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The influence of Endoplasmic Reticulum Stress on the Assembly and Life Cycle of Human Cytomegalovirus

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Thesis Approval

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Dedication

I dedicate this letter to the spirit of my father pure and my mother dear, to my dear husband and my beloved son, to my brothers and sisters and to all who stood beside me and helped me. I especially thank and gratitude dear brother Zaidoun who has long halt beside me to take me to here and it was the best guide and directory.

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 study has not been submitted for a higher degree to any other university or institution.

Signed …….

Maysa Mahmoud Hassan Salah

Date: 13-01-2013

Abstract

In the struggle between viruses and host cells, taking control over the cell's death machinery is very crucial for survival. Several viruses, including human cytomegalovirus (HCMV) induce Endoplasmic Reticulum (ER) stress and activate UPR signaling. While different aspects of ER stress and HCMV were studied earlier, the influence of ER stress on the assembly complex was hardly illuminated. This work focuses on one aspect of the viral life cycle in respect to cellular ER stress. Viral assembly complex is the site, where variable viral proteins are assembled to start the journey of building progeny viruses. Due to the 96 hours long life cycle of HCMV, the virus needs to keep the cell functioning at least for its life cycle period and avoid any detrimental effects of cellular stress. .

We used Thapsigargin as an inducer for ER stress in HCMV infected cells versus mock infected cells. Human foreskin fibroblasts (HFF) were used for infection assays as the main successful model for infection with HCMV lab isolates. Wildtype HCMV lab isolates AD169 and the UL97 deletion mutant (□UL97) was used as HCMV strains. UL97 is the only HCMV kinase, a serine/threonine kinase and the target of anti viral drugs, was shown earlier to be involved in phosphorylation of viral and cellular proteins to facilitate infection. Our investigations showed that ER stress affected the assembly site and viral assembly proteins. Further experiments revealed that the only viral kinase (pUL97) was involved in the modulation of the ER stress leading to the detected consequences on the assembly complex, viral titer and viral load. The consequences on the assembly complex were verified by detecting viral assembly protein pp65 via western blot, which revealed drastic effects on this major tegument protein. The ER stress marker BIP was clearly induced in our system and its induction was proportional with the effects detected in assembly complex.

ER stress is a scientific field of its own; the involvement of viruses with this massive cellular response had shed the light on various aspects in the research of viral/cellular interaction and in the aspect and potential of the infection under such cellular circumstances. The data presented here are a clear evidence for the involvement of viral assembly proteins in the ER stress, modulated by UL97.

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List of Abbreviations

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AC	**Assembly Compartment**
AD169	**Wild type human cytomegalo virus**
APP	**Assembly Protein Precursor**
APS	**Ammonium per sulfate**
ATF6-4	**Activating transcription factor 6-4**
Babta-am	**Bis(2-aminophenoxy)ethane tetraacetic acid**
Bip	**Binding immunoglobulin protein**
Bp	**Base Pair**
BSA	**Bovine Serum Albumin**
Ca+2	**Calcium ion**
CaCo-2	**Intestinal Epithelial Cell Line**
CD8+T cells	**Cytotoxic T Cells**
CDK	**Cyclin-Cyclin-Dependent Kinase**
CERP	**Retino plastoma pocket proteins**
CID	**Cytomegalic inclusion disease**
CNV	**Copy Number Variation**
CREB-2	**cAMP responsive element binding protein**
CTL	**Committee of Testing Laboratories**
Cy2	**Cyanine2**

Cy5	**Cyanine5**
DAPI	**4′, 6-diamidino-2-phenylindole**
DH2O	**Distilled water**
DMEM	**Dulbecco modified Eagle's minimal essential medium**
DMSO	**Dimethyl sulphoxide**
DNA	**Deoxyribonucleic acid**
e IF 2*α*	**Eukaryotic initiation factor**
EBV	**Epstein-Barr virus**
EDTA	**Ethylene diamine tetraacetic acid**
EGFR	**Epidermal Growth Factor Receptor**
EOR	**Over load response**
ER	**Endoplasmic reticulum**
ERGIC	**Endoplasmic Reticulum-Golgi Intermediate Compartment**
FITC	**Fluorescein Isothio cyanate**
Gb	**Glycoprotein B**
Gh	**Glycoprotein H**
GI	**Gastrointestinal**
Gl	**Glycoprotein L**
Grp	**Glucose regulated protein**
GST	**Glutathione S-Transferase**
HAART	**Highly active antiretroviral therapy**
HCMV	**Human Cytomegalovirus**

HFF	**Human foreskin fibroblasts**
HHV	**Human herpes virus**
HIV	**Human immune deficiency virus**
Hpi	**Hours Post Infection**
HRP	**Horse radish peroxidase**
HSPGs	**Heparin sulfate proteoglycane**
HSV	**Herpes Simplex Virus**
ICTV	**International Committee on the Taxonomy of Viruses**
IE1-2	**Immediate early protein-1or 2**
IF	**Immune fluorescence**
IgG	**Immunoglobulin G**
IGIV	**Immune globulin intravenous**
IM	**Infectious mononucleosis**
Irel a and b	**Inositol-requiring enzyme 1-2**
Kbp	**Kilo base pair**
Kd	**Kilo Dalton**
KS	**Kaposi sarcoma**
KSHV	**Kaposi's sarcoma-associated herpes virus**
LB	**Lysogeny broth**
mAbs	**Monoclonal Antibodies**
mC-BP	**Minor Capsid Binding Protein**
MCP	**Major Capsid Protein**
Mcp	**Minor Capsid Protein**

