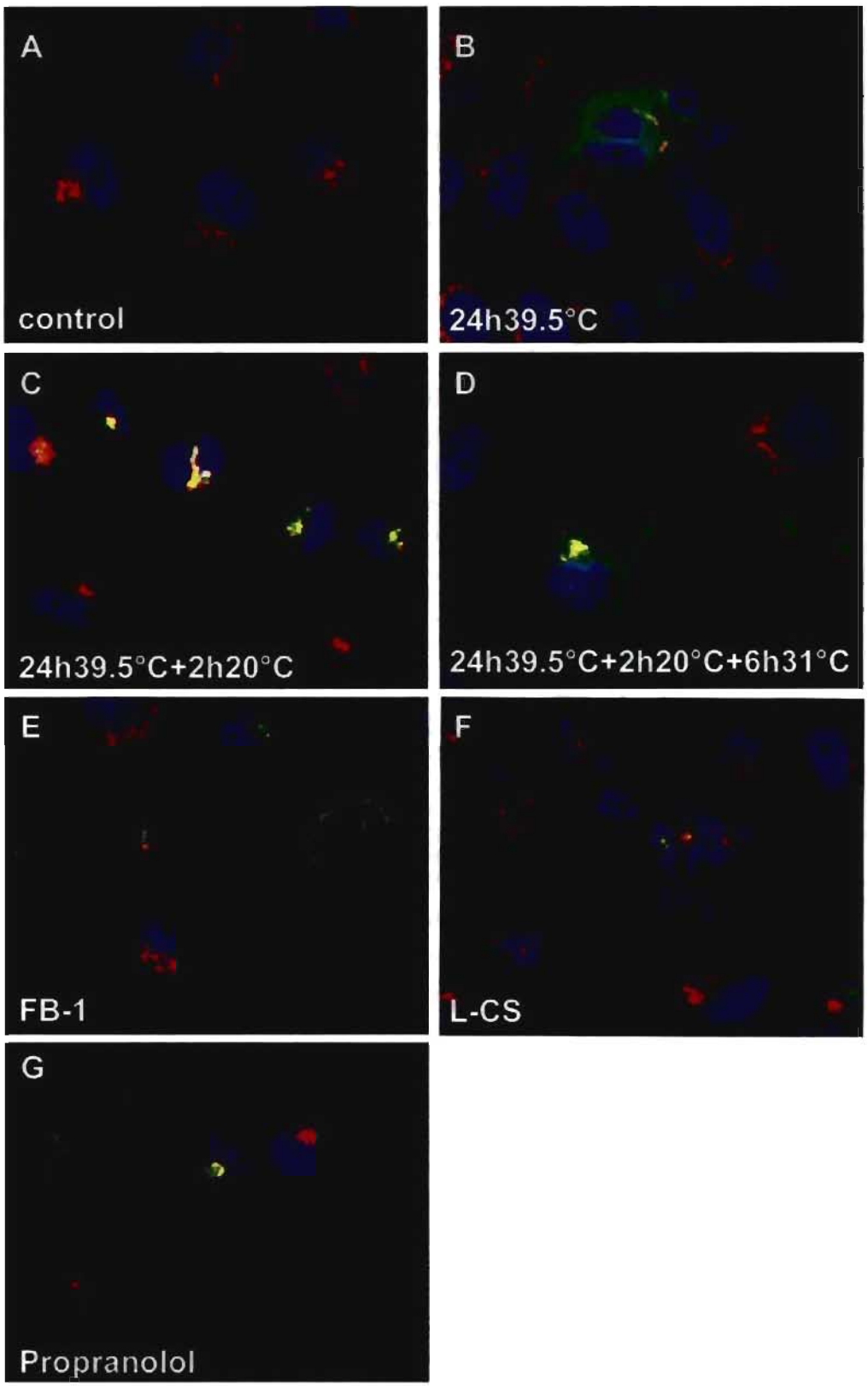
Fig. 1: ***Synchronization of HSV-1 egress to the TGN.*** 143B cells were mock treated (panel a) or infected with V701 at a MOI of 5 (panels b-e) on coverslips in 24-well plates and incubated for 7 hours at 39.5°C to accumulate the capsids in the nucleus. The cells were either fixed immediately (panel b), after a further incubation of 6 hours at 20°C (panel c), an incubation of 12 hours at 31°C (panel d) or an incubation of 6 hours at 20°C followed by another incubation of 6 hours at 31°C (panel e). All samples were stained with Hoechst to label the nuclei (in blue) and ICP5 to label all viral capsids (in green). In panels a-c and e, the TGN was revealed with TGN46 (in red). In panel d, TGN staining was omitted and replaced by biotinylation of the plasma membrane prior to permeabilization and incubation with Alexa 568-streptavidin (in red). Note the effectiveness of the 20°C to retain the virus at the TGN, while the virus readily reaches the cell surface at 31°C (see arrows).



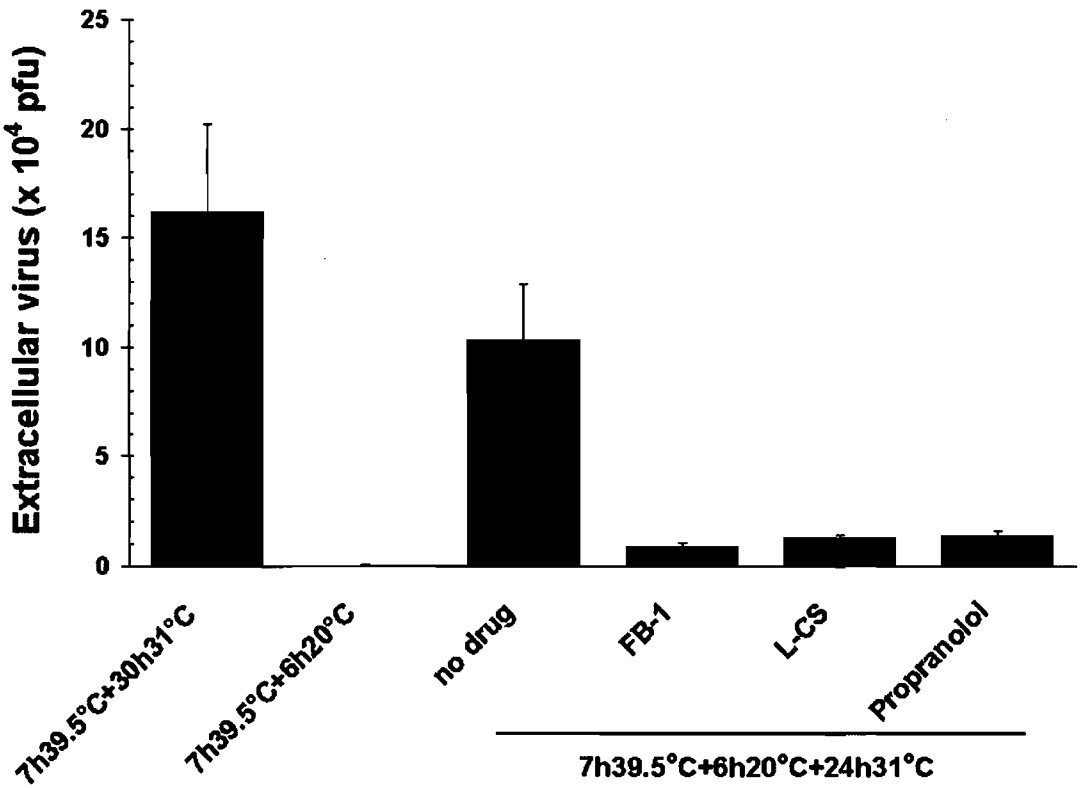
Mihai et al (fig. 1)

