

AL-QUDS UNIVERSITY

FACULIY OF GRADUATE STUDIES

**The Role of Human Cytomegalovirus UL16
Glycoprotein and pp65 Phosphoprotein on Tegument
Site, Membrane and Raft Association in Human
Fibroblast Infected Cells**

BY

Khader Mohamed Ibraheem Zawahreh

Supervisor: Dr. Maysa Azzeh

Jerusalem

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ENDORSEMENT

Thesis Title

The Role of Human Cytomegalovirus UL16 glycoprotein and pp65 Phosphoprotein on Tegument Site, Membrane and Raft Association in Human Fibroblasts Infected Cells

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June, 2009

Dedication

To my lovely wife, Ratebah, now more than ever

Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis has not been submitted for a higher degree to any other university or institution

Khader Mohamed Ibraheem Zawahreh

June, 2009

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List of Abbreviations

CMV	Cytomegalovirus
HCMV	Human Cytomegalovirus
ICTV	International Committee on the Taxonomy of Viruses
Bp	Base Pair
HSV	Herpes Simplex Virus
NIEPs	Non Infectious Enveloped Particles
PP65	Phospho protein 65
MCP	Major Capsid Protein
mcp	Minor Capsid Protein
mC-BP	Minor Capsid Binding Protein
SCP	Smallest Capsid Protein
NLS	Nuclear Localization Sequences
APP	Assembly Protein Precursor
MPR	Maturation Protease Precursor
gB	Glycoprotein B
gH	Glycoprotein H
gL	Glycoprotein L
MICB	MHC Class I-Related Chains
ULBPs	UL16-Binding Proteins
NK Cells	Natural Killer Cells
CD8⁺T cells	Cytotoxic T Cells

ORFs	Open Reading Frams
ΔUL97	UL97 Kinase Deletion Mutant
CDK	Cyclin-Cyclin-Dependent Kinase
TAP	Tandem Affinity Purification
GST	Glutathione S-Transferase
Δpp65	pp65 Deletion Mutant
ERGIC	Endoplasmic Reticulum-Golgi Intermediate Compartment
AC	Assembly Compartment
HF_s	Human Fibroblasts
CaCo-2	Intestinal Epithelial Cell Line
EGFR	Epidermal Growth Factor Receptor
hpi	Hours Post Infection
mAbs	Monoclonal Antibodies
WGA	Wheat Germ Agglutinin
FITC	Fluorescein Isothiocyanate
PBS	Phosphate Buffer Saline
Moi	Multiplicity of Infection
TX	Triton X
BSA	Bovine Serum Albumin
WT	Wild Type
WB	Western Blot

ملخص

عائلة Herpesviruses والتي تندرج تحت الفيروسات التي تحتوي على سلسلة مزدوجة من الحامض النووي الريبوزي منقوص الاوكسجين تصنف الى ثلاث مجموعات رئيسية (الفا , بيتا , جاما) . Human cytomegaloviruses تندرج تحت مجموعة بيتا. HCMV مجموعة تتكون في تركيبها من المادة الوراثية محاطة ب Capsid والذي يحيطه ال Tegument والذي يحاط ب ال Envelope الذي يحتوي بدوره على كمية من البروتينات الكربوهيدراتية.

PP65 هو من البروتينات الرئيسية المكونة ل Tegument و UL16 هو احد بروتينات الغلاف والذي يلعب دور هام وبارز في انتشار الفيروسات من الخلايا المصابة الى الخلايا السليمة من خلال قطع اشارة التواصل بين الخلايا المصابة وجهاز المناعة. UL97 kinase هو انزيم الفسفرة الوحيد الموجود في الفيروس والذي يلعب دور هام في فسفرة البروتينات الخلوية والفيروسية المهمة في دورة حياة الفيروس والنقل عبر الاغشية النووية.

الوظائف الهامة التي تقوم بها UL16 و PP65 كان عامل محفز لنا لدراسة دور هذه البروتينات في دورة حياة الفيروس و كذلك الخصائص البيوكيميائية لهذه البروتينات ودراسة تأثير ارتباطها مع UL97 . من خلال التجارب التي اجريت بواسطة تقنية Immunofluorescence تبين لدينا ان UL16 هو مكون اساسي في عملية بناء الفيروس السيتوبلازمية وتبين ايضا من تجارب Western blot ان هذا البروتين هو مكون للجزء الدهني للغشاء الخلوي Raft association protein وان البروتين pp65 هو بروتين غير مرتبط بالغشاء ولكنه يتعرض للفسفرة بشكل اساسي من قبل ال UL97.

Abstract

Herpes viruses are double stranded DNA viruses, classified under three sub-groups; alpha-, beta- (to which human cytomegaloviruses belong), and gammaherpesviruses.

HCMV structure consists of icosahedral capsid encasing 235-kbp genome. The capsid is surrounded by the tegument, which is surrounded by the envelope. pp65 is a major viral tegument proteins with multiple phosphorylation sites. UL97 is also a tegument protein and encodes the only viral kinase, which is a serine/threonine protein kinase that plays a role in phosphorylation events of viral and cellular proteins. The viral envelope contains a large number of virus-encoded glycoproteins. UL16 is an envelope glycoprotein, which interferes with the immune response by interaction with MICB (MHC Class I-Related chains) ULBPs (UL16-binding proteins).

The critical functions of UL16 and pp65 in viral life cycle encouraged us to study these proteins and their roles in viral assembly, membrane and raft association, and their interaction with UL97 viral kinase in infected HF cells. Furthermore, the influence of cellular kinase inhibitors, wortmannin and polymyxin B, was tested in infected cells.

Our immunofluorescence experiments using UL16 polyclonal antibodies, revealed that UL16 distributed differently in AD169 (*wt*) infected HF cells, compared with Δ UL97 deletion mutant infected cells. UL16 distributed as a “bulb like” compact juxtenuclear structure in (*wt*) viruses infected cells and as a “crown like” diffuse perinuclear structure highly punctuated with vacuoles in Δ UL97 infected cells. UL16 also co-localized with the major assembly site building proteins. Inhibitors of cellular kinase PI-3 and C affected the subcellular distribution of UL16 in (*wt*) virus infected cells as well as the resulted viral titer. These inhibitors did not result in major effects on UL16 subcellular distribution or on the viral titer in Δ pp65 or Δ UL97 infected cells.

Studying the biochemical properties of UL16 and pp65 employing western blot techniques indicated that pp65 is a detergent soluble protein; while UL16 is a detergent insoluble raft associated protein.

Finally we conclude that UL16 glycoprotein is a part of the viral cytoplasmic assembly site and that it is a raft association protein. pp65 is not a membrane associated protein and is subjected to phosphorylation activity target of UL97 viral kinase. Both proteins play major roles in HCMV life cycle and the knowledge gained here contributed to better understanding of both proteins' roles during HCMV infection.

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3.4.2- The influence of variable cellular kinase inhibitors and activators on subcellular distribution of UL16

The influence of NGIC-I on HCMV infected cells encouraged us to test further cellular kinase inhibitors, which may indicate yet unknown roles for cellular kinases in HCMV life cycle. We therefore determined the influence of further cellular kinases on the subcellular distribution of UL16 and on the cytoplasmic assembly site in general. For this wortmannin, a PI-3 kinase inhibitor, Thrombin, a PI-3 kinase activator and Polymyxin B, a cellular kinase C inhibitor were employed. First, drugs were tested using drug inhibition assay (3.4) and finally subjected to IF.

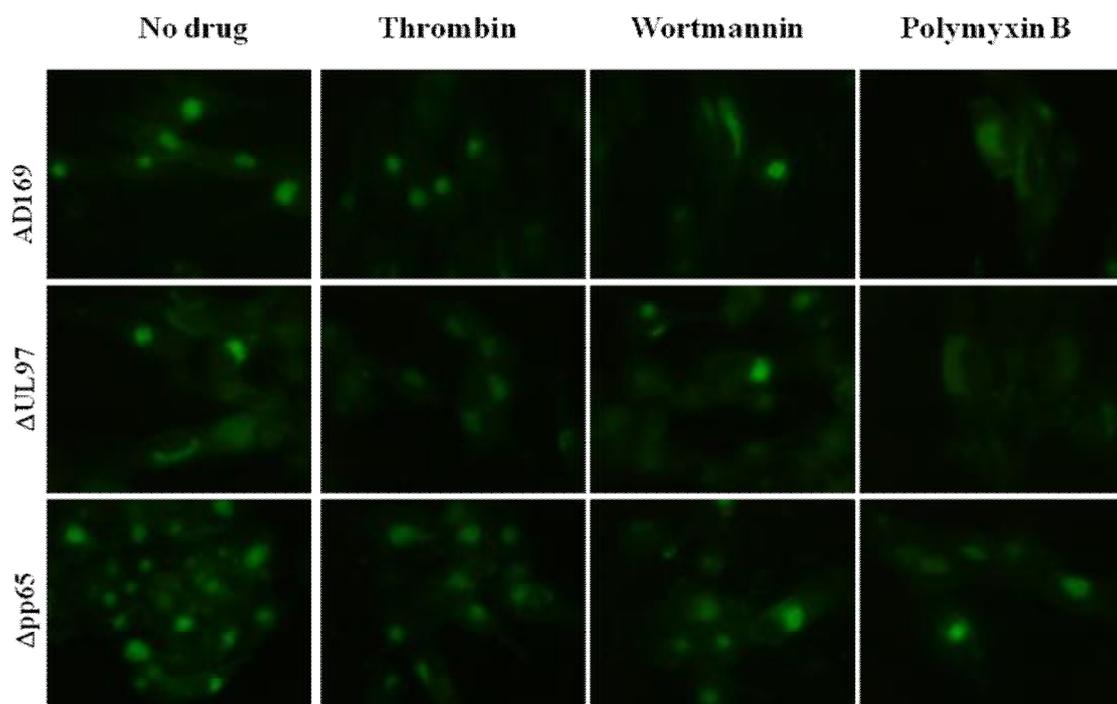


Figure 3.8: Drug inhibition/activation assay. Cells were subjected to drug inhibition assay using Wortmannin (100nM) and Polymyxin B (250 μ g/ml) or activation using Thrombin (IU/ml) 2h after infection. At 96hpi, cells were subjected to IF using UL16 pAb and anti-Rabbit Cy2 (green).

Figure 3.8 demonstrate that Wortmannin and Polymyxin B caused a clear inhibition along with changes in subcellular distribution of UL16 in AD169 infected cells, which is similar to the Δ UL97 infection characteristic. This conversion affected 30-40% of cells (estimated by counting 10 fields with ~1000 cells) compared with 70% in case of NGIC-I inhibition (Azzeh *et al.*, 2006). Thrombin did not result in any changes in the case of AD169, but a partial conversion of the Δ UL97 characteristic subcellular distribution of UL16 in 30% of Δ UL97 infected cells (estimated by counting 10 fields with ~1000 cells). On the other hand, no changes of subcellular distribution of UL16 was detected in both Δ pp65 and Δ UL97 infected cells, when inhibited with Polymyxin B or Wortmannin.

As indicated in figures 3.4-C, 3.5-B and 3.6-B, changes in subcellular distribution of UL16 were consistent with changes in WGA Golgi marker. In order to test, if the drugs are influencing the WGA subcellular distribution in uninfected cells, the drug inhibition assay with infected cells was performed parallel to uninfected cells. Instead of virus containing medium, only medium was added to the uninfected cells (see 3.4). Finally, cells were subjected to IF using the WGA Golgi marker.

Figure 3.9 demonstrates that the addition of the drugs in the current concentration (see figure 3.8, 3.9) did not affect the uninfected cells, since Golgi typical appearance did not alter in drug treated uninfected cells compared with untreated uninfected cells. This result is important since it ensures the fact that changes observed in figure 3.8 are a consequence of drugs' influence only on the infected cells.

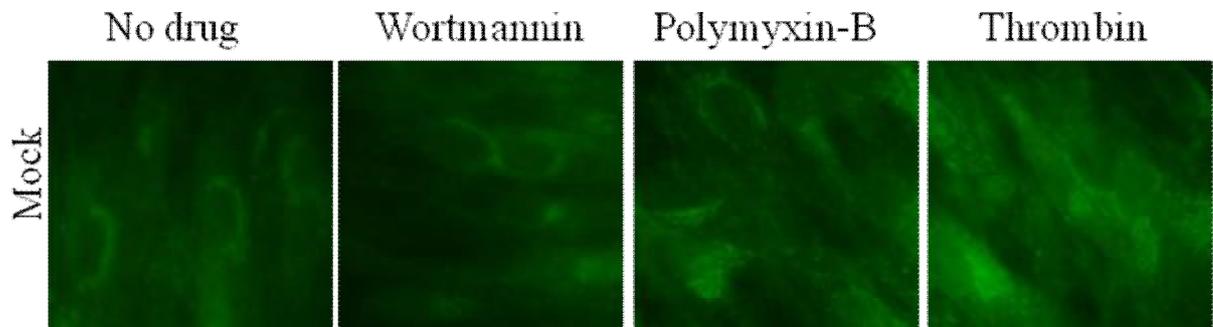


Figure 3.9: Influence of drugs on uninfected cells. Uninfected cells were subjected to drug inhibition assay using Wortmannin (100nM) and Polymyxin B (250 μ g/ml) or activation using Thrombin (IU/ml) parallel to the infected cells in figure 3.8. After 96h, cells were subjected to IF using WGA-FITC Golgi marker (green).