MICB	**MHC Class I-Related Chains**
Moi	**Multiplicity of Infection**
MPR	**Maturational Protease Precursor**
mRNA	**Messenger Ribonucleic acid**
NGIC-1	**Non-glycosidic Indolocarbazole I**
NIEPs	**Non Infectious Enveloped Particles**
NK Cells	**Natural Killer Cells**
NLS	**Nuclear Localization Sequences**
ORFs	**Open Reading Frames**
PBS	**Phosphate Buffer Saline**
PCR	**Polymerase chain reaction**
PERK	**Protein endoplasmic reticulum kinase**
PFU	**Plaque forming unit**
Ph	**Measure of the acidity or basicity of a solution**
Pi	**Post infection**
PP65	**Phospho protein 65**
PUl38	**Viral protein**
RT	**Room temperature**
SCP	**Smallest Capsid Protein**
SDS	**Sodium dodical sulfate**
SERCA	**Sarco/endoplasmic reticulum Ca^{2+}ATPase, is a calcium ATPase type P ATPase**
SR	**Sarcoplasmic reticulum**
TAP	**Tandem Affinity Purification**

TEMED	***N,N,N′,N′*-Tetramethylethylenediamine**
Thap	**Thapsigargine**
TX	**Triton X**
ULBPs	**UL16-Binding Proteins**
UPR	**Unfolded protein response**
V	**Voltage**
VCA	**Viral Capsid antigen**
VZV	**Varicella zoster virus**
WB	**Western Blot**
WGA	**Wheat Germ Agglutinin**
WT	**Wild Type**
XBP-1	**X-box binding protein**
Δpp65	**pp65 Deletion Mutant**
ΔUL97	**UL97 Kinase Deletion Mutant**

Chapter One

Introduction

1.1 Historical background

Herpes Viruses have been prevalent as early as ancient Greek times (Nahmias etal., 1968; Wildy, 1973). Hippocrates described the cutaneous spreading of herpes simplex lesions and scholars of Greek civilization define the Greek word "herpes" to mean "to creep or crawl" in reference the spreading nature of the herpetic skin lesions (Nahmias etal., 1968; Wildy, 1973). In Romeo and Juliet, Shakespeare wrote Queen Mob to say "O'er ladies lips, who straight on kisses dream, which oft the angry Mab with blisters plagues because their breaths with sweetmeats tainted are" (The Tragedy of Romeo and Juliet, act 1 scene4, Mocarski., 2007). Astruc, a physician of the King of France, identified herpes as a cause of genital infection in the 18th century (Hutfield et al., 1966). Ribbert et al. described enlarged cells in the kidneys of a stillborn, without realizing the fact that he had just isolated HCMV infected cells (Ribbert et al., 1904). Vidal was the first to report human-to-human transmission of HSV infections, identifying the necessity of intimate human contact for spread of infection (Wildy, 1973).

1.2 classifications of Herpesviruses

Herpesviruses belong to the order Herpesvirales, which is divided into 3 main families; Herpesviridae under which mammals, reptiles and bird viruses are located, Alloherpesviridae for fish and frog viruses and Malacoherpesviridae for a bivalve virus (Davison, 2010). Herpesviridae is subdivided into 3 subfamilies based mainly on biological properties. Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae (Matthews, 1979). These subfamilies are furthermore divided into different genus.

Herpes viruses are mainly known with common names which are widely accepted, however all these viruses have taxon names (Davison, 2010). The taxon name is an abbreviation of three parts: The first part is the infected host; i.e. human (h), the second is the word herpes (h), the third is virus (v) and finally an Arabic number was added, which doesn't imply any meaning about taxonomic or biological properties of the virus.

1.2.1 Alphaherpesvirinae

The members of Alphaherpesvirinae are distinguished for infect variable hosts, causing efficient destruction of infected cells with relatively short reproductive cycle, and ability to establish latent infections primarily in sensory ganglia. This subfamily contains the genera *Simplexvirus* (HSV-1), *Varicellovirus* (VZV), *Marek's disease-like virus*, and *Infectious laryngotracheitis-like virus.*

1.2.1.1 Genus Simplexvirus

Simplexvirus is a spherical virion, enveloped (envelop contains viral glycoproteins, Fc receptors), 150-200 nm in diameter and double-stranded linear DNA genome of 124-235 kbp. Furthermore, it persist indefinitely in infected hosts Frequently reactivated in immunosuppressed hosts.

The most known herpes viruses belonging to this genus are herpes simplex virus type 1 and type 2. Herpes simplex virus type 1 (HSV 1), taxon Human herpes virus 1 (HHV1) and herpes simplex virus type 2 (HSV 2), taxon human herpes virus 2 (HHV2) are responsible for developing oral herpes (cold sores) and genital herpes infections (Davison, 2010). HSV disease can cause mild illness, not visible in the majority of patients, but also sporadic, severe, and life-threatening disease in infants, children, and adults (Roizman et al., 2007). Human seems to be the main reservoir for transmission of these viruses to other humans, since no animal vectors for human HSV infections were identified yet. Virus is transmitted from infected to susceptible individuals during close personal contact. Primary HSV-1 infections usually occur in early ages

of childhood commonly seen on mouth and lips. Most genital HSV Infections are usually associated with HSV-2 and are acquired through sexual contact (Josey et al., 1966; Parker and Banatvala, 1967; Josey et al., 1972; Deardourff et al., 1974).