Fig. 2: ***Inhibition of TGN to cell surface transport by PKD inhibitors***. 143B cells were mock treated (panel a) or transfected with GFP tagged VSV G ts045 (panels b-g) for 24 hours at 39.5°C to accumulate the protein in the reticulum endoplasmic. They were either fixed immediately (panel b), after a further incubation at 20°C for 2 hours to chase VSV G to the TGN (panel c) or after both the 20°C incubation and a subsequent 6 hour chase at 31°C to let the protein reach the cell surface (panels d-g). To test the impact of PKD inhibitors on TGN to cell surface transport in 143B cells, 25 μg/ml FB-1 (panel e), 1.5 mM L-CS (panel f) or 50 μM propranolol (panel g) was added during both the 20°C incubation and the subsequent 31°C chase. Following their fixation, the cells were stained with TGN46 (red) and Hoechst (blue), while GFP VSV G could be seen in green. The drugs clearly all perturbed VSV G transport from the TGN to the plasma membrane in this cell line.



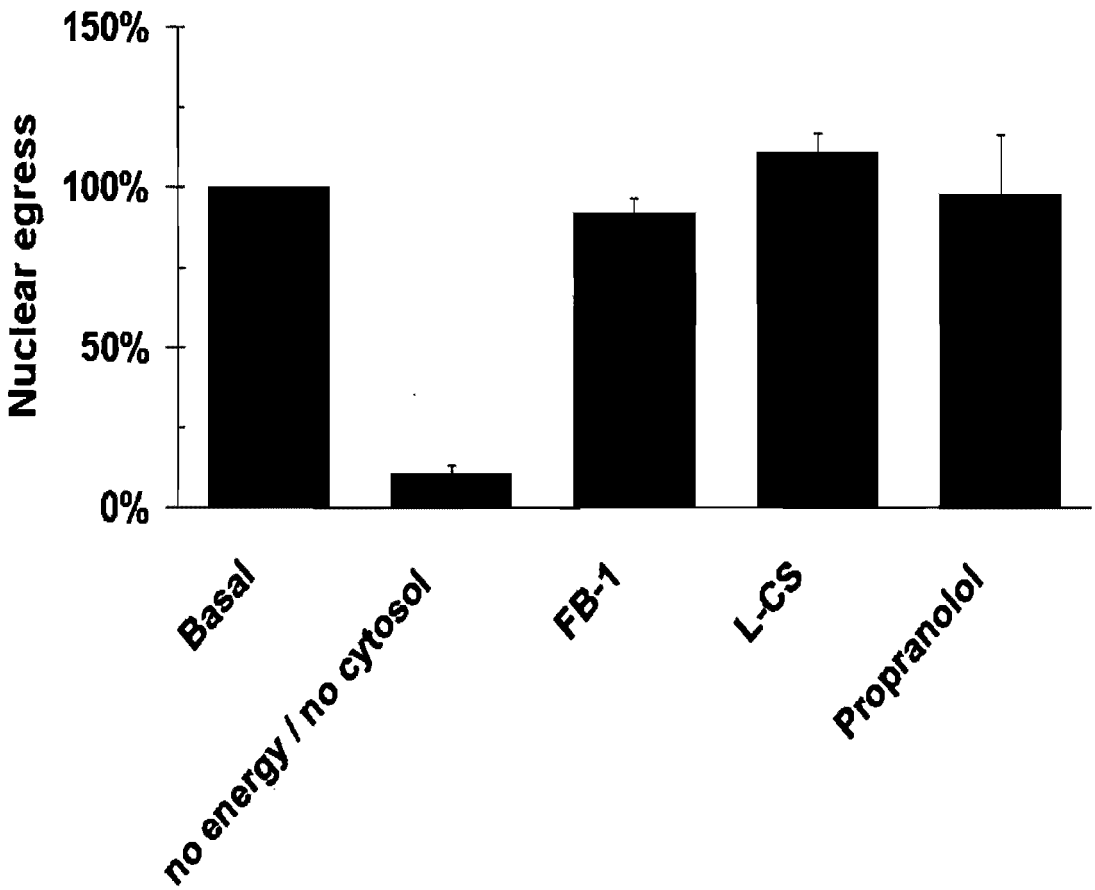
Mihai et al (fig. 2)

Fig. 3: ***Viral egress is hampered by PKD inhibitors.*** 143B cells seeded in 6-well plates were infected with V701 at a MOI of 5 and incubated at the temperatures indicated in the histogram. As above, 25 μg/ml FB-1, 1.5 mM L-CS or 50 μM propranolol was added to the cells when they were switched to 20°C and throughout the chase at 31°C. At the end of the incubation periods, the extracellular medium was titrated on Vero cells. The data represent 3 different experiments, each done in duplicates. The bars indicate the standard deviation of the means. Note that all three PKD inhibitors strongly inhibited viral egress.



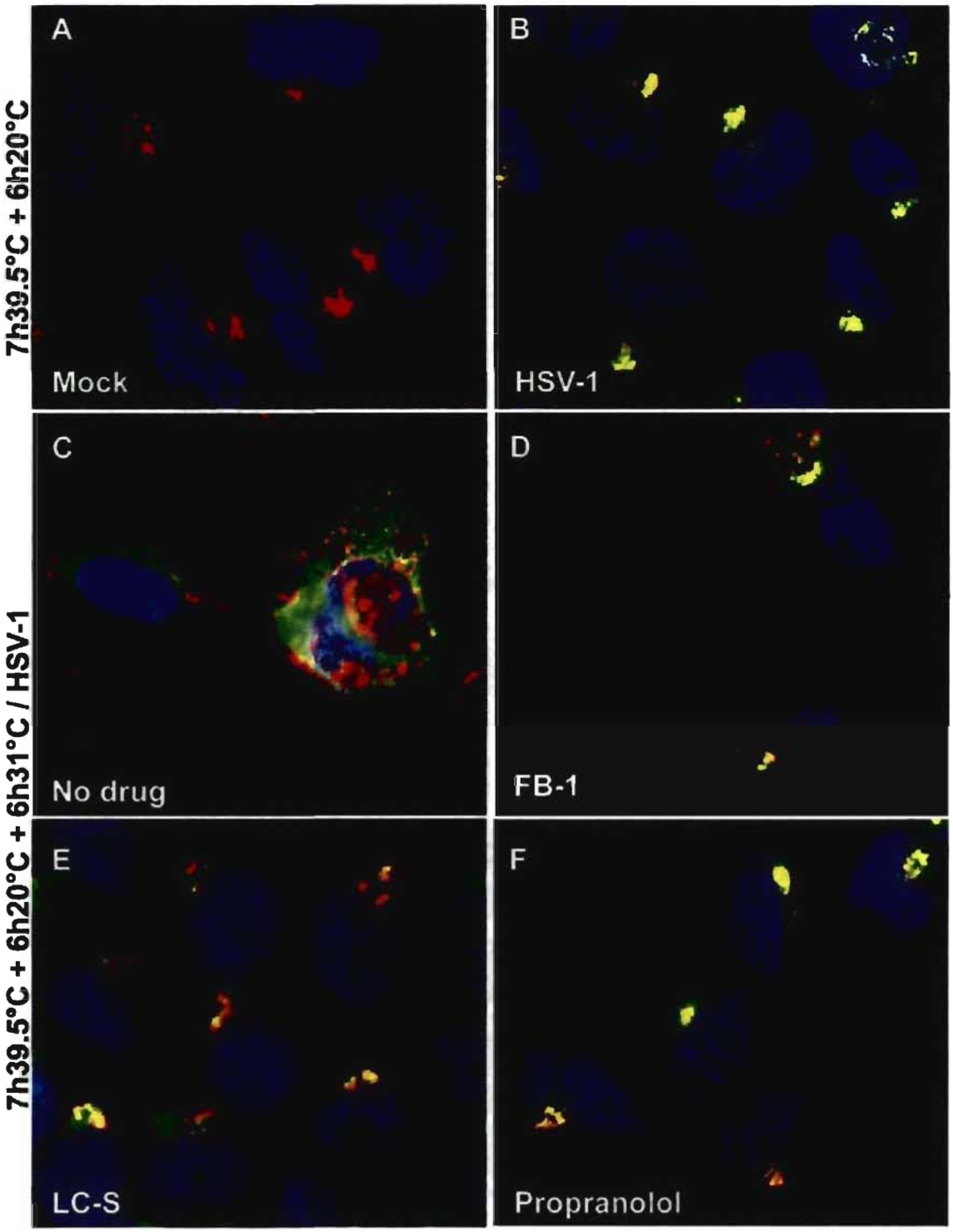
Mihai et al (fig. 3)