3.5 Influence of Wortmannin, Polymyxin B and Thrombin on HCMV viral load

The results obtained from drug inhibition assays of Wortmannin, Polymyxin B and Thrombin on UL16 reflects a direct or indirect effect of these drugs on the viral assembly site. Viral assembly is a part of the viral life cycle and an inhibition or activation of this site or any protein involved in building it may also affect the viral yield. For this, plaque assay was performed using the supernatants of the drug inhibition assay experiments versus supernatant of non inhibited infections. Log 10 of the resulted viral plaque in each test was calculated and a diagram was generated in Excel as early discussed in 2.4 (figure 3.10). The drugs resulted generally in reduction of viral yield compared to untreated

infected cells. This reduction was not very remarkable in Δ UL97 infected cells. Although not visible on the level of viral assembly, Δ pp65 was reduced almost one log by Wortmannin. The results for ADI69 titer were in agreement with those results observed in the IF. AD169 titer was reduced 2 logs when inhibited with Polymyxin B or Wortmannin compared to untreated AD169 titer. Interestingly, although used as an activator, Thrombin reduced the viral titer of AD169 half a log. The average plaque assay results of 3 independent drug inhibition assays and 3 independent plaque assays were utilized to generate the results shown in figure 3.10.

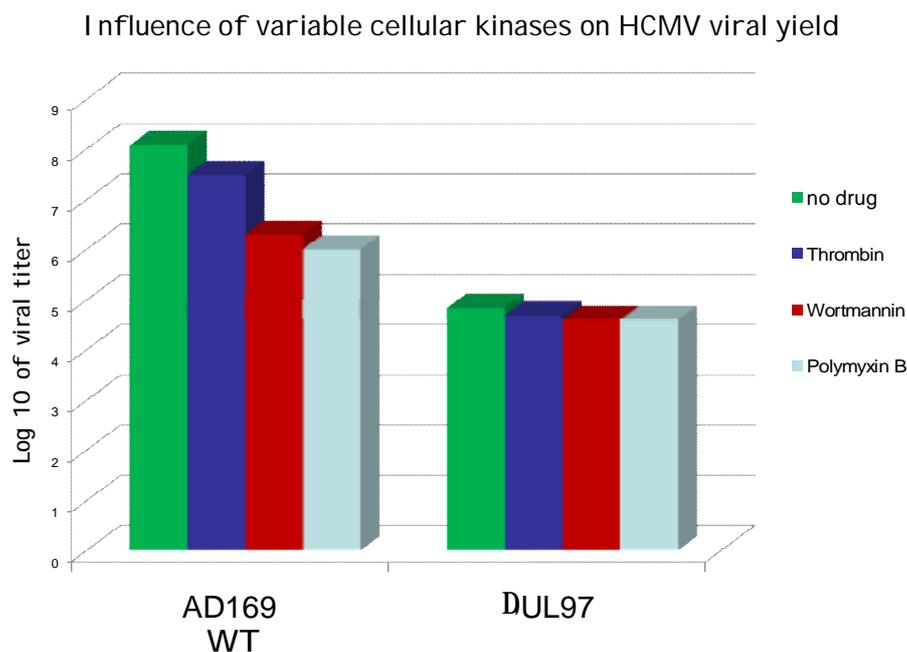


Figure 3.10: Titration of the viral supernatant of drug treated versus untreated cells. At 96hpi viral supernatant was collected prior to IF treatment and subjected to plaque assay. Log 10 of viral titer is given on the Y axis, virus type on the X axis. These titration results were confirmed in 3 independent experiments.

3.6 Membrane association analysis of HCMV viral proteins

The membrane association of any viral protein dictates its cellular localization and is believed to mediate its role in secondary viral envelopment in the cytoplasm. In this work membrane and raft association of UL16 glycoprotein and pp65 were tested.

3.6.1 Membrane association of pp65

Some viral tegument proteins were shown to be raft or membrane associated as in the case of vhs in Herpes simplex virus (Lee *et al.*, 2003). To examine the membrane association of the pp65, we carried out flotation assays (see 2.5) using CHAPS as a detergent and Nycodenz as a density medium. CHAPS is known to float many membrane-associated proteins whereas soluble proteins not associated with membranes remain in the bottom of the gradient. As demonstrated in figure 3.11, pp65 is not membrane associated, since it did not partition in the top of the floatation. The majority of pp65 was detected in fractions 7/8-11/12. Although not membrane associated, we observed a remarkable difference, in marked contrast to AD169, the phosphorylated portion of pp65 in Δ UL97 infected cells was down-regulated compared to the unphosphorylated portion. Therefore, we are now studying the phosphorylation properties of pp65 in the absence of UL97. As a positive control, we used the membrane associated pp28, since it was shown before to partition in the top of the floatation (Azzeh M *et al.*, 2006).

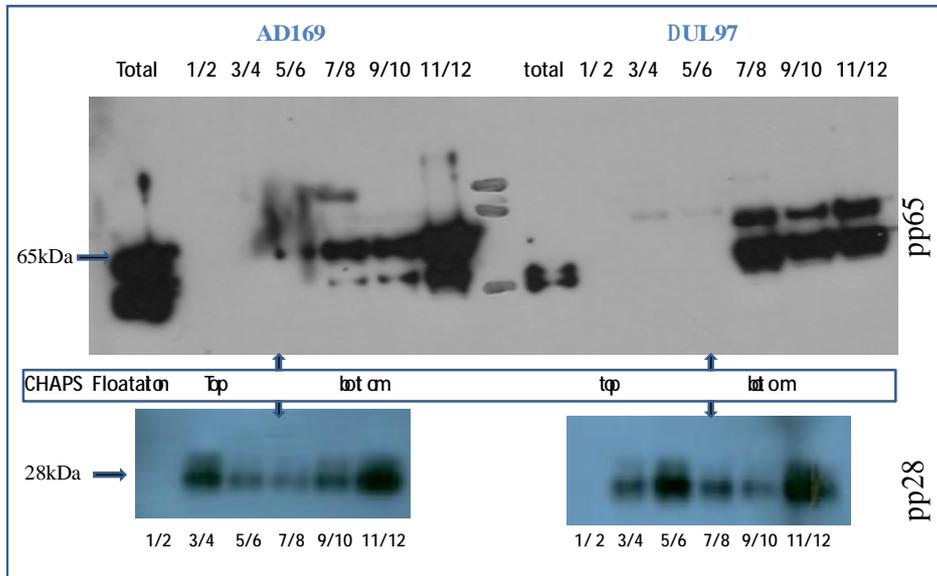


Figure 3.11: Membrane association analysis of pp65. Flotation fraction (numbers above) of CHAPS extracts of cells infected with either AD169 or Δ UL97 were collected from the top of Nycodenz gradient (1st–low density, 12th–high density) and subjected to Western blot analyses using pp65 mAb or pp28mAb.

3.6.2 Raft association of UL16

As Glycoprotein, UL16 is membrane associated; we therefore checked its rafts association. In this case we performed IP after TX-100 floatation, since UL16 was not detectable by direct WB. We succeeded to show the 42kDa band of UL16 in fraction 3/4 at the top of the floatation where the majority of the lower band of double band was clear (figure 3.12). For further studying of UL16, we intend though to order a new preparation

of the antibody from commercial companies. WB using pp28 was performed as negative control, since pp28 is not a raft associated protein (Azzeh *et al.*, 2006).

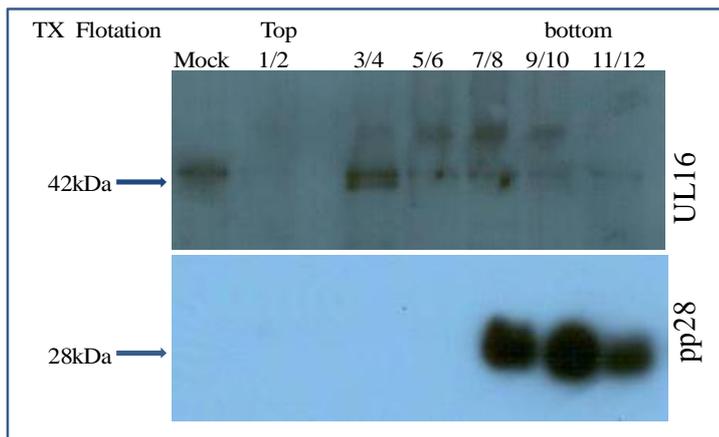


Figure 3.12: Raft association analysis of UL16 protein. Flotation fraction (numbers above) of TX-100 extracts of cells infected with AD169 were collected from the top of Nycodenze gradient (1st –low density, 12th –high density), Immunoprecipitated with A-coupled sepharose and UL16 antibody. Finally samples were subjected to Western blot analysis using UL16 pAb or pp28 mAb as a negative control.

CHAPTER 1

Introduction

1.1 Herpes Viruses

1.1.1 Definition

A typical herpes virion consists of a core, containing a linear double-stranded DNA, an icosadeltahedral capsid of approximately 100 to 110 nm in diameter, containing 162 capsomeres with a hole running down the long axis. An amorphous-appearing, sometimes asymmetric material surrounds the capsid and designated the tegument. There is also an envelope containing viral glycoprotein that forms spikes on the viral surface.

1.1.2 Biological Properties

The known herpes viruses appear to share four significant biological properties:

1. All herpes viruses specify a large array of enzymes involved in nucleic acid metabolism, DNA synthesis, and processing of proteins.
2. The synthesis of viral DNAs and capsid assembly occur in the nucleus.
3. Production of infectious progeny virus is invariably accompanied by the destruction of the infected cell.
4. The herpes viruses examined to date are able to remain latent in their natural hosts. In cells harboring latent virus, viral genomes take the form of closed circular molecules, and only a small subset of viral genes are expressed. Latent genomes retain the capacity to replicate and cause disease upon reactivation.

1.1.3 Classification

The members of the family *Herpesviridae* was initially classified into three subfamilies (i.e., the *Alphaherpesvirinae*, the *Betaherpesvirinae*, and the *Gammaherpesvirinae*) on the basis of biological properties.

1.1.3.1 Alphaherpesvirinae

The members of the subfamily *Alphaherpesvirinae* were classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia. This subfamily contains the genera *Simplex virus* (HSV-1, HSV-2), *Varicellovirus* (VZV), *Marek's disease-like virus*, and *Infectious laryngotracheitis-like virus*.

1.1.3.2 Betaherpesvirinae

A nonexclusive characteristic of the members of the subfamily *Betaherpesvirinae* is a restricted host range. The reproductive cycle is long and the infection progresses slowly in culture. Infected cells frequently become enlarged (cytomegalia) and carrier cultures are readily established. The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidneys, and other tissues. This subfamily contains the genera *Cytomegalovirus* (CMV), *Muromegalovirus* (murine cytomegalovirus), Human cytomegalovirus (HCMV) and *Roseolovirus* (HHV-7).

1.1.3.3 Gammaherpesvirinae

The experimental host range of the members of the subfamily *Gammaherpesvirinae* is limited to the family or order to which the natural host belongs. In vitro, all members replicate in

lymphoblastoid cells, and some also cause lytic infections in some types of epithelioid and fibroblastic cells. Viruses in this group are usually specific for either T or B lymphocytes. Latent virus is frequently demonstrated in lymphoid tissue. This subfamily contains two genera: *Lymphocryptovirus* (EBV), and *Rhadinovirus* (AtHV-2 and SaHV-2).

1.1.4 Nomenclature

The International Committee on the Taxonomy of Viruses (ICTV)-endorsed nomenclature consists of designation of herpes viruses by serial Arabic number and the family (most cases) or subfamily (for primates and some animals) of the natural host of the virus (e.g., *Human herpes virus 7*, *Cercopithecine herpes virus 1*). The species number is not intended to imply anything about the relationship between a virus and other herpes viruses that infect the same host species (e.g., HHV-7 and HHV-8 are members of different subfamilies) or between similarly numbered viruses that infect different host species (e.g., EHV-2 and BHV-2 are members of different subfamilies). Also, viruses are named according to genes and their products for example, glycoprotein B is encoded as UL28 (the position of the gene encoding it) in HSV, U39 in HHV-6B, UL55 in HCMV, orf8 in HHV-8, and so on.

1.1.5 Cytomegalovirus (CMV)

This is a distinct, widely distributed subgroup of herpes viruses that share common growth characteristics and induce a cytopathology involving characteristic nuclear and cytoplasmic inclusions (Smith MG, 1954; Smith MG, 1956; Plummer G, 1973). Viruses in this group were generally known as salivary gland viruses until the common name cytomegalovirus was

proposed by Weller *et al.* (1960) to reflect both virus-induced cytopathic effects and the virus's role in congenitally acquired cytomegalic inclusion disease.

Cytomegaloviruses are the principal members of the betaherpesvirus subgroup. They share several characteristics with other herpes viruses, including virion structure and the ability to establish persistent and latent infections. The CMVs exhibit a number of distinguishing biological characteristics common to the betaherpesviruses, including salivary gland tropism, strict species specificity, and slow growth in cultured cells.

In most areas of the world, HCMV spreads at an early age and infects the large majority of the population. This pattern has been altered by increased hygiene in developed countries, where this virus may reach only 40% to 60% of the population. Thus, in the developed world, a large proportion of the adult population remains susceptible to primary infection, increasing the risk of congenital transmission and subsequent disease. The importance of HCMV as a pathogen has also risen over the past three decades with the increase in organ allografting and immunosuppressive post transplant therapies and the increase in acquired immunodeficiency syndrome (AIDS). These conditions predispose individuals to a primary CMV infection or to reactivation of latent infection, which may lead to fulminate, life-threatening disease that can be difficult to treat despite the available antiviral drugs (Drew WL and Lalezari JP, 1999; Mocarski ES, 1999).