1.2.1.2 Genus Varicellovirus

Varicella-zoster virus (VZV), human herpes virus 3 (HHV3) is the most common virus of genus Varicellovirus. VZV is closely related to the herpes simplex viruses (HSV), sharing much genome homology. VZV virions are spherical and 180–200 nm in diameter. VZV causes Varicella, known as chickenpox, characterized by fever and vesicular rash commonly in childhood age (Sanfillipo et al., 2004). It has high tropism for T lymphocytes, which aids in viral dissemination to skin. Like other herpes viruses, VZV causes latency in cells of the dorsal root ganglia. Later in life, a latent infection of VZV causes herpes zoster، commonly known as shingles. Shingles is known to affect adults and immunocompromised patients of all ages and causes acute pain, accompanied by severe fever (Mocarski et al., 2007).

1.2.2 Betaherpesvirinae

Betaherpesviruses are characterized by their capacity to infect restricted host range, with long reproductive cycle, causing the enlargement of the infected cells. The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidneys, and other tissues. This subfamily contains the genera *Cytomegalovirus* (HCMV), *Muromegalovirus* (murine cytomegalovirus), and *Roseolovirus* (HHV-7). Human cytomegalovirus (HCMV), taxon human herpes virus 5 (HHV5) belongs to the Cytomegalovirus genus and is the virus of interest in this work, see 1.3 below.

1.2.3 Gammaherpesvirinae

This subfamily contains two genera: *Lymphocryptovirus* and *Rhadinovirus*. The host range of the members of the subfamily is limited to the family or order to which the natural host belongs.

In vitro, all members replicate in lymphoblastoid cells, and may 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.

Epstein-Barr virus (EBV); human herpes virus 4 (HHV4) is the main representative *Lymphocryptovirus* genus. EBV is widely spread in the human population with about 90% of persons being seropositive IgG for the viral capsid antigen (VCA) complex (Henle et al., 1969). It is transmitted via oral route. Most children from 1-3 years become infected from other family members (Gratama et al., 1990). Primary infections are normally asymptomatic, however it may cause mild to severe infectious mononucleosis (IM), when it occurs after 10 years of age (Crawford et al., 2006). EBV is mainly associated with nasopharyngeal cancer, Burkitt's lymphoma (tumor of the jaw and face found in children), and oral hairy leukoplakia (Khanna et al., 1995; Al-Kuraya et al., 2006).

Kaposi's Sarcoma-associated herpesvirus (KSHV); human herpes virus 8 (HHV8) is the main representative of the *Rhadinovirus* genus. Initially, it was first suggested that HIV virus was causing KSHVcancer legions, as it was mainly found in HIV patients (Beral et al., 1999). Later, KSHV was identifies to infect the endothelium originating cells known as spindle cells (Boshoff et al., 1995; Staskus et al., 1997; Sturzl et al., 1997), which then produce proinflammatory and angiogenic products (Ensoli et al., 1989). KSHV is known to cause primary effusion lymphoma and some types of multicentric Castleman's diseas.

1.3 Human cytomegalovirus

1.3.1 Structure of human cytomegalovirus

The virion of HCMV consists of an icosahedral capsid encasing a 235-kbp linear genome, surrounded by a tegument or matrix (Wright et al., 1964; McGavran and Smith, 1965) and enveloped in a lipid bilayer carrying a large number of virus-encoded glycoprotein's (Spaete et al., 1994; Britt, 1996). By cryoelectron microscopy, the capsid appears similar to, though larger than, the herpes simplex virus (HSV)-1 capsid (Chen et al., 1999). HCMV has a double-stranded

DNA genome (Richard et al., 1985), capable of encoding more than 200 potential protein products (Hay et al., 1991). A cartoon of the virus particle is shown in figure 1.1

HCMV infected cells generate three different types of particles including:

A- Infectious mature virions.

B- Non infectious enveloped particles (NIEPs), composed of the viral proteins but lack viral DNA.