Fig. 4: ***PKD inhibitors act downstream of nuclear egress.*** Suspension HeLa cells were infected with wild type HSV-1 in the presence of 3H thymidine to label the viral genomes. Eight hours post-infection, the infected nuclei were harvested as previously reported (Remillard-Labrosse et al., 2006). The isolated nuclei were then incubated with cytosol and energy in the absence or presence of 25 µg/ml FB-1, 1.5 mM L-CS or 100 µM propranolol for 6 hours *in vitro*. The capsids released by the nuclei were isolated and quantified by liquid scintillation. Viral egress was normalized to the counts obtained without drug (100%). In contrast, the sample devoid of energy and cytosol represents the background signal. The errors bars depict the standard deviation of the mean (2 experiments, each done in duplicates). None of the drug altered the release of HSV-1 by the nuclei.



Mihai et al (fig. 4)

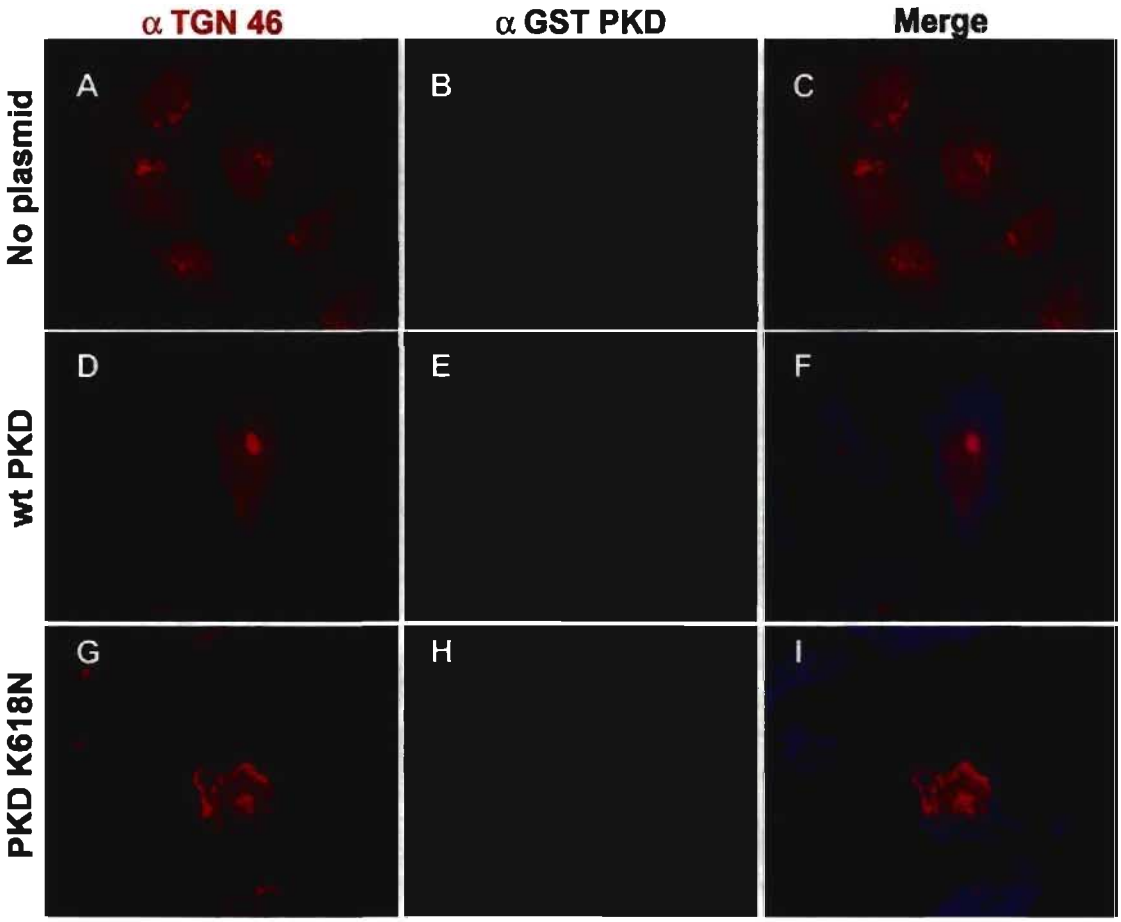
Fig. 5: ***PKD inhibitors trap HSV-1 in the TGN.*** Mock treated (panel a) or 143B cells infected with V701 at a MOI of 5 (panels b-f) were incubated for 7 hours at 39.5°C then hours at 20°C to synchronize the virus in the TGN. They were either fixed immediately (panel b) or after an additional chase of 6 hours at 31°C in the absence (panel c) or presence of 25 μg/ml FB-1 (panel d), 1.5 mM L-CS (panel e) or 50 μM propranolol (panel f). The cells were finally stained with Hoechst (nuclei in blue), TGN46 (TGN in red) or ICP5 (total capsids in green). Note the strong retention of the virus at the TGN by all drugs, under conditions that normally allow the virus to travel beyond the TGN to the cell surface (panel c).