1.2 Epidemiology of HCMV Infection

1.2.1 Distribution, Age, and Demographics

Seroepidemiologic studies show that HCMV is universally distributed among human populations from developed, industrial societies to isolated aboriginal groups (Ahn K *et al.*, 1997; Heise MT *et al.*, 1998). Although the prevalence of HCMV infection increases with age in every studied group, the overall prevalence of infection and the age at initial acquisition of the virus vary greatly according to living circumstances (Bernard N, *et al.*, 2001). In general, prevalence is greater and HCMV is acquired earlier in life in developing countries and in the lower socioeconomic strata of developed countries. Although in the United States and Europe, HCMV infection rates are higher in nonwhites than in whites, racial differences appear to reflect differences associated with socioeconomic status and living circumstances (Bernard N, *et al.*, 2001). Seasonal variation in the incidence of HCMV infection has not been recognized, and epidemics have not been described, aside from reports of very high infection rates in institutional populations such as day care centers.

1.2.2 Transmission

Although HCMV is predictably transmitted in settings where susceptible individuals have frequent contact with body fluids from persons excreting the virus, HCMV is not highly contagious and transmission appears to require direct contact with infectious material (Bernard N, *et al.*, 2001). Following initial acquisition of HCMV, infectious virus is present in the urine, saliva, tears, semen, and cervical secretions for months to years. Not surprisingly, high rates of CMV infection occur in settings where close contact with body fluids is expected, such as

between sex partners, among children in day care centers, and between preschool-aged children and their care givers.

1.3 Clinical Features

1.3.1 CMV Mononucleosis

It has been estimated that HCMV is responsible for 20% to 50% of heterophile-negative mononucleosis and that it accounts for approximately 8% of all cases of mononucleosis (Fons MP *et al.*, 1986). As a rule, acquired HCMV infection in the normal host is clinically silent. Studies of HCMV infection in pregnant women show that less than 5% of acquired infections are symptomatic, and an even smaller percentage are associated with a mononucleosis syndrome (Hirai K and Watanabe Y, 1976). Symptomatic infection is also uncommon in infants who acquire HCMV during birth or from mother's milk and in preschool-aged children who acquire HCMV in day care centers.

1.3.2 CMV Infection in Infants and Children

Although HCMV is frequently spread from mother to infant during birth or through breast feeding, clinical evidence of infection is rare in the healthy, full-term neonate infected intrapartum or postnatal from a maternal source. However, among very low birth weight premature infants acquired HCMV infection is a significant cause of morbidity.

Although HCMV infection in infants has been associated with liver disease (Goedhals D *et al.*, 2008), the risk of disease development is particularly great for newborns with birth weight less than 1,500 g who are born to HCMV-seronegative mothers and are infected via receipt of red blood cell transfusions from seropositive donors (Lehner PJ *et al.*, 1997).

1.3.3 CMV Infection in the Immunocompromised Host

Cytomegalovirus is one of the most common and difficult opportunistic pathogens that complicate the care of immunocompromised patients. Infection is common because it can occur (1) by reactivation of latent virus, (2) by reinfection in patients who have had past infection, and (3) by primary infection. Procedures that are part of the patient's care, such as transfusions or organ transplant, can transmit HCMV at a time when the patient is maximally immunosuppressed. HCMV infection in the immunocompromised patient is difficult because diagnosis requires not only detection of the virus but also determining whether HCMV is causing disease: HCMV shedding and viremia are common in patients with impaired cellular immunity even when disease due to HCMV is not present (Bernard N *et al.*, 2001). In addition, preventive and therapeutic options are few and not completely effective. The severity of HCMV infection roughly parallels the degree of immunosuppression (Garrigue I *et al.*, 2008; Soderberg-Naucler C, 2008).

The most severe infections are seen in recipients of allogeneic bone marrow transplants and in patients with acquired immunodeficiency disease (AIDS) with very low CD4 counts. HCMV disease is also seen in solid organ transplant patients, in patients receiving immunosuppressive chemotherapy for cancer or collagen vascular disease, and in congenital immunodeficiencies. Infection is often clinically silent even in the immunocompromised, and when the disease occurs, its severity ranges from a brief, self-limited febrile illness to multisystem disease that can be life-threatening or debilitating.

1.3.4 CMV Infection and HIV/AIDS

Cytomegalovirus infection is important in patients with HIV infection because it is arguably the leading opportunistic infection among adults with advanced AIDS. Some investigators have found that HCMV infection is associated with clinical progression of HIV infection. Almost all adults and approximately 50% of children with HIV infection have serologic evidence of HCMV infection (Mussi-Pinhata MM *et al.* 1998).

1.4 HCMV Structure

The virion of HCMV consists of an icosahedral capsid encasing a linear genome, surrounded by a tegument or matrix (Wright HT *et al.*, 1964; McGavran MH and Smith MG, 1965) and enveloped in a lipid bilayer carrying a large number of virus-encoded glycoprotein's (Spaete RR *et al.*, 1994; Britt WJ and Mach M, 1996). By cryoelectron microscopy, the capsid appears similar to, though larger than, the herpes simplex virus (HSV)-1 capsid (Chen DH *et al.*, 1999). In thin sections of infected cells or by negative staining, the HCMV envelope appears more pleiomorphic than the envelope of other herpes viruses (Wright HT *et al.*, 1964), allowing intact HCMV virions to be distinguished from other herpes viruses based on morphology. The genomes of the characterized HCMVs are all linear DNA molecules ranging in size from 200 to 240 kbp, which is significantly larger than those of other herpes viruses.

HCMV infected cells generate three different types of particles including:

- Infectious mature virions.
- Non infectious enveloped particles (NIEPs), which is composed of the same viral proteins but lack viral DNA.

- Dense bodies, which are uniquely characteristic of HCMV infection and are non-replicating, fusion-component enveloped particles, composed primarily of the tegument protein pp65.

The quantities of these different HCMV particles are dependent on the viral strain and the multiplicity of infection.

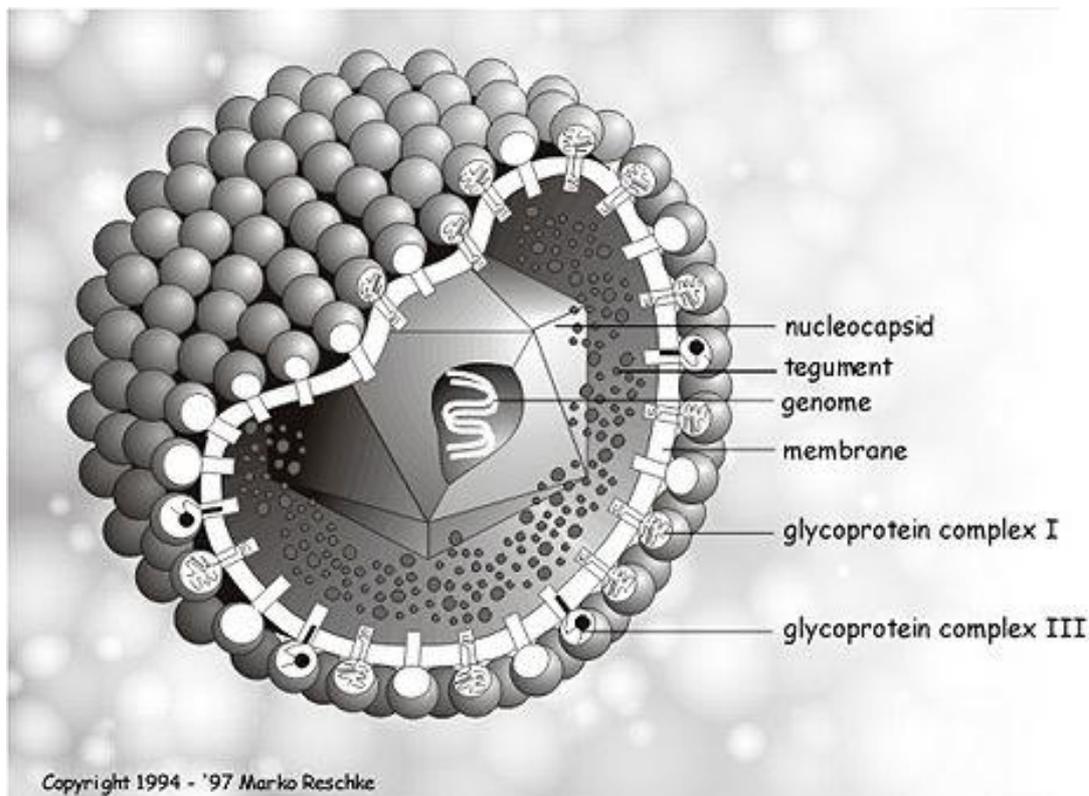


Figure 1.1: The Structure of HCMV virus.

1.5 HCMV Virion Proteins

Purified virus particles of HCMV have been estimated to contain 30 to 40 polypeptides with molecular weights ranging in size from 20 to over 300 KD (Gibson W, 1983; Baldick CJ Jr and Shenk T, 1996).

1.5.1 Capsid Proteins

The capsid is composed of seven proteins:

- 1- Major capsid protein (MCP) is encoded by *UL86* and forms both hexons and pentons
- 2- Minor capsid protein (mCP) encoded by *UL85*
- 3- Minor capsid binding protein (mC-BP) encoded by *UL46*
- 4- Smallest capsid protein (SCP) encoded by *UL48.5* (also called *UL48/49*)
- 5- Three distinct assemblin/assembly protein (AP)-related proteins encoded by *UL80*, *UL80a*, and *UL80.5* associate with capsids (Baldick CJ Jr, Shenk T, 1996; Gibson W, 1996; Gibson W *et al.*, 1996; Chen DH *et al.*, 1999; Trus BL *et al.*, 1999). Also the assemblin/assembly play major roles as scaffolding proteins, which have critical roles in capsid assembly. The primary scaffolding components of cytomegalovirus, is the assembly protein precursor (pAP, pUL80.5) and the maturational protease precursor (pPR, pUL80a), which contains two nuclear localization sequences (NLS1 and NLS2), at least one of which is required in co expression experiments to translocate the major capsid protein (MCP, pUL85) into the nucleus (Nguyen NL *et al.*, 2008).

1.5.2 Envelope Glycoproteins

The envelope of HCMV contains viral proteins embedded in a host lipid bilayer and is derived from intracellular membranes. The acquisition of the envelope and its associated glycoproteins occurs at both nuclear and cytoplasmic sites. Envelope glycoproteins are important targets of the host antibody-mediated immune clearance mechanisms, and many reviews have been written

about the synthesis, mapping, function, and immunogenicity of the more prominent envelope glycoprotein's (Mocarski ES, 1993; Britt WJ and Mach M, 1996). Early studies revealed approximately eight major glycoprotein species in the human CMV envelope (Gibson W, 1983).

1.5.2.1 gB glycoprotein (gpUL55)

Being recognized as the major envelope constituent, and as the most highly conserved glycoprotein in mammalian and avian herpes viruses, gB is a type I integral membrane protein that influences virus binding to and entry into cells, cell-to-cell transmission, fusion of adjacent cells, and targeting of progeny virus to apical membranes for release from polarized cells (Compton T *et al.*, 1993; Navarro D *et al.*, 1993; Bold S, *et al.*, 1996; Tugizov S *et al.*, 1996; Tugizov S *et al.*, 1998; Pietropaolo R and Compton T, 1999; Vanarsdall AL *et al.*, 2008). Glycoprotein B is the major heparin sulfate proteoglycan-binding glycoprotein, and it binds to a 30- to 36-kd cellular protein that is a suspected receptor (Adlish JD *et al.*, 1990; Taylor HP and Cooper NR, 1990).

Glycoprotein B is found on all membranes of infected cells and becomes incorporated into virions as they undergo envelopment at different sites (Sanchez V *et al.*, 2000). Because it is abundant, highly conserved, and the most immunogenic and best-studied envelope glycoprotein, gB has been the principle candidate of subunit vaccine initiatives (Spaete RR *et al.*, 1988; Adler SP *et al.*, 1999; Pass RF *et al.*, 1999).

1.5.2.2 gH (gpUL75)

HCMV gH, or gpUL75, is a 742- to 743-amino-acid envelope glycoprotein (Pachl C *et al.*, 1989), that forms part of a second, relatively abundant glycoprotein complex to which

neutralizing antibodies may be directed (Rasmussen LE *et al.*, 1984). The gH complexes with at least one other herpesvirus-common glycoprotein, denoted gL, facilitates transport to the cell surface (Kaye JF *et al.*, 1992; Spaete RR *et al.*, 1993).

1.5.2.3 gL (UL115)

HCMV gL is encoded by *UL115* and is a 278 aminoacid (32-kd) glycoprotein modified by N-linked and possibly O-linked carbohydrates. Cell-cell fusion is frequently involved in the majority of cells. gB and gH/gL were both necessary and sufficient for fusion, whereas no fusion occurred when either glycoprotein was omitted (Vanarsdall AL *et al.*, 2008).

1.5.2.4 UL16 glycoprotein

The HCMV *UL16* gene encodes a glycoprotein that interferes with the immune response to virus-infected cell. In *vitro*, UL16 interacts with MICB (MHC Class I-Related Chains) and ULBPs (UL16-Binding Proteins). These proteins are ligands for the stimulatory receptor NKG2D, expressed on NK cells and CD8⁺T cells. UL16 expression has been shown to promote intracellular accumulation of MICB, ULBP1 and thus, interferes with the immune response to HCMV-infected cells (Valés-Gómez M *et al.*, 2006).

Several additional viral glycoproteins are likely to be minor envelope constituents but are not apparently associated with any major glycoprotein complexes.

1.5.3 Tegument Proteins

At least 25 proteins are located in the tegument layer between the virion capsid and envelope. The products of 11 open read frames (ORFs) (UL25, UL26, UL32, UL47, UL48, UL48.5, UL82,

UL83, UL85, UL88, UL99) have been detected when virion/dense body polypeptides are electrophoretically separated. All these proteins appear to be both phosphorylated (Roby C and Gibson W, 1986) and highly immunogenic (Landini MP, 1992). These ORFs are all conserved in the betaherpesviruses (Nicholas J, 1996; Rawlinson WD *et al.*, 1996; Vink C *et al.*, 2000).