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

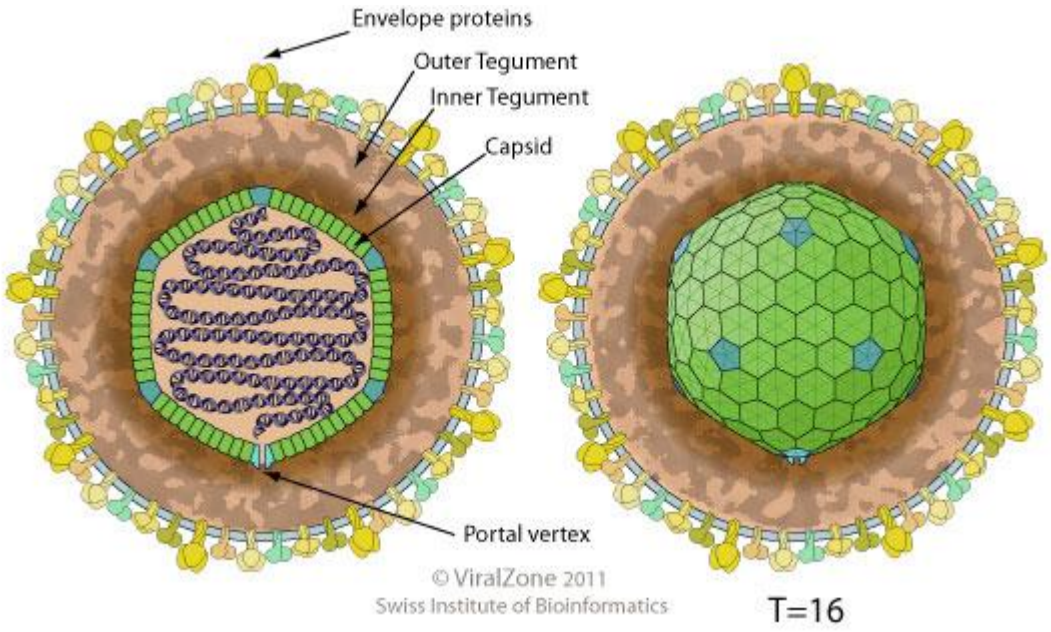


Figure 1.1: A graph of HCMV structure (from. http://viralzone.expasy.org).

1.3.2 Glycoproteins of HCMV

The viral glycoproteins are embedded in the lipid bilayer of the HCMV viral envelope. The acquisition of the envelope and its associated glycoproteins occurs at both nuclear and cytoplasm

sites. The host antibody-mediated immune clearance mechanisms target the envelope glycoproteins (Mocarski, 2007; Britt and Mach, 1996). There are approximately eight major glycoprotein types in the HCMV envelope.

gB; pUL55 is the major envelope component and the most highly conserved glycoprotein in mammalian and avian herpes viruses. gB is a type I integral membrane protein that functions in viral attachment and entry into cells, cell-to-cell transmission, fusion to adjacent cells, and in targeting of progeny virus to apical membranes for release from polarized cells (Bold et al., 1996; Compton et al., 1993; Navarro et al., 1993; Tugizov et al., 1996; Tugizov et al., 1998; Vanarsdall et al., 2008). Cell surface heparan sulfate proteoglycans (HSPGs) is the major receptor for gB (Adlish et al., 1990; Taylor and Cooper, 1990).

The HCMV UL16 gene encodes a glycoprotein which interferes with the immune response to virus-infected cell. UL16 expression has been shown to promote intracellular accumulation of MICB, ULBP1 (Valés-Gómez et al., 2005).

Several additional viral glycoproteins are likely to be minor envelope constituents but are not apparently associated with any major glycoprotein complexes. HCMV gH, or gpUL75, is part of a second, relatively abundant glycoprotein complex to which neutralizing antibodies may be directed (Rasmussen et al., 1984). gH complexes with at least one other herpesvirus-common glycoprotein, denoted gL, which facilitates the transport of gH to the cell surface (Kaye et al., 1992; Spaete et al., 1993). Finally, gL, encoded by UL115, is a glycoprotein modified by N-linked and possibly O-linked carbohydrates. Cell-cell fusion frequently involved in the majority of cells and gB and gH/gL were both necessary and sufficient for fusion, whereas no fusion occurred when either glycoprotein was omitted (Vanarsdall et al., 2008).

1.3.3 Tegument proteins

The tegument appears to be a relatively amorphous virion region located between the capsid and envelope. The virion tegument contains most of the virion proteins as well as a selection of viral and cellular RNA making up about 40% of the total virion mass as well as the overwhelming majority of the dense body mass (Mocarski et al., 2007).

There are at least 25 proteins located in the tegument layer between the virion capsid and envelope. These proteins are the products of 11open read frames (ORFs) (UL25, UL26, UL32, UL47, UL48, UL48.5, UL82, UL83, UL85, UL88, UL99). All of These ORFs are conserved in the betaherpesviruses (Gompels et al., 1995; Nicholas, 1996; Rawlinson et al., 1996; Vink et al., 2000), although only a small number is herpesvirus-common.

1.3.3.1 pp65

PP65 is the major tegument protein, and it is the target antigen used for rapid diagnosis of HCMV clinical infection by antigenemia assays. 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 as a primary target for phosphorylation in vitro by the virion-associated protein kinase (Somogyi et al., 1990). The formation of tegument aggregates appear to be dependent on pp65 since they were not induced in cells infected with a pp65 deletion mutant (Prichard et al., 2005).

1.3.3.2 UL97 kinase

UL97 is a protein kinase encoded by (HCMV) is an important target for antiviral drugs (Kamil and Coen , 2007). UL97 has a multi-step role in both early and late phases of the viral life cycle, namely DNA replication, capsid maturation and nuclear egress (Azzeh et al., 2006). 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 in the wild type to diffuse perinuclear structures punctuated by large vacuoles in ΔUL97 virus (Azzeh et al., 2006). UL97 protein was shown to have activities similar to cellular cyclin-cyclin-dependent kinase (CDK) complexes. UL97 phosphorylates and inactivates retinoblastoma tumor suppressor, stimulated cell cycle progression in mammalian cells, and rescued proliferation of Saccharomyces cerevisiae lacking CDK activity. UL97 represents a functional ortholog of cellular CDKs that is independent of normal CDK control mechanisms (Hume et al., 2008). UL97 kinase plays an important role in the acquisition of tegument during virion morphogenesis

in the nucleus and this activity represents an important step in the production of mature virus particles (Prichard et al., 2005).