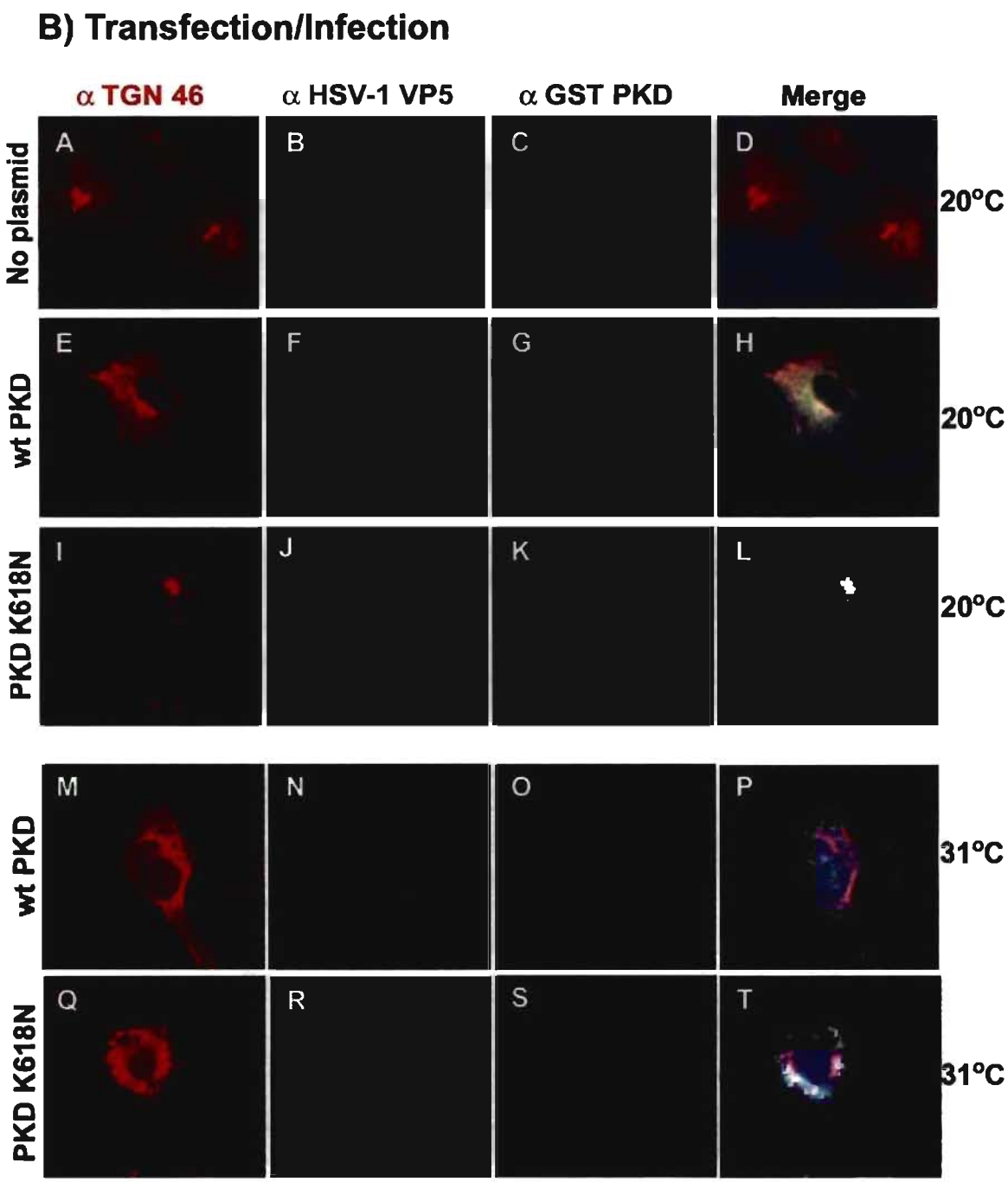
Mihai et al (fig. 5)

Fig. 6: ***HSV-1 co-localizes with PKD K618N induced tubules at the TGN.*** 143B cells were transfected without plasmid, with wild type PKD (wt PKD) or kinase dead PKD (PKD K618N) for 24 hours at 37°C as indicated to the left of the panels. In part "A) Transfection", all the cells where shifted to 20°C for 6 hours before being mounted for immunofluorescence (see below). In part "B) Transfection/Infection", the cells were subsequently mock treated (panels a-d) or infected with V701 at an MOI of 5 (panels e-t) for 7 hours at 39.5°C to synchronize the infection. They were then shifted to 20°C for 6 hours (panels a-l) or 31°C for 9 hours (panels m-t) before fixation. All samples were finally mounted for immunofluorescence (TGN labelling with TGN46 in red; mature capsids labelled with 8F5 in green; GST PKD labelled in blue with antibody against GST). Note that the nuclei were not stained with Hoechst to avoid confusion with the GST labelling. Note the specificity of the viral and PKD antibodies, the presence of long tubules induced by the kinase dead PKD and the strong association of virus with these tubules.

A) Transfection

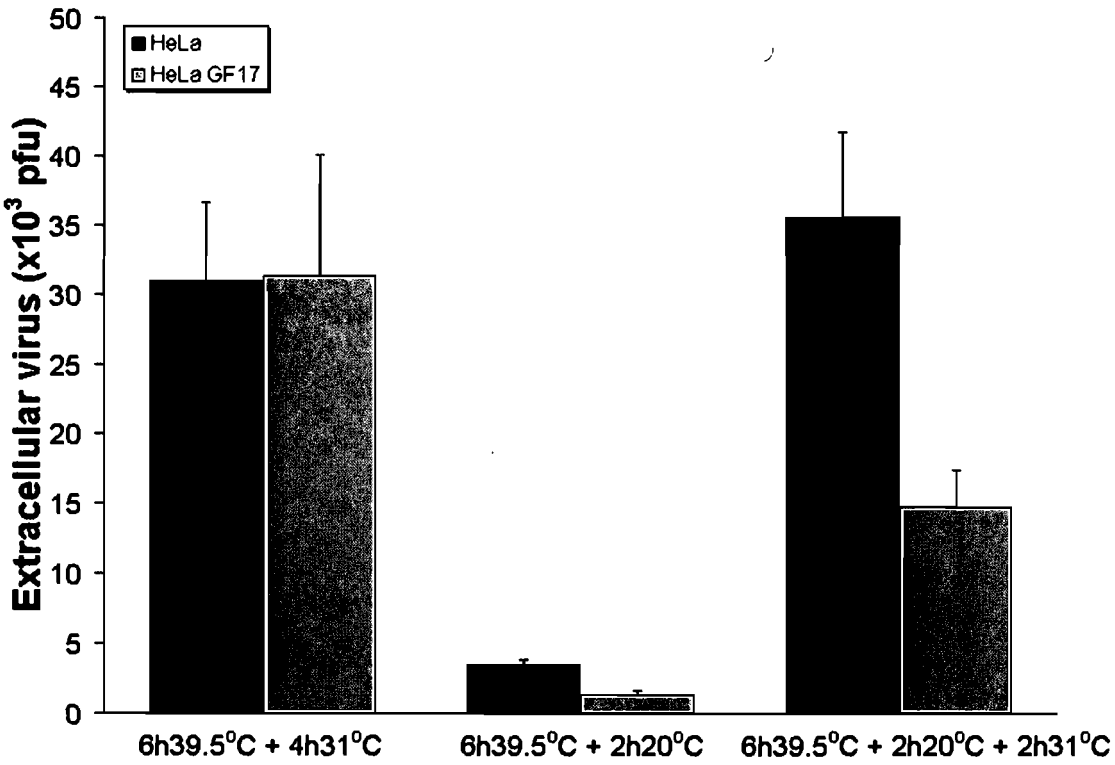


Mihai et al (fig. 6A)