1.5.3.1 pp65 and pp150

Two tegument proteins, pp150 (ppUL32 or basic phosphoprotein), a 1,048-amino-acid (150 to 155Kd) protein, (ppUL83 or lower matrix protein), a 561-amino-acid (65 to 68Kd) protein, are the most abundant proteins made during replication.

pp65 is the major tegument protein, and the target antigen in antigenemia assays used for rapid diagnosis of HCMV clinical infection. The large amount of pp65 produced by laboratory strains of virus is associated with abundant dense body production. Although all tegument proteins are phosphorylated, pp65 is a major phosphate acceptor in infected cells. As well it is the primary target for phosphorylation *in vitro* by the virion-associated protein kinase (Somogyi T *et al.*, 1990). The analysis by mass spectrometry demonstrated that the HCMV tegument aggregates are formed principally of the tegument proteins pp65 and ppUL25 but also contained additional virion structural proteins including the major capsid protein (Prichard MN *et al.*, 2005).

Immunoblotting experiments confirmed that the formation of the tegument aggregates appeared to be dependent on pp65, since it was not induced in cells infected with a recombinant virus with this open reading frame deleted (Prichard MN *et al.*, 2005).

The other major tegument phosphoprotein, pp150, is the product of UL32 (Jahn G, *et al.*, 1987). It is incorporated preferentially into virions rather than dense bodies. It is estimated to make up 20% of the virion mass (Benko DM *et al.*, 1988). pp150 is a prominent betaherpesvirus-

conserved virion tegument protein, that accumulates within a cytoplasmic inclusion adjacent to the nucleus at late times during infection (AuCoin D P *et al.*, 2006).

1.5.3.2 UL97 kinase

This is a protein kinase encoded by HCMV. It is an important target for antiviral drugs (Kamil JP and Coen DM, 2007). Studies of HCMV UL97 kinase deletion mutant (Δ UL97) indicated a multi-step role for this kinase in early and late phases of the viral life cycle, namely, in DNA replication, capsid maturation and nuclear egress (Wolf *et al.*, 2001). The absence of UL97 kinase activity results in a modified subcellular distribution of the viral structural protein assembly sites, from compact structures impacting upon the nucleus to diffuse perinuclear structures punctuated by large vacuoles (Azzeh M *et al.*, 2006). Also UL97 protein has activities similar to cellular cyclin-cyclin-dependent kinase (CDK) complexes (Sanchez *et al.* 2004; Hume AJ *et al.*, 2008). UL97 phosphorylate and inactivate the retinoblastoma tumor suppressor, stimulated cell cycle progression in mammalian cells, and rescued proliferation of *Saccharomyces cerevisiae* lacking CDK activity (Hume AJ *et al.*, 2008). UL97 is not inhibited by the CDK inhibitor p21 and lacks amino acid residues conserved in the CDKs that permit the attenuation of the kinase activity (Hamirally S *et al.*, 2009). Thus, UL97 represents a functional ortholog of cellular CDKs that is independent of normal CDK control mechanisms (Hume AJ *et al.*, 2008). UL97 kinase plays an important role in the acquisition of tegument during virion morphogenesis in the nucleus. This activity represents an important step in the production of mature virus particles (Prichard MN *et al.*, 2005).

Recombinant HCMV that does not express UL97 kinase activity exhibit a distinctive plaque morphology characterized by the formation of highly refractile bodies late in infection. These

structures were also observed in infected cells treated with the UL97 kinase inhibitor maribavir (Prichard MN *et al.*, 2005).

Both UL97 kinase activity and the LxCxE RB (retinoblastoma pocket proteins) binding motif are required for the phosphorylation and stabilization of RB in infected cells. This effect can be antagonized by the antiviral drug maribavir (Prichard MN *et al.*, 2008).

1.5.3.3 pp28 (ppUL99)

The (HCMV) UL99 open reading frame encodes a 190aminoacid myristoylated and phosphorylated tegument protein, pp28, that is. pp28 is essential for the assembly of infectious virus. Nonenveloped virions accumulate in the cytoplasm of cells infected with recombinant viruses with a UL99 deletion. pp28 is localized to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) in transfected cells, while in infected cells, it is localized together with other virion proteins in a juxtannuclear compartment termed the assembly compartment (AC) (Seo J Y and Britt W J, 2006).

1.5.3.4 Other HCMV tegument proteins

The tegument also contains a number of functionally uncharacterized proteins. A virion associated protein implicated in genome encapsidation is pp130 (ppUL56) (Bogner E *et al.*, 1993; Bradshaw PA *et al.*, 1994). Based on drug resistance studies, it was proposed that pp130 may complex with the CMV terminase (ppUL89) to package viral DNA during virion maturation (Krosky PM *et al.*, 1998). One additional protein, pp67, which includes some UL65 sequence (Davis MG and Huang ES, 1985), has not been consistently observed in characterized virus strains. The association of other tegument proteins encoded by UL25, UL26, UL47, and UL88

products with the tegument has been determined by sequencing proteins directly isolated from virus particles.

1.5.4 The Interaction Between pp65 and UL97

A recombinant HCMV, NTAP97, which expresses a tandem affinity purification (TAP) tag at the amino terminus of UL97, was used to obtain UL97 protein complexes from infected cells. pp65, the 65-kDa virion tegument phosphoprotein, specifically co purified with UL97 during TAP, as shown by mass spectrometry and Western blot analyses (Kamil JP and Coen DM, 2007). Reciprocal coimmunoprecipitation experiments using lysates of infected cells also indicated an interaction between UL97 and pp65. Moreover, in a glutathione S-transferase (GST) pull-down experiment, purified GST-pp65 fusion protein specifically bound *in vitro*-translated UL97, suggesting that UL97 and pp65 do not require other viral proteins to form a complex (Kamil JP and Coen DM, 2007). Notably, pp65 has been previously reported to form unusual aggregates during viral replication when UL97 is pharmacologically inhibited or genetically ablated. A pp65 deletion mutant (Δ pp65) was observed to exhibit modest resistance to a UL97 inhibitor (Prichard MN *et al.*, 2005).

A stable protein-protein interaction between pp65 and UL97 may be relevant to the incorporation of these proteins into HCMV particles during virion morphogenesis. This might have potential implications for immunomodulation by HCMV, and may also be a mechanism by which UL97 is negatively regulated during HCMV replication (Kamil JP and Coen DM, 2007). Morphologically similar aggregates could be reproduced in nuclei of uninfected cells by over expressing pp65. Their formation was prevented by co-expressing the UL97 kinase. Inhibition

of UL97 kinase activity with maribavir or mutation of an essential amino acid in the kinase abolished its ability to prevent this aggregate formation (Prichard MN *et al.*, 2005).

1.6 The interaction between HCMV and cellular kinases

HCMV "ensures" that the cellular machinery efficiently supports viral reproduction by reprogramming the cellular factors, such as regulatory protein kinases towards virus-specific regulatory pathways (Fortunato EA *et al.*, 2000; Bain M and Sinclair J, 2007). Few examples of interaction between HCMV and cellular kinases are documented. HCMV infection stimulates PI(3)K (Phosphatidylinositol 3-Kinase) activities, and this important for HCMV to usurp PI(3)K signal transduction pathways to induce the unique polarization of HCMV-infected monocytes needed for the earliest steps in the viral dissemination and persistence strategy (Chan G *et al.*, 2009). Also the same with protein kinase C, when endothelial cells were treated prior to infection with phorbol myristoyl acetate, an activator of protein kinase C, the number of HCMV-positive cells increased two to three times. On the other hand, pretreatment of the cells with RO 31-8220, a specific protein kinase C inhibitor, or with staurosporine, a general protein kinase inhibitor, resulted in a decreased infection level (Slobbe-van Drunen ME *et al.*, 1997). The activities of CDKs 1, 2, 7 and 9 are crucial for HCMV replication (Bresnahan W A *et al.*, 1997; Chen Z *et al.*, 2001; Sanchez V *et al.*, 2001; Sanchez V *et al.*, 2003; Sanchez V and Spector DH, 2006; Kapasi AJ and Spector DH, 2008; Hamirally S *et al.*, 2009; Rechter S *et al.*, 2009). In the course of infection, they phosphorylate the tegument protein pUL69 (Rechter S *et al.*, 2009) and mediate phosphorylation of the tegument protein pp65 (Sanchez V *et al.*, 2007). On the other hand, the tyrosine kinase affects virus/cell fusion (Keay S and Baldwin BR, 1996). HCMV

utilize the PI3 kinase pathway to inhibit apoptosis (Yu Y, and Alwine JC, 2002) and to induce viral replication (Johnson RA *et al.*, 2001).

1.7 General Considerations in HCMV Growth and Lifecycle

Human CMV exhibits a highly restricted host range in cell culture. Primary differentiated human fibroblasts (HFs) from skin or lung show the greatest susceptibility to viral infection. During natural infection, fibroblastic, epithelial, macrophage, smooth muscle, and endothelial cell types support productive replication (Sinzger C *et al.*, 1995). Although HFs are commonly used to isolate and propagate HCMV in culture, studies have clearly shown that freshly isolated HCMV replicates preferentially on endothelial cell types (Waldman WJ *et al.*, 1991; Woodroffe SB *et al.*, 1997; Sinzger C *et al.*, 1999; Riegler S *et al.*, 2000). Undifferentiated, transformed, or aneuploid human cells, including almost all cell lines, are nonpermissive (LaFemina RL and Hayward GS, 1988). The host cell restriction to HCMV replication observed in nonpermissive cells is the result of a postpenetration blockage of viral gene expression and not to a failure to enter cells; virus has never been adapted to replicate in any completely nonpermissive cell type. HCMV replication in an intestinal epithelial cell line (CaCo-2) has been associated with this host cell's undifferentiated state (Esclatine A *et al.*, 2000). Certain astrocytoma cell lines, such as U373MG, although fully permissive (Duclos H *et al.*, 1989), do not support viral replication to the high levels observed with fibroblasts. HFs that have been immortalized with the human papillomavirus type 16 E6/E7 genes are just as permissive as primary HFs (Compton T, 1993; Greaves RF *et al.*, 1995), and such cells have proved to be invaluable as a basis for the construction of complementing cell lines for the isolation of viral mutants (Mocarski ES *et al.*, 1996; Greaves RF and Mocarski ES, 1998).

Attachment and penetration at the cell surface (Compton T *et al.*, 1992) is rapid and efficient in permissive as well as in nonpermissive cell types, suggesting that cellular receptors for CMV are widely distributed. Addition of exogenous heparin, or treatment of cells with heparinase, blocks viral attachment and implicates the major role of cell surface proteoglycan heparan sulfate in the initial interaction between virus and cell (Kimpton CP *et al.*, 1989; Neyts J *et al.*, 1992), as is the case for other herpes viruses. Initial interaction with heparan sulfate is followed quickly by heparin-independent adsorption and penetration steps that employ widely distributed but poorly characterized receptors (Adlish JD *et al.*, 1990). The efficiency of penetration depends on the presence of these receptors (Nowlin DM *et al.*, 1991; Sinzger C *et al.*, 2008). Initial interest in a 30- to 36-kd gB-binding cell surface protein that appeared to be annexin II, has been tempered by the observation that annexin II does not have to be present on cells for entry. Interest in a gH-binding phosphoprotein which may be important in penetration (Keay S and Baldwin B, 1991), has led to the partial cloning of a CMV fusion A receptor (CMVFR) (Baldwin BR *et al.*, 2000; Compton T, 2004) although its full characterization remains to be completed.

A number of mechanisms for entry have been proposed for cellular and viral functions that may influence the efficiency of entry into cells. Heparan sulfate proteoglycan, annexin II, and CD13 (amino peptidase N) are reported to be associated with HCMV entry into human fibroblast cell. Also epidermal growth factor receptor (EGFR) serves as a receptor for HCMV (Compton T, 2004).

Attachment to the cell surface is followed by penetration, mediated by fusion of the virion envelope and the cell surface in a pH-independent manner, a process that probably involves the gH/gL complex (Vanarsdall AL *et al.*, 2008). Viral nucleocapsids rapidly make their way to the nucleus. The tegument protein ppUL83 can be detected within 20 to 30 minutes following

exposure to a virus inoculum, and expression of *ie1/ie2* gene products follows shortly thereafter (Dal Monte P *et al.*, 1996). Several studies have shown that CMV (or CMV glycoprotein) binding to cells is sufficient to initiate a signaling cascade even in the absence of viral penetration (Boldogh I *et al.*, 1991; Yurochko AD *et al.*, 1997; Zhu H *et al.*, 1997; Boyle KA, *et al.*, 1999; Nguyen NL *et al.*, 2008). Viral attachment may therefore stimulate specific cellular processes that facilitate viral replication, or they might be part of some other host cell response to viral infection. Low-speed centrifugation (centrifugal enhancement) increases the efficiency of adsorption and penetration of murine or human CMV (Woods GL *et al.*, 1987; Agha SA *et al.*, 1988) by stabilizing weak interactions or encouraging fusion. Productive replication in permissive HF cells follows a pattern of coordinate expression with viral genes assigned to different kinetic classes depending on time of expression and sensitivity to inhibition of protein synthesis or viral DNA synthesis.