UL97 phosphorylates the viral proteins UL44 (a DNA polymerase accessory protein (Krosky et al., 2003a, b), and pUL69 (a tegument protein and pluripotent regulator) (Thomas et al., 2009) as well as the cellular proteins like p32 (Marschall et al., 2005), which facilitates viral infection. UL97-deleted HCMV are severely impaired in their replication, which may be the result of defects in viral DNA synthesis and particularly in nuclear export of viral capsids (Krosky et al., 2003a, b).

1.3.3.3 pp28 (ppUL99)

PP28 is a 190-amino-acid tegument protein encoded by the UL99 open reading frame, which is myristoylated and phosphorylated (Sanchez et al., 2002a). PP28 is essential for assembly of the 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 juxtanuclear compartment termed the assembly compartment (AC) (Sanchez et al., 2002b; Azzeh et al., 2006; Seo and Britt, 2006).

1.3.3.4 pp150 (ppUL32)

The major tegument phosphoprotein, pp150 (ppUL32 or basic phosphoprotein), is the product of UL32 (Jahn et al., 1987). It is incorporated preferentially into virions rather than dense bodies, and it makes up about 20% of the virion mass (Benko et al., 1988).

1.3.4 Capsid

These viruses have a doughnut shaped capsomere about 100-200 nm in diameter with an icosahedral nucleocapsid. The latter contains 162 capsomeres.

The capsid is composed of seven proteins: Major capsid protein (MCP) which is encoded by UL86 and forms both hexons and pentons, minor capsid protein (mCP) encoded by UL85, minor capsid binding protein (mC-BP) encoded by UL46, smallest capsid protein (SCP) encoded by UL48.5 (also called UL48/49), and three distinct assemblin/assembly protein (AP)-related proteins encoded by UL80,UL80a, and UL80.5 associate with capsids (Gibson, 1996; Gibson et al., 1996; Baldick and Shenk, 1996; Chen, et al., 1999; and Trus, et al., 1999). 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), both contains two nuclear localization sequences (NLS1 and NLS2),where at least one seems required to translocate the major capsid protein (MCP, pUL85) into the nucleus (Nguyen et al., 2008).

1.4 Life cycle of HCMV

The HCMV life cycle begins with the attachment of the virus to the cell surface. Initially, HCMV binds to cell surface heparin sulfate proteoglycans (HSPGs) as mediated via two viral envelope glycoproteins; major glycoprotein (gB) and glycoprotein M (gM) (Kari and Gehrz, 1992; Compton et al., 1993). HSPGs are not sufficient to stabilize the interaction; therefore, cellular epidermal growth factor receptor (EGFR) was suggested to be responsible for the stable interaction (Wang et al., 2003; Wang et al. 2005). However, recent studies suggest that the HCMV binding event involves viral glycoproteins including gB interacting with β1 and β3 heterodimers without the involvement of EGFR (Isaacson et al., 2007; Feire et al., 2010). After binding to the cell surface, the virus penetrates the host cell membrane after fusing with it using the virion envelope by a process not fully understood which involves the gH/gL complex of glycoproteins expressed on the surface of the virus (Keay and Baldwin 1991; Milne et al., 1998). This fusion event results in the release of the nucleocapsid into the cytoplasm where it is quickly transported to the nucleus through intact microtubule networks utilizing a process that also requires actin depolymerization (Jones et al., 1986; Ogawa-Goto, 2003; Wang et al., 2005). The nucleocapsid then transfers the viral DNA genome into the nucleus by a not fully understood mechanism (Wang et al., 2005).

Viral DNA expression in the nucleus begins with immediate early (IE), or α genes, which are expressed with the assistance of tegument proteins. The viral life cycle takes approximately seventy two hours, hereby genes are referred to time point of their expression during this long life cycle (Salvant et al., 1998). Immediate early genes are those expressed at early stages like IE1, IE2 are referred to as. Delayed early genes are expressed within the first 48 h of infection, like the tegument protein pp65. Late and true late genes are those expressed at late stages of infection, like the tegument protein pp28. Most early gene products function to replicate viral DNA and their expression depends on the expression of IE gene products. Viral DNA replication results in the generation of long concatemeric DNA molecules, which provide the templates for late (L) or γ gene expression (Salvant et al., 1998). Virion structural components, virus assembly and egress genes are the products of L genes (Mocarski et al., 2007). Late gene expression occurs once the virus begins replicating its genome and these genes encode for structural components of the virion. Nucleocapsid particles are assembled within the nucleus and then acquire their tegument and envelope by yet undefined pathway. The mature virus buds off from the cell into the surrounding area and can infect other permissive cells.

At latent stage, HCMV does not produce infectious virus and can last for the life of the host. The immune system is unable to clear latent infection. At reactivation stage, the virus switches from latent state back to a productive infection state (Mocarski et al., 2007), shedding of virus in saliva, urine or other body secretions. CMV DNA is typically detected in 2% to 5% of the population in saliva (Miller et al., 2005) or urine (Mehta et al., 2000) and, therefore, virus must either reside in these sites or be sporadically reseeded from a latent reservoir. Reactivation from latency causes most of the serious CMV disease in immunocompromised individuals such as transplant recipients.