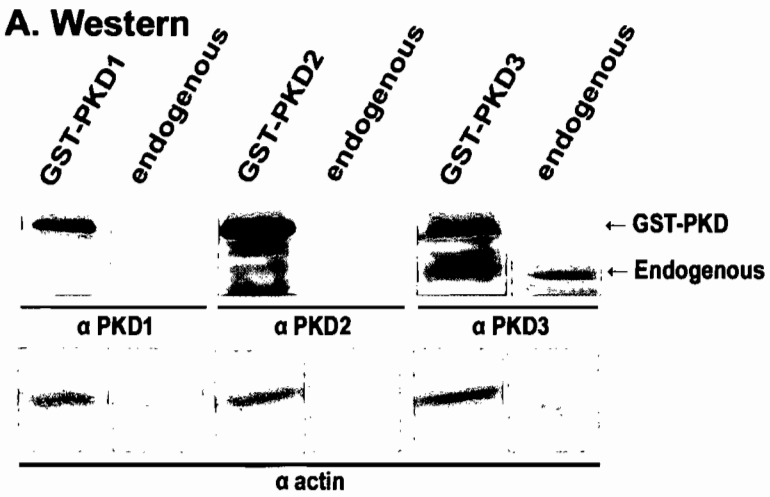
Mihal et al (fig. 6B)

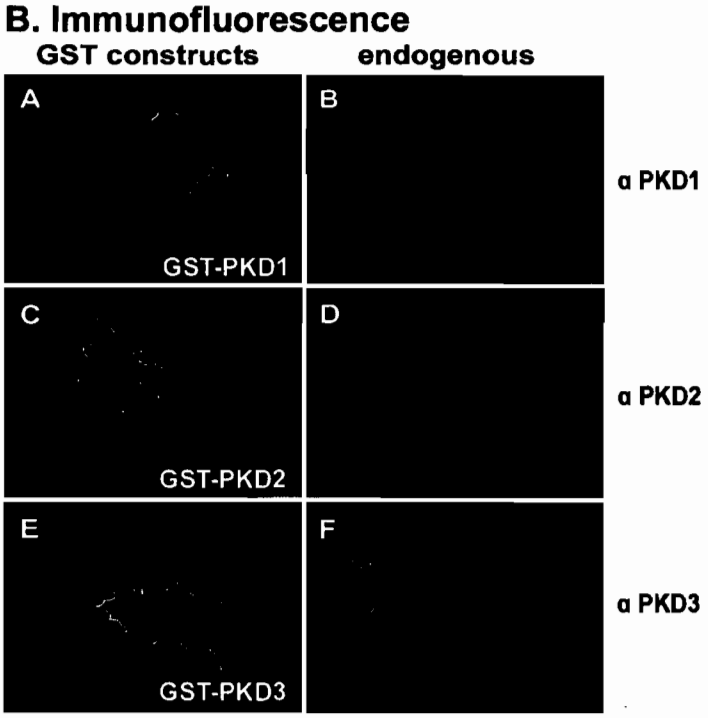
Fig. 7: ***Cell line expressing kinase dead PKD mutant has reduced viral output.*** HeLa and HeLa GF17 cells were infected with V701 at a MOI of 5. They were incubated 6 hours at 39.5°C to synchronize the infection. Three subsequent scenarios were tested and indicated below each bar, including a 4 hour incubation at 31°C to release the virus (total viral release), a 2 hour incubation at 20°C (TGN block) or 2 hours at 20°C with a subsequent chase of 2 hours at 31°C. The extracellular medium was collected, the virus concentrated and titered by plaque assay on Vero cells (see Experimental Procedures). The data represents a typical experiment performed in duplicates. On average, HeLa GF17 cells released 60.6% + 2.7 less virus in the medium compared to control HeLa cells (two independent experiments).

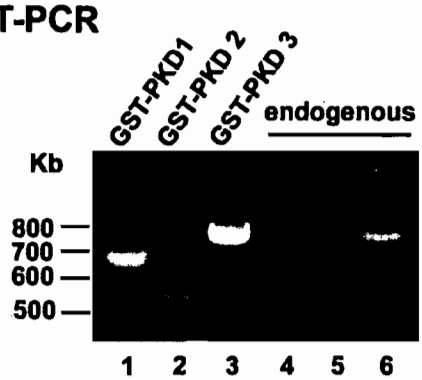


Mihai et al. (fig. 7)

Fig. 8: ***143B cells solely express PKD3.*** To determine the presence of the different PKD isoforms, 143B cells were analyzed by Western blotting and immunofluorescence using PKD specific antibodies or RT-PCR with PKD specific primers (see Materials and Methods). Controls included 143B cells transfected with pME-Py-GST-PKD1, 2 or 3 constructs to insure that all reagents worked as anticipated. A) Western blots using equal number of cells in each lane (~ 40 μg) were performed with anti PKD1, 2, 3 antibodies as indicated below each blot. An actin immunoblot served as loading control. Lanes labeled with GST-PKD1, 2 or 3 represent cells transfected for 24 hours with the respective construct. B) Immunofluorescence using the above PKD antibodies as indicated to the right of the panels. In panels a, c and e, the cells were transfected for 24 hours with pMEPy- GST-PKD plasmids as indicated. In panels b, d and f, untransfected cells were analyzed to determine the endogenous expression of PKD. The staining obtained with the GST antibody perfectly co-localized with the anti PKD labelling in transfected cells, confirming the specificity of the antibodies (data not shown). C) RT-PCR performed with mRNA isolated from mock treated or GST-PKD transfected cells. Lane 1: GST-PKD1 transfected cells probed with PKD1 primers, lane 2: GST-PKD2 transfected cells probed with PKD2 primers, lane 3: GST-PKD3 transfected cells probed with PKD3 primers, lanes 4-6: Untransfected cells respectively probed with PKD1, 2 and 3 primers. Each primer set amplifies the respective PKD sequence (data not shown). Note the detection of endogenous PKD3 in the three assays, but the absence of endogenous PKD1 and PKD2.