Although the replication cycle of HCMV is slow, requiring 48 to 72 hours to begin the release of progeny (Stinski MF, 1983), virion tegument proteins influence this earliest phase of infection, and viral functions expressed early play regulatory roles later in infection (Kamil JP and Coen DM, 2007). The switch from early phase to late phase is delayed until 24 to 36 hours post infection (hpi), with maximal levels of virus release starting 72 to 96 hpi. Once maturation starts, infected fibroblasts continue to produce the virus at peak levels for several days.

During productive infection in HF cells, a gradual increase in HCMV DNA synthesis is observed starting as early as 14 to 16 hpi, and is accompanied by a stimulation of host cell metabolism. Viral replication and maturation parallel accumulation of viral DNA replication functions, features suggesting that the prolonged replication cycle of this virus may result from this slow accumulation of essential gene products. HCMV infection does not inhibit general cellular

metabolism but rather stimulates both RNA and protein synthesis (Bain M and Sinclair J, 2007; Sanchez V and Spector DH, 2008).

CHAPTER 2

2. Materials and Methods

2.1 Cell line and Cell Culture

Human foreskin fibroblasts (HF) are a primary cell line isolated from new born male's foreskin. Passage 7 of HF cells was kindly provided by Dr. Nina Mayorek, Hebrew University and cultured in culture flasks (Easy Flask, 25 cm², 75 cm², 175 cm², Nunc, Denmark) in complete DMEM medium. Complete DMEM medium contains DMEM (01-055-1A, Beit Haemek), 100 units/ml Penicillin+100µg/ml Streptomycin mixture (03-031-5C, Beit Haemek), 20mM L-Glutamine (03-020-1A, Beit Haemek) and 10% heat inactivated serum (1:1 mixture of New born bovine serum, 04-121-1A and Fetal bovine serum, 04-122-1A, Beit Haemek). Cells were cultivated in 96% humid 5% CO₂ incubator at 37°C (Hera cell incubator).

2.1.1 Propagation and Passage of HF Cells

HF cells were propagated by splitting into 2 or more culture flasks, or into different well plates. For this, culturing complete medium was removed from confluent cells in flask and replaced with 0.25% Trypsin-EDTA (03-052-1A, Beit Haemek). 1 ml was used for trypsinization of confluent cells grown on 25 cm² flask, 2 ml for confluent cells on 75 cm² flask and 4 ml for confluent cells 175 cm² flask respectively. After 2 minutes incubation, complete medium was added to the non adhering trypsinized cells, gently mixed and distributed to the new flasks. The new cells are one passage higher than the one they were split from. Since HF is a primary cell line, it was propagated up to passage 18 in our lab. Experiments were performed on passages 10-18.

2.1.2 Freezing and Thawing of HF Cells

Cells can be kept for years if frozen in liquid nitrogen. HF cells were frozen at low passages (6-12). Freezing medium (50% complete medium, 40% serum, 10% DMSO, dimethyl sulphoxide, Sigma, D2650) is required to keep the cells well maintained while freezing and able to grow once thawed. Freezing proceeded as followed:

For a medium flask, 75cm²

- 1- The medium was removed
- 2- 2 ml 0.25% Trypsin was added
- 3- 6 ml complete medium was added to the detached cells in Trypsin and mixed well
- 4- Mixture was equally distributed into 2x 15mL tubes
- 5- Tubes were centrifuged at 1500 rpm for 10 min
- 6- The supernatant was discarded carefully
- 7- Pellet in each tube was gently resuspended in 1.5 ml freezing medium
- 8- Resuspended cells were transferred into cryotube (Simport, Canada)
- 9- Cells in cryotubes were first frozen at -70°C and transferred a day later to liquid nitrogen (slow freezing).

For thawing, cells in cryotubes were thawed rapidly and transferred to 25 cm² culture flasks containing 5ml complete media. After 24 hours, complete media was replaced with new complete media to remove DMSO residuals. Cells are usually ready to split and passage 48 hours after thawing.

2.2 Viruses

HCMV strain AD169 (wt-HCMV; American Type Culture Collection) and pp65 deletion mutant (Δ pp65) were a kind gift from Prof. Bodo Plachter (Institute of Virology, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany). The UL97 deletion mutant, Δ UL97, was kindly provided by Dr. Mark Prichard (Dept of Pediatrics, University of Alabama school of Medicine, Birmingham, Alabama, USA).

2.2.1 Propagation of Viruses

Viruses were propagated in human foreskin fibroblast (HF) culture. Initially HF was infected with AD169, Δ pp65 or Δ UL97 at low multiplicity of infection (moi). moi represents the number of viruses divided by the number of cells. In this work viruses were propagated in 175 cm² flasks (3×10^7 HF cells). 4 ml viral suspension (virus stock diluted in complete medium) at moi of 0.001 were added to the cells and incubated in CO₂ incubator. 2 h after infection, viral suspension was removed and replaced by 30 ml complete medium. The infection period at this stage is referred to as post infection (pi). At 10 days pi, viruses were harvested by collecting the 30 ml supernatant in a sterile 50 ml tube and replaced with fresh 30 ml complete medium for further incubation. Viral harvest was centrifuged for 10 min at 1500 rpm to get rid of cell debris. The clear supernatant was transferred to a new 50 ml tube, 1% DMSO was added, the mixture was finally aliquoted in cryotubes and frozen at -70°C. At 14 days pi, a viral re-harvest is performed as above and the highly infected cells were discarded.

2.2.2 Viral Titration and Plaque Assay

Plaque assay technique was employed to determine the viral titer (viral count/ml). An aliquot of frozen viral harvest (see 2.2.1) was thawed and viral dilutions of 10^{-1} - 10^{-8} (titrations) were prepared step wise (1:10 dilution steps) in complete medium. 90-100% confluent HF cells cultured in 24 well plates (Nunc, Denmark) were infected with 0.2 ml of either the undiluted viral stock, or one of the 10^{-1} - 10^{-8} viral dilutions. The dilutions depend on the virus; wild type virus AD169 demands higher dilutions than deletion mutants. Four wells in each column of the 24 well plate were infected with the same viral dilution, so that 4 controls of each dilution were guaranteed.

Meanwhile, 2.5% agarose was dissolved in sterile dH₂O (distilled water) and cooled down to 50°C. Complete medium was also pre-warmed up to 50°C. 2h after infection, viral media were removed from each of the 4 wells in one column and replaced with 1 ml 0.25% agarose containing complete medium (2.5% agarose diluted in the pre-warmed complete medium). The same is repeated for the rest of the wells in each column. This procedure has to be done pretty fast to avoid polymerization of the agarose. Finally, plates are left at room temperature (RT) for 30 min to allow agarose to polymerize and then transferred to the cell culture incubator. One week later, another 1 ml agarose-containing medium is added following the procedure above. The agarose in the medium should hinder virus progeny from spreading to neighboring cells and allows counting the plaques in the correct dilutions resulting from the infection independently.

At 2 weeks pi, viral plaques are counted in those wells with those viral dilutions, which allow counting separate plaques. Those wells, where viral dilutions used allow counting of 1-20 plaques are usually chosen to determine viral titer.

The viral titer, also referred to as plaque forming unit (pfu) was calculated as followed:

No. of plaques X Dilution factor X 5 = viruses/ml

Factor 5 is to calculate the amount /ml, since only 0.2 ml viruses were added to each well, multiplication by 5 gives the amount per 1ml.

i.e. 10 plaques were counted in dilution 10^{-5} , then there are $10 \times 10^5 \times 5 = 5 \times 10^6$ viruses/ml

2.3 Immunofluorescence (IF)

Cells grown on 8-well glass slides (Labtek chamber slide w/cover, #177402, Nalge Nunc International, Naperville, IL 60563) were infected at a (moi) of 0.5 pfu/cell. Supernatant was removed at 96h pi (unless otherwise indicated). Cells were washed three times with 1X PBS (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 , 0.24g KH_2PO_4 in 1000 ml dH_2O , pH 7.4) and fixed with 3.7% paraformaldehyde (diluted in PBS) for 30 min at RT. After three washes with PBS, cells were permeablized with 0.1% Triton X-100 (diluted in PBS) for 1-2 min at RT. Cells were then washed for five times with PBS and blocked with 1% bovine serum albumin (BSA, 0175, AMRESCO Inc., USA, prepared in PBS) for 60 min at RT. First antibody (see 2.3.1) was diluted in 0.5% BSA and incubated with cells for 2 hrs at RT or over night at 4°C . Cells were washed 5 times with PBS. The last wash was left on cells for 30 min. The secondary antibodies including the Golgi marker (see 2.3.1) were diluted at a proper concentration in 0.5% BSA and incubated with the cells 30-60 min in the dark by covering the plates with aluminum paper or by placing them in a dark room. Before the last wash, the nucleus was stained with $1\mu\text{g/ml}$ 4', 6-diamidino-2-phenylindole, 2HCl (DAPI, 268298, Calbiochem, Germany) for 10 min at RT. Cells were washed 5 times with PBS. Finally, cells were covered with mounting anti-fading solution (0.5% g n-propyl gallate; P3130, Sigma, Germany; 100mM Tris pH 9 , 70% glycerol). The wells were carefully peeled off, covered with a cover slip (24X50 mm, Menzel-Gläser) and gently pressed to

get rid of excessive mounting solution. The edges of both surfaces of the slide and cover slip 4 were carefully glued with colorless nail polish.

If co-localization IF is to be performed, first antibodies, secondary antibodies or secondary antibody with Golgi marker were added as a mixture to the cells.

2.3.1 Monoclonal, and Polyclonal Antibodies and Fluorescing Markers

Variable monoclonal and polyclonal antibodies were used in this work to perform the immunofluorescence (IF), Western blot (WB) and immunoprecipitation (IP) assays. Primary mouse monoclonal antibodies (mAbs) against HCMV pp28 (CA004-100), pp65 (CA003-100), and glycoprotein B (gB, CA005-100) were purchased from Virusys Corporation (Sykesville, MD, USA). The polyclonal antibody UL16 (pAb UL16) was kindly provided by Mar Valez, Dept of Pathology, University of Cambridge, UK. Secondary antibodies, Goat anti Rabbit-Cy2 (111-225-144), Goat anti Rabbit-Cy5 (111-175-144), Goat anti-Rabbit-HRP (111-035-003), Donkey anti-Mouse-Cy2 (715-225-150), Donkey anti-Mouse-Cy5 (715-175-150) were purchased from Jackson Immunoresearch (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Goat anti-Mouse-HRP was kindly provided by Prof. Moshe Kotler, Dept of Pathology, Hebrew University, Jerusalem. All secondary antibodies are highly cross-absorbed. Golgi marker Wheat germ agglutinin (WGA, L4895) coupled to fluorescein isothiocyanate (FITC) was obtained from Sigma (St Louis, Missouri, USA). All antibodies were reconstituted according to supplier, aliquoted in 50µl volumes in eppendorf tubes and frozen at -70°C. For current use, antibodies were kept in -20°C.

2.3.2 Visualizing staining and processing images of IF experiments

Olympus BX60 IF microscope was used for all IF experiments. U-MWIB2 mirror unit was used to visualize Cy2 staining, U-MWU2 for DAPI and U-MWIY2 for Cy5 staining respectively. Cell images were always captured through the 100X lens with Olympus DP71 camera (unless otherwise indicated) and “analySIS LS report” software. Precise single cell images were captured using the extra focus tool (FS) on the BX60 microscope. Images were processed and merged using “Adobe Photoshop” program.

2.4 Drug Inhibition Assay

To investigate the influence of drugs (i.e. cellular kinase inhibitors or activators) on HCMV life cycle, they were added to HCMV infected cells for a 96 hpi. In this work drug inhibition assay was performed to detect structural changes in HCMV infected cells caused by the drug using the IF technique. For this, cells grown on chamber slides (see 2.3) were infected with HCMV (AD169, Δ UL97 or Δ pp65). Two hours after infection, complete medium containing drug in appropriate concentration (see 2.4.1) was added to the cells. At 96hpi supernatant was frozen at -70°C to perform plaque assay and cells were subjected to IF as in 2.3.

Frozen supernatants of drug inhibition experiments versus no inhibition were thawed and subjected to plaque assay as in 2.2.2. A diagram was used to demonstrate the influence of a drug on the viral titer. Log 10 of resulted viral titers were calculated and used in a the diagram as the y-axis against the virus type and drug type in the x-axis (see 3.9, page 48). Results shown in diagram were replicable in 3 independent experiments.

2.4.1 Antiviral drugs, Cellular Kinase Inhibitors and Activators

The antiviral drug NGIC-I (MBS481500, Calbiochem, Germany) was reconstituted in DMSO at 5 mM stock solution, aliquoted and frozen at -20°C. NGIC-I was used at 0.5µM final concentration (Azzeh *et al.*, 2006) diluted in complete media. Wortmannin, (W1628, Sigma, Germany) was reconstituted in DMSO at 10mM stock solution and used at 100 nM final concentration (diluted in complete media). Thrombin (Baxter, USA) was delivered as 1000U/ml stock solution and used at 1U/ml (diluted in complete media). Polymyxin B sulfate (50mg/ml solution, 420413, Calbiocmem, Germany) was used at final concentration of 250 µg/ml (diluted in complete media).

2.5-Flotation Assay

Flotation assay was performed in order to study the membrane or raft association of viral proteins investigated in this work. Such biochemical properties are detectable using specific detergents, which are known to float membrane or raft associated proteins. Triton[®] X-100 (TX-100, 93443, Sigma, Germany) for example is known to float raft associated proteins, since these proteins are insoluble in it. On the other hand, CHAPS (C5070, Sigma, Germany), a non-denaturing zwitterionic detergent floats membrane associated proteins, while soluble proteins remain at the bottom of the gradient (Rouvinski *et al.*, 2003, see below for gradient). Floating is connected with the technique used to perform this assay. Cells or infected cells are lysed with a buffer containing this detergent and subjected to density gradient centrifugation, all under cold condition, cellular and viral proteins, which float in the lowest concentration on the top of the gradient are considered raft associated proteins if cells are lysed with TX-100 and membrane associated if cells are lysed with CHAPS (see figure 2.1).