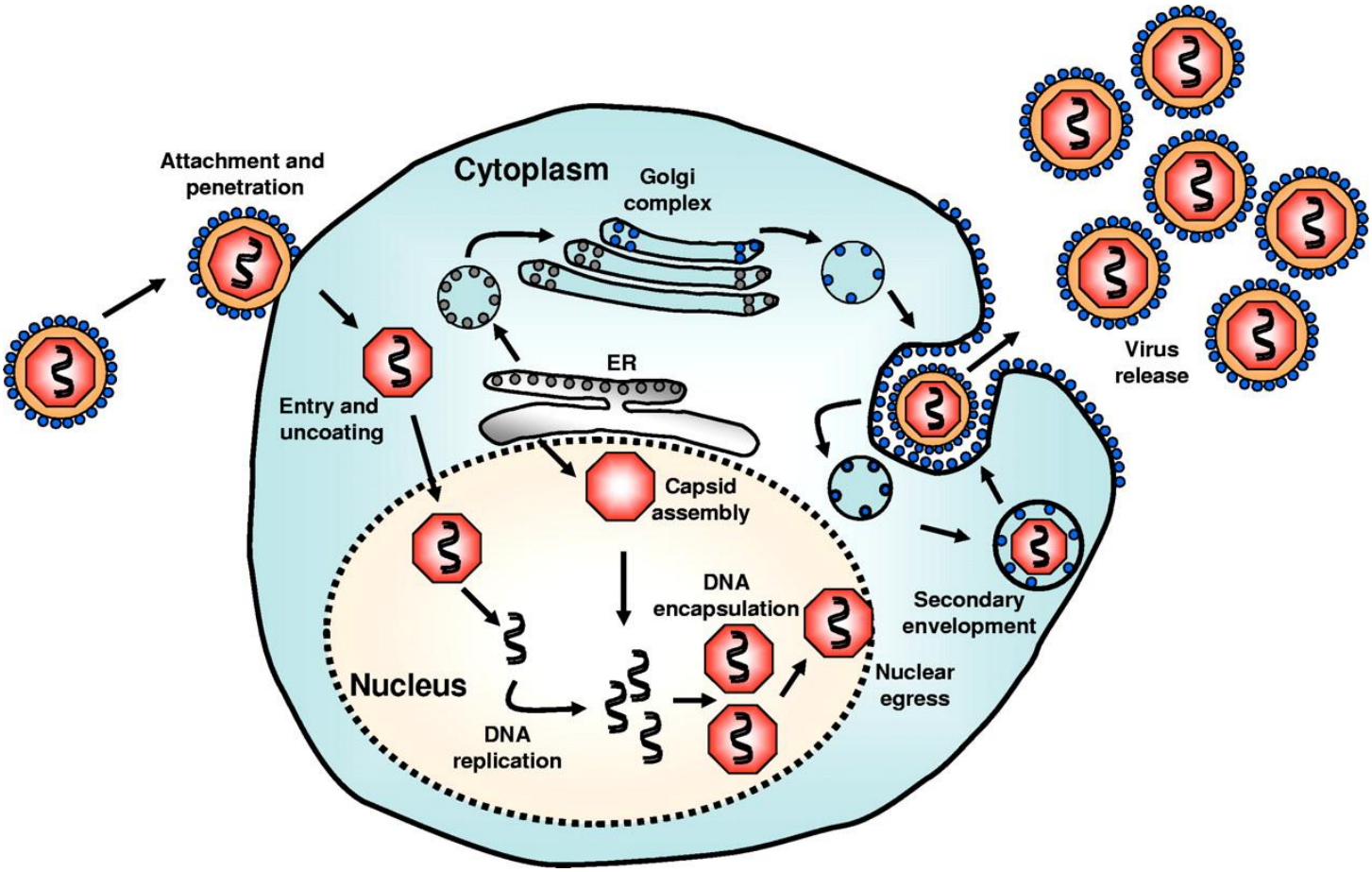


Figure 1.2: **Summary of the HCMV life cycle** (Crough and Khanna, 2009). The viral entry begins as an attachment to the plasma membrane via viral glycoprotein and cell surface receptors, which facilitates the releasing of nucleocapsids to the cytoplasm, which is then translocated to the nucleus initiating viral DNA replication followed by capsid assembly and integration of the DNA in the capsid (Encapsidation). This capsid buds from the nucleus as the virus gets the primary envelopment. Once transported to the cytoplasm, the virus undergoes secondary envelopment in the endoplasmic reticulum (ER)-Golgi intermediate compartment. Final envelopment occurs as a complex two-stage process, which is followed by exocytosis (virion release).

1.4.1 HCMV egress and assembly

Following HCMV DNA synthesis, encapsidation proteins recognize this concatemeric template. Capsid localization, packaging, and cleavage of viral DNA are regulated by the conserved viral protein kinase, pUL97 as well as by cellular kinases, which may carry out redundant functions (Mocarski et al., 2007). Upon the formation of the nucleocapsid, a complex two-stage envelopment and egress process starts in the nucleus and leads to virion release by exocytosis at the plasma membrane (Mettenleiter, 2004; Britt, 1996). This is a two-stage envelopment/deenvelopment/re-envelopment process (Sanchez and Spector, 2002; Mocarski et al., 2007). Here, nuclear egress starts with primary envelopment at the inner nuclear membrane, followed by a de-envelopment event at the outer nuclear membrane and release of the nucleocapsid into the cytoplasm (Mocarski et al., 2007). Secondary envelopment occurs in the cytoplasm at ER-Golgi intermediate compartment (ERGIC) membranes building a cytoplasmic assembly complex (AC).