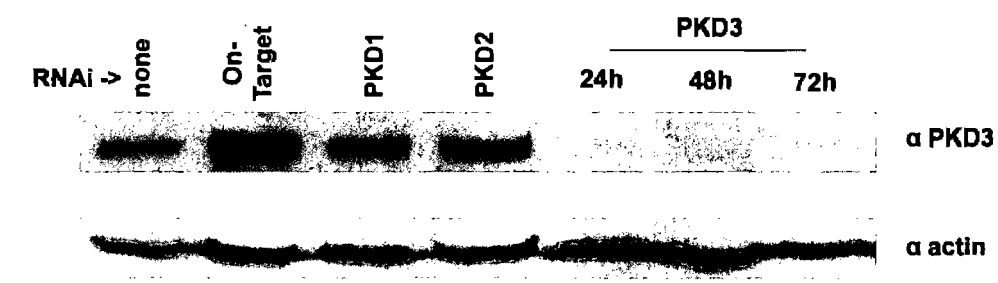






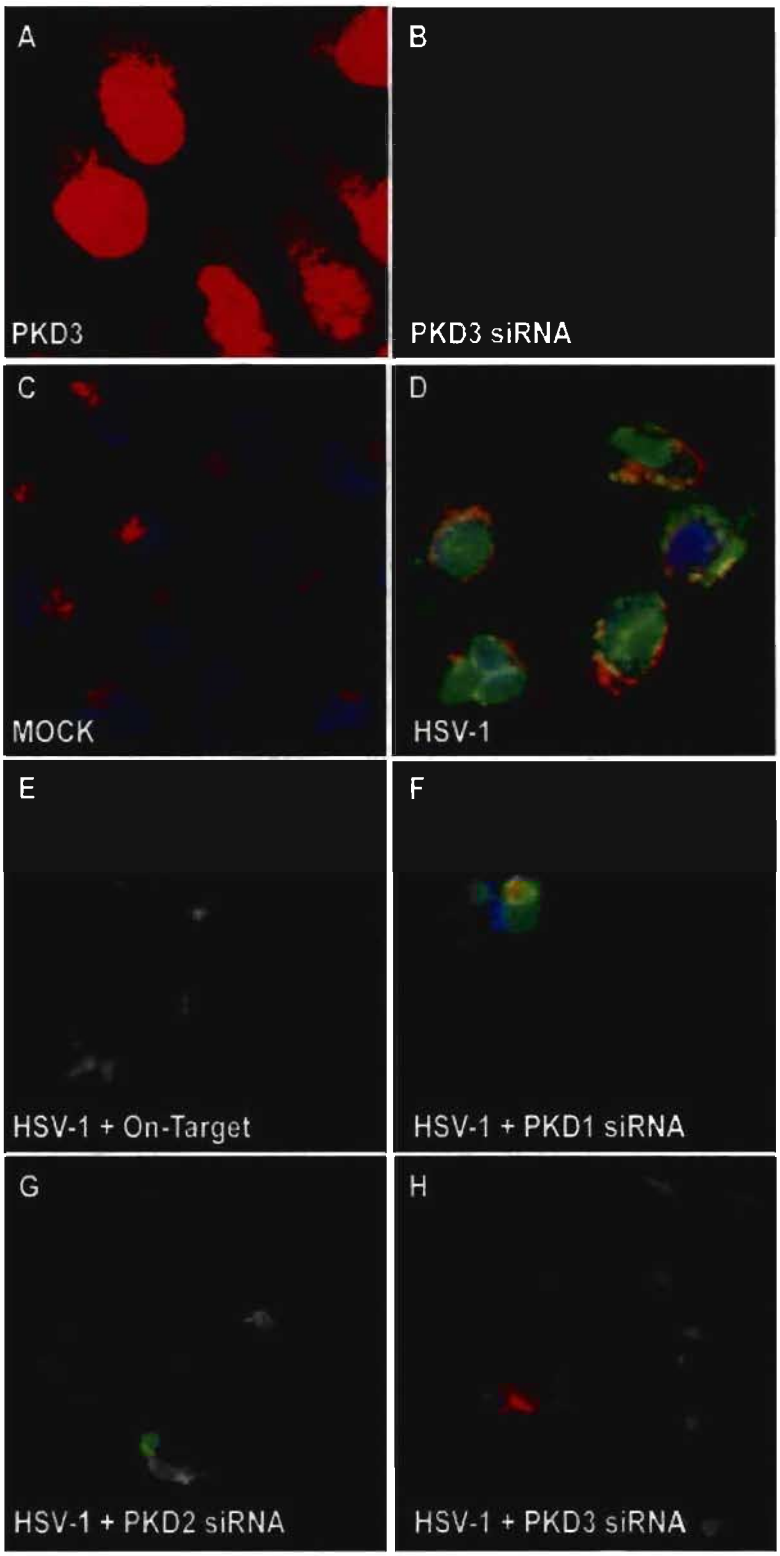
Mihai et al (fig. 8)

Fig. 9: ***Inhibition of PKD3 protein expression by siRNA.*** A) To determine the efficacy of siRNA against their respective targets, 143B cells were co-transfected for 48 hours with pME-Py-GST-PKD 1, 2 or 3 in the presence or absence of siRNA, as indicated. Equal numbers of cells (approximately 40 μg) were analyzed by Western blotting with PKD specific antibodies or actin as a loading control (as indicated below each blot). Note the efficient down expression of each PKD isoform by its respective siRNA and the very strong down regulation of PKD3 by its siRNA but not PKD1, PKD2 or the control On- Target siRNA. B) The endogenous levels of PKD3 were examined in the presence or absence of various siRNA. For the controls (mock transfected, On-Target, PKD1 and PKD2 siRNA), the cells were analyzed 48 hours post transfection. Cells transfected with the PKD3 siRNA were analyzed 24, 48 or 72 hours post transfection.



Mihai et al (fig. 9)

Fig. 10: ***Trapping HSV-1 at the TGN by siRNA targeted against PKD3.*** 143B cells were transfected with PKD3 siRNA (panels b, h), control siRNA (panels e-g) or lipofectamine only (panels a, c, d) as indicated in each panel. Panels a-b were analyzed by immunofluorescence 24 hours post transfection for endogenous PKD3 expression using a PKD3 antibody to evaluate the efficacy of the siRNA. The remaining panels were either mock treated (panel c) or infected with V701 (panels d-h) and further incubated 7 hours at 39.5°C, then 9 hours at 31°C. Panels c-h were stained with TGN46, 8F5 (mature HSV- 1 capsids) and Hoechst (nucleus).



Mihai et al (fig. 10)

Chapter IV Discussion

PKD regulates TGN to plasma membrane HSV-1 transport

As shown in the HSV-1 literature, viral glycoproteins travel though the biosynthetic pathway and join HSV-1 capsids at the TGN. The main objective of this research is to determine the mechanism of how the mature re-enveloped capsids bud at the TGN membranes, before reaching the plasma membrane. In order to achieve this goal, we used a HSV-1 mutant as well as knowledge gathered along the time, about different pathways used by cellular proteins in their travel from TGN to the plasma membrane. Given the very fast HSV-1 life cycle our approach was to synchronize the HSV-1 life cycle with the thermosensitive HSV mutant V701 (Register et al., 1996). This mutant encodes a thermosensitive UL26 protease which is essential for capsid maturation, and DNA encapsidation (Church and Wilson, 1997). When cells are infected with such mutant at the nonpermissive temperature of 39.5°C, they accumulate immature procapsids in the nucleus (Church and Wilson, 1997). At the permissive temperature of 31°C, mature capsids form and are released from the nucleus in a synchronized wave (Church and Wilson, 1997; Turcotte et al. 1995). Similarly, the intracellular transport is temperature-dependent and it can be reversibly stopped at the TGN by incubation at 20°C (Griffiths, et all., 1985, Turcotte et al., 2005). At this temperature capsids leave the nucleus and accumulate at the TGN at 20°C.

The above tools allow a better monitoring of viral transport. An additional tool is the 143B cell line, which presents a Golgi apparatus and TGN structure more resistant to the HSV-1 infection (Campadelli-Fiume et al., 1993, Turcotte et al., 2005). It is with such cells that Turcotte et al (2005) demonstrated that the TGN is the site of HSV-1 capsid reenvelopment, as confirmed in this study (fig. 1).