Flotation assay

TX100 Flotation: raft association

CHAPS Flotation: Membrane association

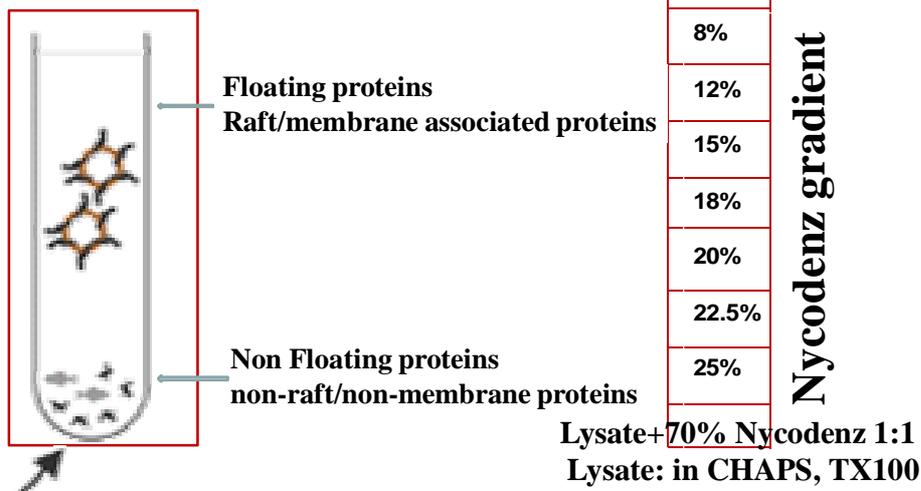


Figure 2.1: Technical Demonstration of Floation Assay

Practically the flotation assay was performed under cold conditions (ice) as followed:

1. Four big flasks (3×10^7 HF cells) were infected with AD169, Δ UL97, Δ pp65 or MOCK at low moi: 0.15 for 2h at 37°C in 5% CO₂ incubator
2. Virus containing medium was replaced with 30 ml fresh complete medium and incubated at 37°C in 5% CO₂ incubator
3. At 6 days pi, medium was removed and cells were washed with cold (4-8 °C) PBS (5 ml each flask), and a last wash for 10 min at 4°C.

4. 1.5 ml PBS was added to each flask and the cells were scratched down with a rubber policeman in 2 pre-cooled sterile eppendorf tube (equal amounts)
 5. Cells in eppendorf tubes were centrifuged for 10 min at 4°C, 3000rpm.
 6. The supernatant was discarded **very carefully** in order not to disturb the cells
 7. The pellets of the same mother flask were resuspended in 1210 µl TNE lysis buffer (25 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA +1% CHAPS or 1% TX-100)
 8. Cells were incubated 30 min at 4°C on ice
 9. Lysates were centrifuged 10 min at 4°C , 3000 rpm
 10. 990 µl of each tube was transferred to a pre-cooled 5 ml Beckman tube
 11. 990 µl 70 % Nycodenz (D2158, Sigma) in TNE was added to each pre-cooled 5 ml Beckman tube and mixed gently to avoid bubbles
 12. The gradient was prepared by stacking the Beckman tubes of step 11 with 440 µl of different cold Nycodenz concentrations (prepared in TNE buffer) starting with the highest; 25% and ending with the lowest; 8%
- Start 25% 22.5% 20% 18% 15% 12% 8% end**
13. The tubes were centrifuged in swing TSL 28 Beckman rotor in 80K Beckman centrifuge for 3 h at 4°C and 55000 rpm without break
 14. 13 eppendorf tubes for each sample were prepared in order to collect the different fractions
 15. 420 µl of each fraction was aliquoted carefully into each tube
 16. The aliquots were frozen at -70°C
 17. Floating proteins were detected using Western blot assay (see 2.6)

2.6-Western Blot (WB)

WB is a method to detect proteins in a sample using mono- or polyclonal antibodies, which binds specifically to this protein/s. In this work BioRad vertical gel electrophoresis system was used (165-3302 Mini-PROTEAN 3 Electrophoresis Module, BioRad, CA, USA).

2.6.1- Preparation of the polyacrylamide Gels

10% Resolving gel for 2 gels:

- 7.9 ml dH₂O
- 6.7 ml 40% Polyacrylamide (PAA, Beit Haemek)
- 5 ml 1.5 M Tris pH 8.8.
- 0.2 ml 10% SDS (in dH₂O)
- 0.2 ml 10% ammonium per sulfate (APS, prepared in dH₂O and frozen until use)
- 8 µl TEMED (*N,N,N',N'*-Tetramethylethylenediamine, T9281, Sigma, Germany)

Solution was loaded bubble free into the casted 1.5 mm casting glass of BioRad set (see above) until 2 cm below the fore glass of the set.

Once polymerized, 5% stacking solution was prepared:

- 1.4 ml dH₂O
- 0.33 ml 40 % PAA
- 0.5 ml 1 M Tris (pH 6.8).
- 0.02 ml 10% SDS
- 0.02 ml 10% APS
- 0.002 ml TEMED

Mixture was added bubble free until the top of the fore glass of the BioRad casting system and 1.5 mm comb was placed between the glasses carefully.

Once polymerized glass plates with gels and combs were casted in BioRad vertical casting system, SDS- Running buffer (25 mM Tris base, 220 mM Glycin, 0.1% SDS) was placed between the plates and outside them in the tank. Combs were removed carefully and slots were inspected and polymerized gel pieces in the slots were removed carefully if found.

2.6.2-Sample Preparation

1- 30 μ l of floatation aliquots (see step 16, 2.5) were aliquoted in new Eppendorf tubes

2- 10 μ l of 4x Lamlli buffer (20mM Tris, pH 6.9, 4% SDS, 40% Glycerol, 0.004% Bromphenol blue+ freshly added 8.4% β -Mercaptoethanol) was added to each eppendorf tube

3- Samples were denaturated at 95° C for 10 min and kept on ice

4- Samples were carefully loaded into slots along with 2 μ l pre-stained protein marker (SM0441 Fermentas, Canada)

5- Proteins were run at 80 V (constant Ampere) for 30 min then at 130 V until the smallest marker's protein (20kDa) reached the bottom of the gel

2.6.3-Blotting:

1- 8.5 X 6.5 cm PVDF membrane (1 per gel) and 8.5 X 6.5 cm Whatmann paper (4 per gel, 580x580mm thick Whatmann)

2- PVDF membrane was activated: 5 min incubation methanol followed by washing in dH₂O and finally well covered in transfer buffer (3g Tris base, 14.4g Glycin, 800ml dH₂O and 200 ml Methanol). Whatmann paper was also well covered with transfer buffer

3- Stacking gel was separated from Resolving gel by cutting

4- Blot sandwich was prepared as following

2whatmann ⇨ Membrane ⇨ Gel ⇨ 2whatmann

5- Blotting 30 min, 17V, constant Ampere in semi dry blotter

6- Membranes were blocked in 1% skim milk for 30 min at RT (shaking) or over night at 4°C

7- Skim milk was replaced by first antibody (see 2.3.2) diluted in PBS and incubated for 2h shaking at RT

8-Membranes were washed 3 times (15 min each) with PBS

9- Secondary antibody (see 2.3.1) was diluted 1:10000 in PBS 30 min at RT shaking

10- Membranes were washed 3 times (15 min each) with PBS, last wash was kept until ECL reaction was performed (see 2.6.4)

2.6.4- ECL Reaction

ECL reaction and developing of the membranes was performed directly at the Legal Medicine center of Al-Quds University. Materials needed below were all carried there to.

1-Two 15 ml tubes (tube A and tube B) were equally filled with 6ml 100mM Tris pH 8.5

2- 3.3 µl 30% H₂O₂ were added to tube A

60 µl Luminol (250mM in DMSO, A8511, Sigma, Germany) + 26.6 µl p-coumaric acid (90mM in DMSO, C9008, Sigma, Germany) were added to tube B

- 3- Solutions in tubes A+B were added simultaneously to the membranes and hand shaken with membranes for 1 min.
- 4- Solutions were poured off
- 5- Membrane was placed in a Nylon file
- 6- In dark room, membranes were covered with X-ray negative film and casted for 30 seconds to 5 min
- 7- Negative was developed. If signals were strong, a shorter incubation of the membrane with negative was performed. If signals were week, a longer incubation was performed and re-developed
- 8-Membranes were sealed in Nylon file and kept in -20°C or directly stripped (see 2.6.5)

2.6.5 Membrane Stripping

If another protein with another antibody need to be detected, membranes were stripped, which is a method used to denaturate already bound antibodies to allow the access and binding of new antibodies. For this, the membrane was treated for 5 min with 300mM NaOH (in dH₂O) shaking, washed 5 times (15 min each) with PBS and re-blocked with 1% skim milk.

2.6.6 Gel Staining

In order to check proteins directly on the gel, it was stained after blotting with coomassie blue staining solution. Gel was destained with destaining buffer until the protein bands were visible.

Coomassie staining solution: 0.25 g Coomassie brilliant blue (R250, Sigma, Germany) dissolved in 90ml methanol: H₂O (1:1 v/v) + 10 ml Glacial acetic acid.

Destaining buffer: methanol: acetic acid 1:1

2.7 Immunoprecipitation (IP)

Some proteins cannot be directly detected in WB and therefore have to be immune-precipitated first before being subjected to WB. This was the case with the UL16 polyclonal antibody (pAb UL16). The following steps were performed to immune-precipitate the UL16 in floatation fractions:

- 1- 1 μ L pAb UL16 + 99 μ L PBS were mixed and added to 50 μ l protein A-coupled Sepharose
- 2- The mixture was rocked over night at 4°C using our self constructed machine in Figure 2.2. This is the step, by which the Ab was coupled to the Sepharose beads
- 3- Mixture was washed 3X with 700 μ l (each time centrifuged at 3000 rpm for 1 min)
- 4- 100 μ l of aliquoted floatation fraction was added to the mixture
- 5- The mixture was rocked for 3h at 4°C
- 6- The mixture was re-washed as in step 3
- 7- 17 μ l of 4x lamlli + β -mercaptoethanol (see 2.6.2) were added to the precipitate and subjected to the denaturation step for 10 min at 95°C
- 8- Supernatant (15 μ l) was subjected to WB as in 2.6.2

CHAPTER 3

3. Results

3.1 Subcellular distribution of UL16 glycoprotein in WT, Δ UL97, and Δ pp65

HF infected cells

HCMV UL16 gene encodes a glycoprotein that interferes with the immune response to the virus-infected cell, UL16 may be distributed to an unexpected localization within the cytoplasm of infected cells to fulfill its function. For this, HF cells grown on 8-well chamber slides were MOCK infected, or infected with either AD169 (wild type), Δ UL97 or Δ pp65 deletion mutants at a MOI of 0.5. At 96 hpi. The cells were stained for UL16 using anti UL16 polyclonal antibody followed by Cy-2 conjugated anti Rabbit and analyzed by immunofluorescence.

As clearly demonstrated in figure 3.1 and 3.2, UL16 distributed similarly to pp28 as compact juxtannuclear "bulb" structure in AD169 and in Δ pp65 infected HF cells. On the other hand, UL16 assumed a diffuse perinuclear, vacuoles' rich, "crown" structure in Δ UL97 deletion mutant infected HF cells (figures 3.1, 3.2). Interestingly, these vacuoles, elsewhere referred to as aggregates (Prichard M *et al.*, 2006), did not stain for UL16, as was the case for pp28 (figure 3.2, Azzeh M *et al.*, 2006).

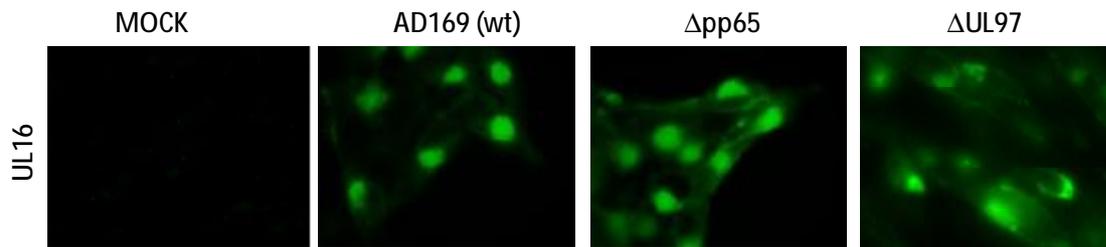


Figure 3.1: Subcellular distribution of UL16 glycoprotein in HCMV infected HF cells.

HF cells were infected with AD169, Δ UL97 or Δ PP65 at moi of 0.5 and treated as described in 2.3. UL16 was stained using pAb UL16 (Rabbit) followed by anti-Rabbit Cy2 secondary antibody (green staining). Images were captured with Olympus DP11 digital camera.

3.2 Correlation between subcellular distribution of UL16 and nuclear structure in HF infected cells

Not only was the subcellular distribution of UL16 very distinguishable in HCMV infected cells, but also the nuclear shape. The particular "bulb" structure of UL16 assumed a compact distribution in the center of "kidney" shaped nucleus in AD169/ Δ PP65 infected HF cells (figure 3.2). Over 70% of nuclei in AD169/ Δ PP65 infected cells assumed the "kidney" shape. This percentage was determined by counting DAPI stained nuclei in 10 different fields (in ~1000 cells) under the IF microscope using the 40X lens. On the other hand, the nuclear shape of Δ UL97 infected cells remained oval as in MOCK infected cells, but was enlarged and punctuated by vacuoles of different sizes, similar to the cytoplasmic ones. Neither the cytoplasmic vacuoles nor the nuclear ones stained for DAPI or UL16 as shown previously for pp28 (Azzeh M *et al.*, 2006). The nuclei of Δ pp65 infected cells did not show any difference to AD169 infected cells.