The cytoplasmic assembly complex (AC) in HCMV-infected cells is a juxtanuclear structure adjacent to the concave invaginations of the kidney shaped nucleus (Azzeh et al., 2006). The morphology of AC depends on pUL97; it is compact "bulb" -like in wt-HCMV, but diffuse, "crown" -like and rich in vacuoles in the absence of pUL97 (Azzeh et al., 2006). Inhibition of UL97 kinase activity using Maribavir or indolocarbazole NGIC-1 resulted also in the diffuse, "crown" -like and rich in vacuoles AC structure (Prichard et al., 2005; Azzeh et al., 2006). The AC consists of viral tegument and glycoproteins (Sanchez et al., 2000a ; Prichard et al., 2005; Azzeh et al., 2006) and cytoskeletal filaments, which radiate from a microtubule organizing center into the AC (Sanchez et al., 2000a; Das and Pellett, 2007). Markers of the Golgi apparatus, trans-Golgi network and early endosomes co-localize to the AC (Sanchez et al., 2000a; Sanchez et al., 2000b; Homman-Loudiyi et al., 2003; Azzeh et al., 2006; Das and Pellett, 2007). The pUL97 is considered to play a role in phosphorylation of tegument proteins to facilitate their incorporation into virions, yet the molecular mode of interaction of the different components remains undefined.

1.5 Diseases caused by HCMV

Congenital cytomegalovirus (CMV) infection is the most common and the most important of all congenital infection (Murphsu et al., 1998). Hereby, the maternal immune status plays the major role in likelihood of congenital infection and the extent of disease in the newborn. If primary maternal infection occurs during pregnancy, vertical transmission of HCMV is 15-20% higher than recurrent infection (Revello and Gerna, 2002). Most infants that have a congenital cytomegalovirus infection are born to women with recurrent HCMV infection. Although most of these infants are clinically healthy at birth, they may have subtle growth retardation and are at risk for neurodevelopmental sequelae (Fowler et al., 1996). The major consequence of congenital cytomegalovirus infection is sensorineural hearing loss. Approximately 15% of these infants will have unilateral or bilateral deafness due to the fact that this deficit may develop after months or even years after birth (Fowler et al., 1999).

Cytomegalic inclusion disease (CID) is the most severe case of congenital CMV infection and comprises approximately 10% of infants with congenital infections with clinical evidence at birth CID is the result of primary HCMV infection.

Perinatal acquisition of cytomegalovirus usually occurs via infected secretions of HCMV in the birth canal or via breastfeeding (Mocarski et al., 2007). Maternal breast milk should be subjected to viral shedding detection, when babies suffer from low birth weight (Schleiss et al., 2006).

Typical cytomegalovirus mononucleosis is a disease found in young adults (Henle et al., 1969) which leads to fever and severe malaise, an atypical lymphocytosis and mild elevation of liver enzymes. Although cytomegalovirus mononucleosis may be transmitted via blood transfusion or organ transplantation, cytomegalovirus mononucleosis is usually acquired via person-to-person transmission (Akhter et al., 2012)

Immunocompromised patients have high risk of getting infected by cytomegalovirus. HCMV infection in these patients can be an acquired via transfusion or organ transplant from a seropositive donor or by reactivation of latent virus infection. Mixed Infections may arise, with

donor and recipient isolates both present (Revello and Gerna, 2002). The most important clinical manifestations consist of pneumonitis, GI disease, and retinitis.

Human Cytomegalovirus pneumonitis is a major cause of pneumonitis in immunocompromised patients such as in HIV infection, congenital immunodeficiency, malignancy, and solid organ or bone marrow transplant (Schleiss et al., 2006). The mortality rate is high in bone marrow transplant recipients (Schleiss et al., 2008). Cytomegalovirus is capable of causing a multiple GI tract diseases which include esophagitis, gastritis, gastroenteritis, pyloric obstruction, hepatitis, pancreatitis, colitis, and cholecystitis. The symptoms may that include nausea, vomiting, dysphasia, epigastria pain, icterus, and watery diarrhea.

Cytomegalovirus retinitis was the most common cause of blindness in adult patients with acquired immunodeficiency syndrome (AIDS).

1.6 HCMV antiviral drugs

Nucleoside analogues aciclovir, adefovir, cidofovir, ganciclovir, penciclovir and valaciclovir, and the pyrophosphate analogue foscarnet are the antiviral agents currently approved by the FDA for treatment of CMV (Mocarski et al., 2007). These drugs reduce or eliminate viremia or HCMV shedding and prevent or control CMV disease in specific situations in immunocompromised patients. A potential for significant toxicity is possible during treatment, therefore, use should be limited to patients with disabling or life-threatening disease (Eddleston et al., 1997). Antiviral treatment of severe congenital HCMV infection with ganciclovir is based on evidence that it prevents late onset or progressive hearing loss (Mocarski et al., 2007). Valganciclovir (Valcyte) is an antiviral drug that is also effective and is given orally (Borthwick, 2005).