An important component of the fission machinery that acts at the TGN is PKD (Jamora et al. 1997, 1999, Baron & Malhotra 2002, Liljedahl et al. 2001, Maeda et al. 2001, Yeaman et al. 2004). As described in the introduction, this is inactive in cytosol and, after its recruitment to the TGN membrane, participates in the formation of distinct vesicular cargos (Baron and Malhotra, 2002; Maeda et al., 2001). The PKD recruited at the TGN operates in a feed-back process by increasing the DAG level and by modifying the TGN membrane organization (Goni & Alonso 1999, Burger 2000, Shemesh et al. 2003). Also, it was demonstrated that if DAG synthesis is reduced, PKD recruitment to the TGN is hampered and cargo transport to the cell surface is inhibited (Baron & Malhotra 2002). Several approaches were therefore used in this study to block the PKD function, including pharmacological reagents that block DAG synthesis, a dominant negative PKD mutant (PKD-K618N) and RNAi targeting PKD.

Pharmacological reagents to study HSV-1 egress include Fumonisin B 1(FB-1), l-cycloserine (L-CS), and propranolol. They are known as interfering in different stages of sphingolipids synthesis (Baron and Malhotra, 2002; van Ooij et al., 2000). Figure I 10 shows their mechanism of action. For example, FB1 produced by Fusarium moniliforme is a mycotoxin whose structure resembles sphingosine (Solomon J. C., et al., 2003). Studies indicate that FB1 inhibits sphingolipid biosynthesis by inhibiting ceramide synthase, the final step in ceramide synthesis (Wang et al., 1991). FB1 also interacts with the binding sites for sphinganine and fatty acyl-coenzyme A (CoA) in a competitive manner (Merrill et al. 1993). In this way, FB1 decreases DAG production in Golgi membranes and blocks PKD recruitment to the TGN. The second reagent, l-cycloserine is an inhibitor that blocks serine palmitoyltransferase activity, and decreases both sphingosine and ceramide levels (Hinkovska-Galcheva V. et al. 2003). The third reagent, propranolol, inhibits PC conversion into phosphatidic acid (PA) and affects the DAG production at the TGN (Brindley & Waggoner 1998, Pyne et al. 2004, Baron & Malhotra 2002).

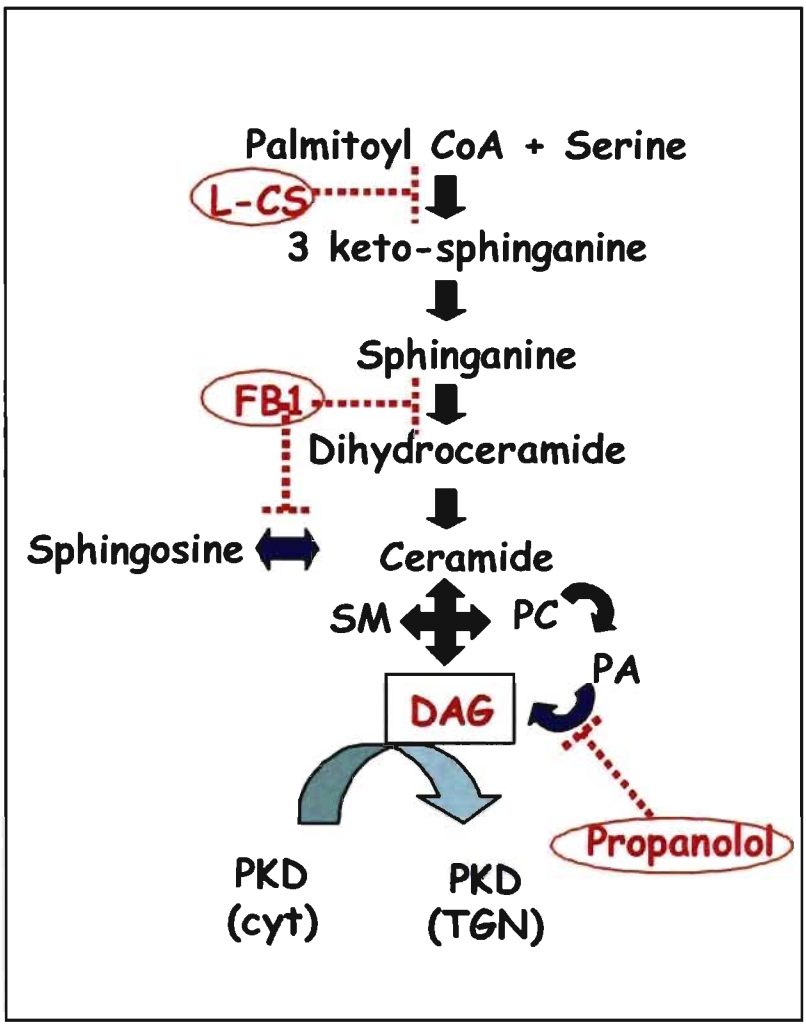


Figure: 1 9. PKD inhibitors block TGN to plasma membrane transport, Liebisch G. Schmitz and G. Hoekstra D. 2004 (Taken from Roger Lippe presentation)

Cyt: Cytoplasmic
DAG: Diacyl Glycerol
PKD: Protein kinase D
L-CS: L-cycloserin

FB1: Fumonisin B1
PC: Phosphatidyl cholin
PA: Phosphatidic acid
SM: Sphingomyelin

An essential step was to confirm the effect of FB1, l-cycloserine, and propranolol against the PKD function in 143B cell line. To this end, we used the well studied VSV G *ts*O45 marker. This is a transmembrane mutant viral protein that accumulates in the ER at 39.5°C and reaches the cell surface when the temperature is shifted to 31°C. Moreover, it is blocked at the TGN at 20°C (Griffiths, G., et al. 1985; Turcotte et al. 2005). Finally, this protein is dependent on PKD to exit the TGN (fig. 2). Using this tool, our results indicated the inhibitors indeed block the VSV G inside the TGN as expected (). The next step was to determine if PKD is also involved in HSV-1 capsids transport to the plasma membrane or whether the virus utilises an alternative transport pathway. The results show FB-1, L-CS and propranolol do not have any impact on the egress of intranuclear capsids into the cytoplasm (fig. 4). In contrast, the three independent inhibitors of DAG synthesis all prevented the release of HSV-1 from the TGN to the extracellular environment (fig. 3, 5); indicating PKD is involved in HSV-1 capsid transport at a late step. Nonetheless, since DAG inhibitors may not specifically target PKD, other evidences were necessary to confirm the implication of PKD in cargo virus transport from the TGN.

The kinase dead PKD (PKD K618N) transfected in 143B cells for 24 hours prior to infection with V701 prevents HSV-1 capsids to arrive at the cell surface (fig. 6). These results were further confirmed in the established HeLa GF17 cell line, which constitutively expresses PKD K618N. As shown in fig. 7, the kinase dead PKD present in HeLa GF17 cells decreased the viral output in the extracellular environment. Finally, the same effects on HSV-1 egress were observed when PKD expression was inhibited with synthetic siRNA in 143B cells (fig. 10). The results present strong evidence of PKD implication in capsid exit from the TGN to the cell surface. Our studies have further proved that these effects were due to PKD3, the only isoform expressed in 143B cells (fig. 8). Hence, RNAi against the other PKD isoforms, respectively, PKD1 and PKD2 or in the presence of "On-Target" control did not have any effect on viral egress (fig 10). As we know from the literature, the PKD mutation in the HeLa GF17 cells is leaky (Liljedahl et al., 2001) and the PKD K618N tubules are able to detach from the TGN membranes at higher temperature (Liljedahl et al., 2001). Furthermore, HeLa cells express both PKD 2 and 3 (Yeaman C. et al., 2004). Consequently, we cannot formally exclude the possibility that the other two isoforms exist might also participate in HSV-1 egress in other cell types (Yeaman C. et al., 2004; Hausser et al., 2005; Maier et al., 2007).