3.3 Co-localization of UL16 glycoprotein with pp28, pp65, gB, and Golgi apparatus

3.3.1 Co-localization of UL16 glycoprotein with pp28, pp65, gB, and Golgi in HF cells infected with AD169

Here we examined whether the disparate UL16 patterns in cells infected with AD169 might reflect a different organization of cellular organelles using antibodies against variable HCMV proteins and cellular Golgi. Azzeh and Sanchez (Azzeh M *et al.*, 2006; Sanchez V *et al.*, 2000) had shown earlier that different HCMV tegument and glycoproteins co-localize to the juxtannuclear assembly. Here we examined whether UL16 co-localizes to that same site. At 96hpi, HF cells infected with AD169 were stained with UL16 pAb, co-stained with either gB mAb, pp28 mAb, pp65 mAb, or WGA-FITC and counterstained with DAPI. Stained cells were analyzed by immunofluorescence microscopy.

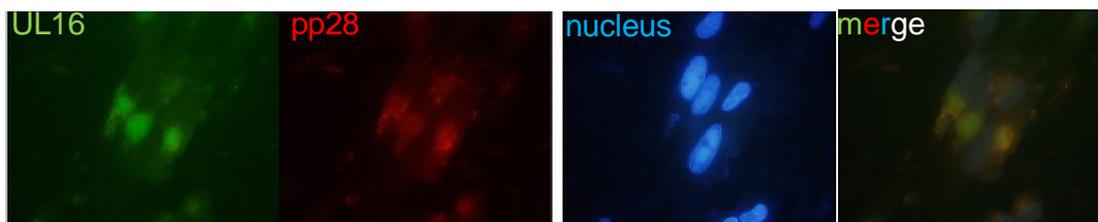


Figure 3.4 -A: Co-localization of UL16 with PP28 in AD169 infected HF cells. HF cells were infected with AD169 at moi 0.5 and subjected to IF as described in 2.3. Cells were then stained with pp28 mAb (mouse) and UL16 pAb (rabbit) followed by anti-mouse Cy5 (red) and anti-Rabbit Cy2 (green). DAPI exerts the blue staining of the nucleus. Images were captured with Olympus DP11.

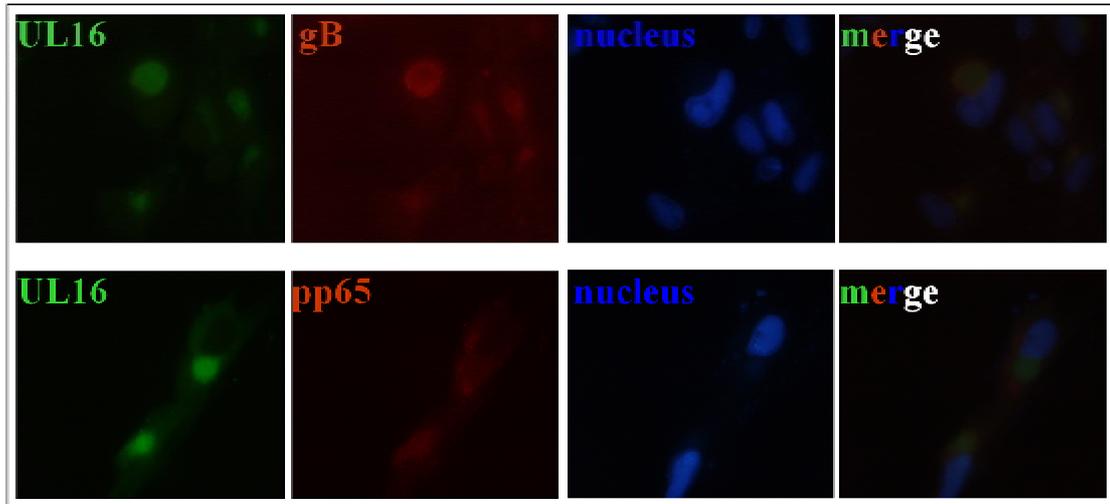


Figure 3.4-B: Colocalization of UL16 with gB or pp65 in AD169 infected HF cells.

Infected HF cells were stained with either gB and UL16 or pp65 and UL16 followed by anti-mouse Cy5 secondary Ab (red staining) and anti-Rabbit Cy2 (UL16 green staining). DAPI exerts the blue staining of the nucleus. Images were captured with Olympus DP11.

Figures 3.4 A and B, demonstrate that UL16 co-localized to 90% with pp28, gB and pp65, which indicates that UL16 is a part of the assembly site and may play a role in building the assembly site.

A previous study demonstrated that UL16 co-localized with ER and TGN markers (Vales-Gomez M, *et al.*, 2005). Here we examined its co-localization with Golgi using a lectin Golgi marker (FITC-conjugated WGA). WGA lectin binds to clustered terminal N-acetylneuraminic acid residues and to N-acetylglucosamine-containing oligosaccharides on proteins (Bhavanandan and Katlic, 1979) and decorates distal Golgi cisternae, the *trans-Golgi* network (Tartakoff and Vassalli, 1983; Virtanen *et al.*, 1980). In uninfected fibroblasts, WGA stained perinuclear structures that were reminiscent of the classical Golgi organization, but strikingly modified by viral infection (Azzeh *et al.*, 2006). First, the WGA staining intensity in AD169 infected cells

was stronger than in uninfected fibroblasts. Most interestingly, the WGA pattern assumed either “bulb” or “crown” shapes in AD169 or Δ UL97 infected cells accordingly with tegument protein pp28 (Azzeh M, *et al.*, 2006).

Our experiments demonstrate that the glycoprotein UL16 distributes similar to subcellular distribution of pp28 and FITC-conjugated WGA, which means that UL16 glycoprotein may play a role in inducing a profound reorganization of WGA-positive structure (figure 3.4-C).

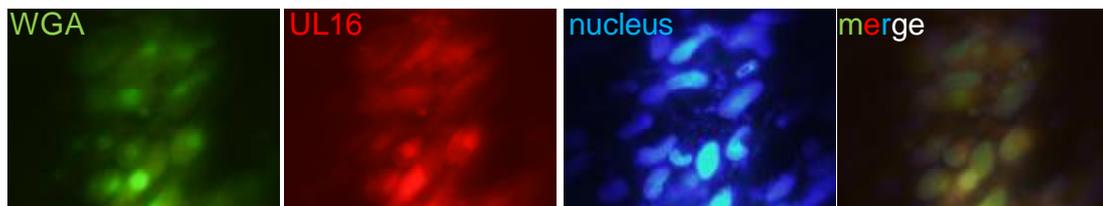


Figure 3.4-C: Co-localization of UL16 with WGA in AD169 infected HF cells. HF cells infected with AD169 were stained with UL16, followed by a mixture of anti-Rabbit Cy5 antibody (red staining of UL16) and FITC-conjugated WGA (green). DAPI exerts the blue staining of the nucleus. Images were captured with Olympus DP11.

3.3.2 Co-localization of UL16 glycoprotein with pp28, WGA in Δ UL97 infected

HF cells

The effect of the deletion mutant of UL97 on UL16 distribution, viral assembly, and the organization of infected cells' organelles was investigated. For this, HF cells infected with Δ UL97 were stained with UL16 pAb and co-stained with either pp28 mAb or WGA-FITC, and analyzed by IF (figure 3.5 A and B).

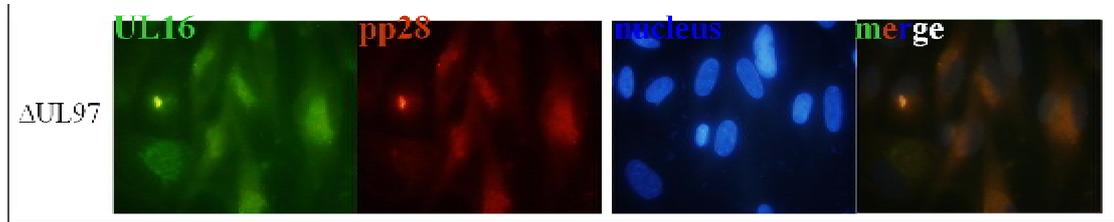


Figure 3.5 A : Colocalization of UL16 glycoprotein with pp28 in HF cells infected with DUL97. HF cells were infected DUL97 at moi of 0.5 and subjected to IF (see 2.3). Cells were co-stained with UL16 pAb and pp28 mAb, followed by the secondary antibodies anti-Rabbit Cy2 (green) and anti-Mouse Cy5 (red). DAPI exerts the blue staining of the nucleus. Images were captured with Olympus DP11.

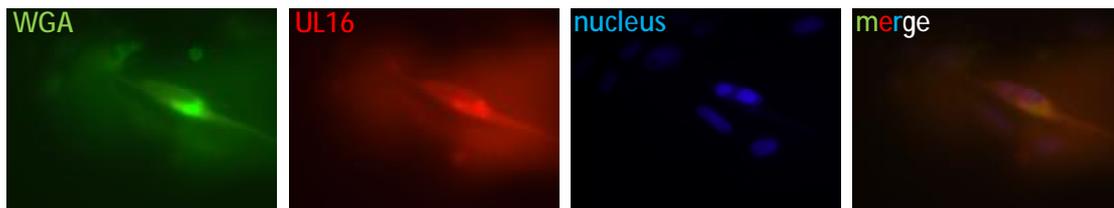


Figure 3.5 B : Colocalization of UL16 glycoprotein with Golgi in HF cells infected with DUL97. HF cells were infected DUL97 at moi of 0.5 and subjected to IF (see 2.3). Cells were stained with UL16 pAb followed by secondary antibody anti-Rabbit Cy5 (red) and WGA-FITC (green). Nucleus was counter-stained with DAPI. DAPI exerts the blue staining of the nucleus. Images were captured with Olympus DP11.

UL16 glycoprotein co-localized to 90% with pp28 and WGA Golgi marker at the assembly site in Δ UL97 cells. These results indicate that the lack of the UL97 kinase does not affect the distribution of UL16 or its co-localization with pp28 or Golgi.

3.3.3 Co-localization of UL16 glycoprotein with pp28 and Golgi in HF cells infected with Δ PP65

The impact of pp65 deletion on subcellular distribution of variable HCMV proteins is not well studied yet. Here we studied the influence of pp65 on UL16, pp28 and Golgi.

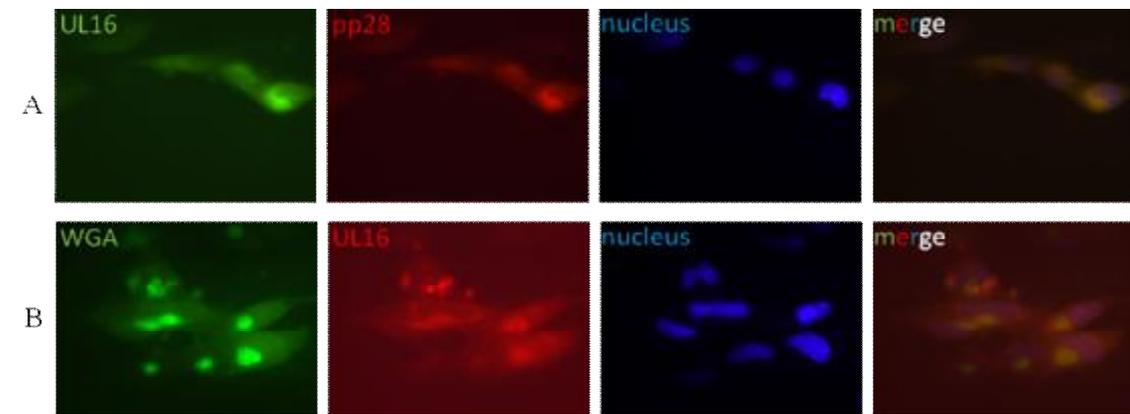


Figure 3.6 A/B : Colocalization of UL16 glycoprotein with pp28/Golgi in HF cells infected with Dpp65. HF cells were infected DUL97 at moi of 0.5 and subjected to IF (3.2). Cells were co-stained with UL16 pAb and pp28 mAb in A and only with UL16 pAb in B. Secondary antibodies anti-Rabbit Cy2 (green) and anti-Mouse Cy5 (red) were added in A, anti-Rabbit Cy5 (red) and WGA-FITC (green) in B. Nucleus DAPI exerts the blue staining of the nucleus. Images were captured with Olympus DP11.

UL16 glycoprotein co-localized with pp28 and WGA Golgi marker at the assembly site in Δ pp65 infected cells (figures 3.6 A and B).

3.4 Influence of cellular kinases on subcellular distribution of UL16

HCMV UL97 kinase has several functions in early and late phases of the viral life cycle, namely, in DNA replication, capsid maturation, nuclear virion morphogenesis and egress (Azzeh *et al.*, 2006, Wolf *et al.*, 2000). Here we studied the effect of variable cellular kinases on UL16 subcellular distribution, cytoplasmic assembly site and viral growth.

3.4.1- The influence of NGIC-I inhibitor on subcellular distribution of UL16

Indolocarbazole NGIC-I, an inhibitor of cellular protein kinases C, A and G, was shown to specifically inhibit the kinase activity of the UL97 *in vitro*, (Marschall *et al.*, 2001, Azzeh M, *et al.*, 2006). We employed this inhibitor to detect the possibility of an effect on UL16 subcellular distribution. 0.5 μ M NGIC-I was added to cells grown on chamber slides 2h post infection with either AD169, Δ pp65, Δ UL97 or MOCK (empty vector) infected. At 96hpi IF was performed with UL16 pAb. Figure 3.7 shows a conversion of the “bulb” like subcellular distribution of UL16 in AD169/ Δ pp65 infected cells into the Δ UL97 characteristic “crown” like, punctuated with vacuoles. In addition, the nuclei lost the typical kidney shape to assume a more oval shape, which includes vacuoles as well. By contrast, NGIC-I exerted no additional effects on either the UL16 subcellular distribution or the nuclear shape in cells infected with Δ UL97. Taken together, these results strongly suggest that it is the lack of UL97 kinase activity that causes the abnormal UL16 pattern in cells infected with Δ UL97 (Figure 3.7).

CHAPTER 4

4. Discussion

The glycoprotein encoded by the HCMV *UL16* gene has been shown to be involved in the escape of the virus-infected cells from attack by the immune system. Specifically, expression of UL16 leads to the accumulation of ligands for the activating receptor NKG2D inside the cell. The mechanism, by which this process is driven, is still unknown (Vales-Gomez M, *et al.*, 2005). Here, we further investigated the cellular interaction and biochemical properties of UL16 in order to understand the role of this glycoprotein in HCMV assembly and viral exit, particularly its possible, yet not studied raft association. pp65 is a well studied HCMV tegument protein, because of its interaction with variable HCMV proteins, cellular proteins, as well as the fact that its abundance is utilized in several experiments for vaccination (Schleiss M R *et al.*, 2007). UL97 is the only kinase encoded by the HCMV and plays a major role in the efficiency of viral replication (Prichard MN *et al.*, 1999).

4.1 Subcellular distribution of UL16 in HCMV infected HF cells

In AD169 (wt) infected HF cells UL16 distributed as compact juxtenuclear structure (figure 3.1). In Δ UL97 infected cells, UL16 distributed as diffuse perinuclear, vacuoles rich structure (figure 3.1), also the nuclear shape changed from typical kidney shape in AD169 infected cells to oval enlarged shape punctuated by vacuoles (figure 3.2). The fact that UL16 staining in Δ pp65 infected cells behaved similarly to AD169 was not exactly expected, since pp65 is an abundant tegument protein that plays variable function in viral

life cycle. These results though prove that pp65 does not play a role in building the assembly site, nor does it affect its structure. It is also a further proof for the unique effect of UL97 on infected cells, since only the UL97 deletion mutant resulted in the “crown” like distribution of tegument and glycoproteins. On the other hand, UL97 plays a major role in nuclear egress, which seems to be responsible for the invagination of the nucleus leading to the kidney shape of the nucleus as assessed in few studies (Prichard *et al.*, 2005; Azzeh *et al.* 2006, Das *et al.*, 2007). Our results provide a further evidence for the critical role of UL97 kinase in nuclear egress and its visible impact on nuclear shape as clearly demonstrated in figures 3.2 and 3.3. This is in agreement with the previous studies (Prichard *et al.*, 2005; Azzeh *et al.* 2006, Das *et al.*, 2007) and the very recent finding of Hamirally S *et al.* (2009).

4.2 UL16 glycoprotein co-localized to pp65, gB, pp28, and WGA in both *wt*-HCMV and UL97 deletion mutant

The results of the subcellular distribution of UL16 obtained gave a hint of strong relationship of the UL16 with the viral assembly site, elsewhere referred to as assembly complex. Therefore we studied the role of UL16 glycoprotein in the cytoplasmic assembly process and the late step of viral maturation. This was approached using IF co-localization experiments of UL16 with early (pp65) and late viral proteins (pp28 and gB) and a Golgi marker. All these viral proteins contribute to the viral assembly site (Sanchez V *et al.*, 2000; Azzeh M, *et al.*, 2006).

UL16 glycoprotein co-localized to the major viral assembly site building proteins in both AD169 and UL97 deletion mutant (figure 3.4 A, B, C and 3.5 A). Also viral infection induced a profound reorganization of WGA-positive structure. We found that UL16 glycoprotein co-localized with these cellular structures (figure 3.5 B). These results indicate that UL16 is a part of the HCMV cytoplasmic assembly process and late steps of viral maturation. Since UL16 is a part of a viral assembly site and its intracellular localization with Golgi was clear, we propose that UL16 plays a major role in the viral cytoplasmic assembly pathway.

Our data provides the first evidence for UL16 role in HCMV viral assembly, but also its possible direct interaction with the HCMV protein kinase, the UL97. This important finding is a first step towards understanding the mechanism(s) of interaction of the UL16 protein with other viral proteins and possibly HCMV maturation process.

4.3 Inhibition of UL97 kinase with NGIC-I resulted in changes of the subcellular distribution of UL16 glycoprotein.

Our observations show that the deletion mutant of UL97 resulted in an abnormal distribution of UL16 glycoprotein in HF infected cells (figure 3.1). In order to see if this effect is due to the lack of the kinase activity of the UL97 gene product, we employed the indolocarbazole NGIC-I, shown to inhibit the kinase activity of the UL97 in vitro (Marschall *et al.*, 2001).

Treatment of *wt*-HCMV- infected HF cells with NGIC-I converted the UL16 distribution from the typical “bulb like” compact juxtenuclear structure to “crown like” diffuse perinuclear Structure highly punctuated with vacuoles (figure 4.7). In addition, the nuclei lost the typical kidney shape to assume a more round, regular shape. By contrast, NGIC-I exerted no additional effect on either the UL16 subcellular distribution or the nuclear shape in the cells infected with Δ UL97. Taken together, these results strongly suggest that it is the lack of UL97 kinase activity that causes the abnormal UL16 pattern in cells infected with Δ UL97.

We conclude that UL97 kinase play a major role in HCMV life cycle late steps through phosphorylation, which is in agreement with Kamil J P and Coen D M (2007). This also leads to the conclusion that UL16 is indirectly affected by the activity of UL97. We propose that protein-protein interaction between UL16 and UL97 kinase take place during late infection. This conclusion has to be further investigated and would add a new impact to the roles of UL97 kinase activity in viral assembly and on the proteins of the viral assembly complex.

4.4 The structural changes of the HCMV assembly site are not only affected by the UL97 kinase, but also different cellular kinases

Our earlier data strongly suggested that the UL97 kinase activity is essentially required for the formation of the compact viral assembly site. In order to find out if other cellular

kinases may contribute to the viral assembly complex and viral load. We employed cellular kinase inhibitors. The inhibitors selected were Wortmannin, Polymyxin B and the activator Thrombin.

The effect of these inhibitors/activator was tested on cells infected with AD169, Δ p65 or Δ UL97 and the results are discussed for each virus below.

a) wt-HCMV

wt-HCMV was affected when treated with Wortmannin (phosphatidylinositol 3-kinase inhibitor). 30% of the infected cells showed the subcellular distribution of UL16 glycoprotein known for Δ UL97 infected cells (figure 3.8). At the same time, *wt*-HCMV titer was reduced by 2 logs compared to untreated. Johnson RA *et al.* (2001) proposed that PI3-K mediates HCMV-induced activation of host cell mitogenic pathways, also demonstrated later by Lee GC *et al.*, (2006). Interestingly, the data by Johnson RA *et al.* (2001) provided strong evidence for the importance of PI3-K activation in initiation of HCMV viral DNA replication and completion of viral life cycle. Hereby we are the first to identify the one possible step in viral life cycle affected by the PI3-K.

Polymyxin B revealed similar changes in UL16 subcellular distribution to those exerted by Wortmannin. The effect of Polymyxin B on HCMV life cycle by itself was not reported yet, but one study showed that PKC may facilitate HCMV/cell membrane fusion (Keay S and Baldwin B, 1996).

Thrombin (PI3-K activator) did not show detectable influence on AD169 assembly site or the viral load. The activity of Thrombin does not seem to be inhibiting or activating any process in HCMV life cycle, at least in *wt*-HCMV life cycle.

b) Δ UL97:

Interestingly nor Wortmannin or Polymyxin B showed any detectable effect on the subcellular distribution of UL16 glycoprotein or on the viral titer in HF cells infected with Δ UL97. Thrombin, on the other hand induced the *wt*-HCMV subcellular distribution of UL16 glycoprotein in 20% of infected cells. This may be not very significant, but this result is in agreement with that revealed in case of Wortmannin inhibition in *wt*-HCMV infected cells. This means that the influence of Thrombin in *wt*-HCMV was not detectable, even if it was there. Another possibility is that a direct or indirect interactor with Thrombin may had been activated only when the viral kinase was missing.

c) Δ pp65:

None of the drugs showed a major effects when cells were infected with Δ pp65, but the viral load of Δ pp65 was slightly affected by the variable inhibition activities of the kinase (data not shown).

Our cellular kinase inhibition assays suggest that cellular kinase may play a role in the assembly site pathway or may have a viral protein substrate among proteins of the assembly complex. We conclude that the cellular PI3-K, PKC and viral (UL97) kinases

are involved in phosphorylation events necessary for building the assembly site. Inhibiting any of the above kinase seems to have an impact on the structural distribution of the assembly site. The fact that no change was observed when viral infection was performed with Δ pp65 may indicate a role of pp65 with cellular kinase activity. It is worth studying which step of the cascade reaction of these cellular kinases play the major role in building the viral assembly site to be more specific.

4.5 pp65 is not a membrane associated protein

Our results show that pp65 is a soluble protein and not associated with the membrane. Although it is an abundant protein, it is not essential for viral replication or infection, but some studies proposed its strong connection with UL97 kinase (Prichard M N, *et al.*, 2005; Kamil J P and Coen D M, 2007). Interestingly, our data propose that the phosphorylation of pp65 is predominantly controlled by the viral UL97 kinase, since the phosphorylated portion of pp65 was markedly reduced in Δ UL97 infected cells. This proposal is in agreement with the model suggested by Prichard MN *et al.* (2005). Prichard MN *et al.* (2005) proposed a model in which the UL97 kinase influences the physical properties of pp65 by direct phosphorylation, which is important for the assembly and possibly nuclear egress of the nuclear form of the virion particle. A more recent study was able to detect a stable protein-protein interaction between pp65 and UL97 as revealed by co-IP experiments (Kamil J P and Coen D M, 2007). Kamil J P and Coen D M (2007) concluded that the UL97-pp65 interaction may be essential for direct localization of pp65 during assembly by UL97 or the other way round. Kamil J P and

Coen D M (2007) referred to an unpublished data done at their lab, where they observed the phosphorylation of recombinant pp65 by UL97 in *vitro*. pp65 is required for the incorporation of pUL69 and pUL97 into the virus particle and for viral growth in macrophages (Chevillotte M et al., 2008).

Our results and those discussed in this section strongly suggest that pp65 interacts with UL97 not only for phosphorylation events but also for facilitating essential roles of pp65 and of UL97 in HCMV viral life cycle.

4.6 UL16 is a lipid raft associated protein

Lipid rafts are called detergent-insoluble glycolipid-enriched complexes (GEMs) or Detergent Resistant Membranes (DRMs). The first appearance of lipid rafts, or lipid rafts-like structure, was occasionally observed by cryo-electronic microscopy in 1980s as cavity, such as caveolae. The functional roles of lipid rafts include facilitating the signaling proteins on and off through forming a liquid ordered phase. Lipid rafts are also involved in virus entry and assembly (Luo C *et al.*, 2008). Lipid rafts in Vero E6 cells are involved in the entry of severe acute respiratory syndrome coronavirus (SARS-CoV) (Lu Y, *et al.*, 2008). Also HIV entry in macrophages is dependent on intact lipid rafts (Carter GC, *et al.*, 2009). All these publications indicate the major role of lipid rafts in virus entry and assembly. Some HSV proteins were found to be lipid raft associated like UL11 (Koshizuka T *et al.*, 2007), UL56 (Koshizuka T *et al.*, 2002) and vhs of UL41 (Grace E *et*

al., 2003). One study demonstrated that glycoprotein B (of HSV) associated with lipid rafts during HSV cell entry (Bender FC *et al.*, 2003).

None of the HCMV proteins was associated with lipid rafts until today, but a single study showed that HCMV entry and interferon (IFN) signaling are coordinated processes that require cholesterol-rich microdomains (Juckem LK *et al.*, 2008). This study also highlights the complexity of innate immune responses at the earliest points in HCMV infection. UL16 glycoprotein has been shown to be involved in the escape of virus-infected cells from attack by the immune system, the intracellular distribution of UL16 has been found to co-localize with WGA and major HCMV proteins, which indicated the critical role of this protein in cytoplasmic assembly and maturation of the virus (figure 4.3A, B, C). Also UL16 trafficked through the ER, TGN, and inner nuclear membrane and to the plasma membrane (Vales-gomez M, *et al.*, 2006). All these biological properties of UL16 glycoprotein and its important role in cytoplasmic assembly discussed in this work, pointed out its possible association with lipid rafts. As shown in figure 3.12, the 42kDa band of UL16 was detectable in fractions $\frac{3}{4}$, at the top of the flotation. This indicated the lipid raft association of UL16. This result is the first evidence for raft association of HCMV viral proteins. Whether this raft association of UL16 is necessary to facilitate its critical role in the immune response or the viral entry still needs to be further studied.

Finally our finding contributed to the understanding of the biochemical properties and roles of pp65 tegument protein and UL16 glycoprotein. Based on these data, we have

established a new field of investigation for UL16 and pp65 and provided evidence for their essential role in viral life cycle. Our UL16 data enlarges the number of viral proteins involved in HCMV cytoplasmic assembly. Being a participant of the assembly complex, UL16 may have a critical role as an immune interactor, facilitating the viral assembly complex. Finally, our data also emphasizes the unique interaction between UL97 and pp65, which is essential for the roles of both proteins and their contribution in viral life cycle.

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