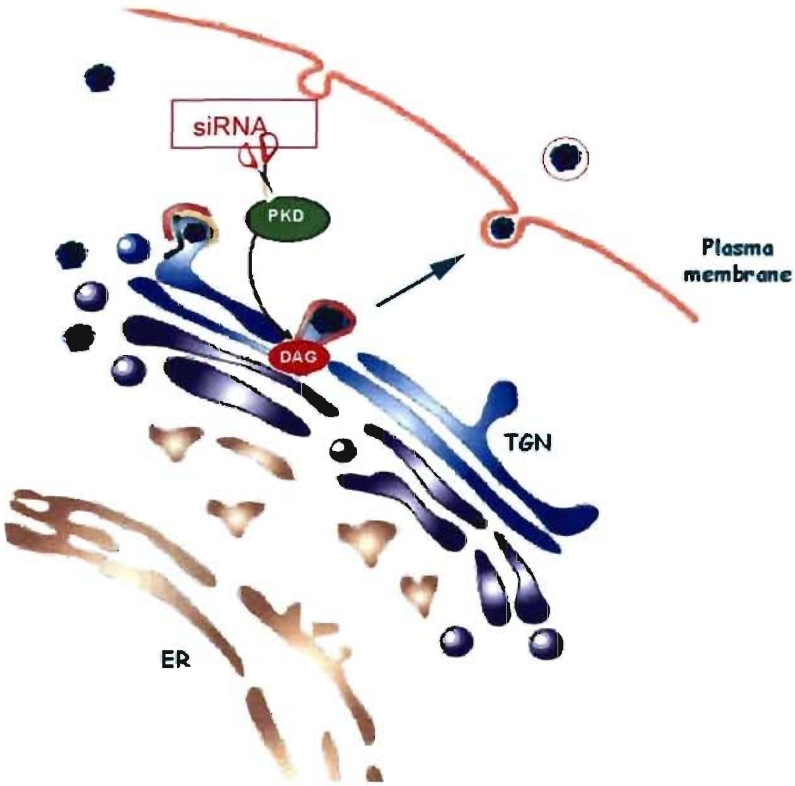


Figure I 10: siRNA PKD3 block the virus containing vesicles from the TGN to the cell surface (adapted from Roth M. 2004 and modified by CM).

8. Model for HSV-1 egress pathway

In conclusion, it is possible to imagine a working model of HSV-1 egress from TGN to plasma membrane (figure I 12). In this model, the envelope of the perinuclear virus fuses with the outer nuclear membrane releasing de-enveloped capsids in the cytoplasm, which then traffic to a cytoplasmic compartment, likely the TGN/endosome. In parallel, the viral tegument proteins and glycoproteins synthesized in the ER arrive independently at the TGN/endosome. There, they associate with naked capsids forming the mature enveloped infectious particles. As a result of the budding mechanism, a tubulo-reticular domain in the TGN is formed. PKD is then recruited by DAG at the TGN and regulates

scission of these tubular structures, resulting in virus containing vesicles that travel to the plasma membrane. Finally, these vesicles fuse with the cell plasma membrane and infectious enveloped virus is released in the extracelular medium. More analyses are now necessary to determine if the virus travels in the same vesicles as cellular proteins or whether it monopolizes this machinery.

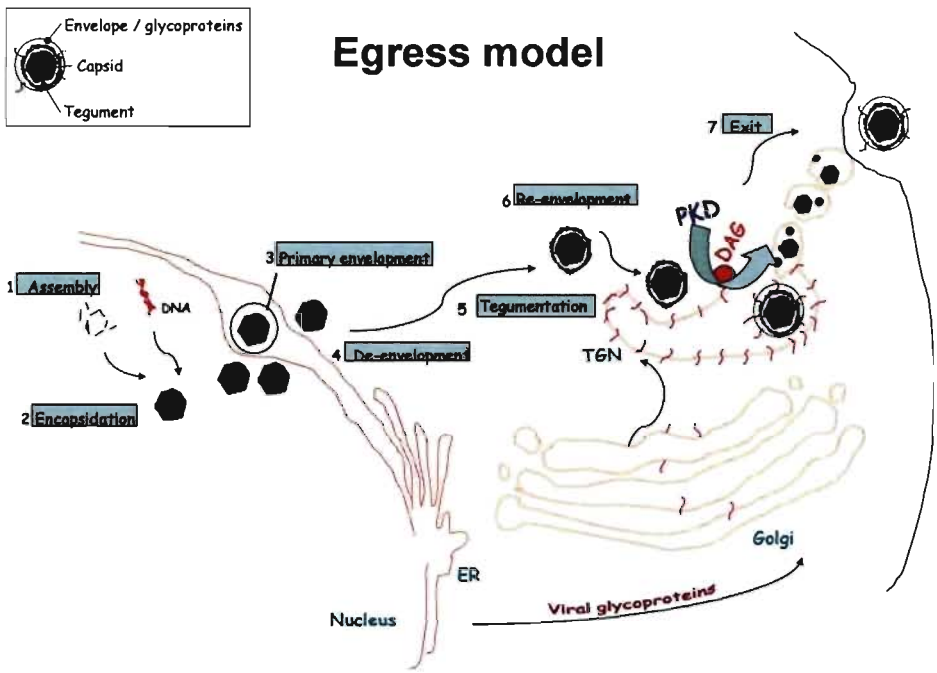


Figure I 11: Model for HSV-1 egress (Adapted from Sophie Turcotte, modified by CM)

Conclusions

The literature mentions a vesicular egress of HSV-1 from the site of re-envelopment to the cell surface. However, there was no evidence regarding the mechanism by which HSV-1 reached the cell surface from the TGN. In the host secretory protein transport, PKD is an important mediator for the formation and detachment of vesicles from the TGN membranes. In this study, we demonstrate that HSV-1 virions utilise the same PKD machinery used by host proteins in their transport from the TGN to the plasma membrane. For instance, three independent DAG inhibitors block viral egress from the TGN to the plasma membrane. Furthermore, the inactive PKD (PKD K618N) also hampered viral transport to the cell surface despite of a temperature dependent mutation. Finally, the same effects have been obtained with siRNA specific for PKD3. Summing up, the presented data are pointing to one conclusion; the PKD3 plays a central role in HSV-1 egress.

It will be very interesting to determine if the virus travels in exactly the same tubules as cellular cargo or if it monopolizes this machinery. Video-microscopy and EM might bring useful information about it. Given a clear modulation by PKD of small cargos such as VSV G and very large cargos such fully assembled virions, it will be interesting to monitor additional small and large cargos by these techniques. Another challenge will be to examine the impact of the various PKD substrates in viral egress. Finally, PKD is meant to specifically regulate the exit of basolateral cargos. It will thus be interesting to determine if it can modulate apical transport as well. In the long run, the hope is to use this knowledge about the life cycle of HSV-1 to ultimately prevent the spread of the virus among the neighbouring cells.

In summary, this study suggests new ways to block the intracellular transport of HSV-1 virions and their spread reduction.

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