The therapeutic effectiveness of these drugs is frequently compromised by the emergence of drug-resistant virus isolates. A variety of amino acid changes in the UL97 protein kinase and the viral DNA polymerase have been reported to cause drug resistance. Therefore, Foscarnet or

cidofovir are only given to patients with CMV resistant to ganciclovir, because foscarnet may cause nephrotoxicity (Borthwick, 2005).

Cytomegalovirus Immunoglobulin Intravenous (Human) (CMV-IGIV) is an immunoglobulin G (IgG) containing a standardized amount of antibody to Cytomegalovirus which may reduce the risk of CMV-related disease and death in some of the highest-risk transplant patients (Scott et al., 2006). Maribavir, a benzimidazole L-riboside, a promissing antiviral compound was a in clinical development (Lu and Sun, 2004) and was shown to inhibit both UL97 kinase and viral DNA synthesis (Evers et al., 2004; Krosky et al., 2003). Maribavir has undergone phase I and II clinical trials and phase III prophylaxis trials in solid organ and stem cell transplant recipients (Lu and Sun, 2004; Winston et al., 2008). In February 2009, ViroPharma announced that Phase III study failed, as no significant difference between maribavir and a placebo in reducing the rate at which CMV DNA levels were detected in patients (www.viropharma.com/)

To date all Anti HCMV drugs targeted the viral DNA polymerase which have high toxicity. Once resistance emerges, treatment chances are very narrow. A very recent promising drug, AIC246, was introduced recently by AiCuris company (AiCuris GMBH, Germany) is currently in clinical phase II. AIC246 has a different mode of action – targeting viral terminase complex – and was tested successfully in HCMV infected patients and specifically in those infected with ganciclovir resistant strains (Lischka et al., 2011; Goldner et al., 2011; Kaul et al., 2011).

1.7 Epidemiology of Human CMV Infection

Seroepidemiologic studies show that HCMV is universally distributed among human populations from developed, industrial societies to isolated aboriginal groups (Ahn, et al., 1997; Heise, et al., 1998). In general, HCMV seroprevalence is high and acquired earlier in life in developing countries and in the lower socioeconomic strata of developed countries (Mocarski et al., 2007). Studies that have included both sexes consistently report slightly higher prevalence rates in women than in men (Demmler et al., 1985; Balcarek et al., 1990; Hecker et al., 2004). The prevalence of HCMV was reported to be 95%-99% in Turkey (Hizel et al., 1995), in Benin (Rodier et al., 1995), in South Korea (Sohn et al., 1992) and Japan (Hirota et al., 1992) compared

to lower prevalence (50%-70%) in Europe and the USA (Stagno et al., 1986; Natali et al., 1997; 4076, Gratacap-Cavallier et al., 1998).

Although HCMV is predictably transmitted in settings where susceptible people have frequent contact with body fluids from persons excreting virus, HCMV is not highly contagious and transmission appears to require direct contact with infectious material (Mocarski et al., 2007). Following initial acquisition of HCMV, infectious virus is present in urine, saliva, tears, semen, and cervical secretions for months to years. High rates of HCMV infection occur in settings where close contact with body fluids is expected, such as between sex partners (Chandler et al., 1985; Stanberry et al., 2004), among children in day care centers, and between preschool-aged children (Pass et al., 1984; Murph et al.,1986; Adler, 1988).

1.8 ER stress

The endoplasmic reticulum (ER) is a central organelle of each eukaryotic cell as the place of lipid synthesis, protein folding and protein maturation. Proteins of the plasma membrane, secreted proteins as well as proteins of the Golgi apparatus and lysosomes fold into their tertiary and quaternary structure in the ER. The ER is the major signal transducing organelle that senses and responds to changes of homeostasis. A quality control mechanism exists in the ER to ensure the success of proteins processing in the ER. Proteins which have been improperly folded or modified are selected. The accumulation of misfolded proteins causes ER stress. ER stress leads to activation of a complex signal transduction cascade; the unfolded protein response (UPR) (Harding et al., 2001). Metabolic conditions (e.g., glucose deprivation) and infection by viruses are physiological conditions which induce ER stress and UPR cascade (Li et al., 1992; Tardif et al., 2002).

Activation of the UPR to eliminate misfolded proteins in the ER can occur by two ways:

1- Upregulating the expression of chaperone proteins and degradation factors to refold or eliminate misfolded proteins (Rutkowski and Kaufman, 2004)
2- Attenuating translation to reduce incoming protein traffic in the ER (Rutkowski and Kaufman, 2004)

Different cellular genes are upregulated to participate in the cell recovery from ER stress. Nevertheless, if the cell fails to recover from ER stress, it undergoes apoptosis.

1.8.1 ER stress and viruses

In order for viruses to maintain cellular infection, a high demand on cellular nutrients must be available (Alwine, 2008). An increase in glucose uptake and manipulation of the cell cycle are essential to grant the virus its optimal infection are essential (Alwine, 2008). Above all, the virus have to control cell apoptosis to complete its infection cycle successfully (Alwine, 2008). Cellular responses signal the cell when potential danger exists to initiate conditions to survive stress, otherwise cells undergo apoptosis.

Viral replication elicits cellular responses, such as endoplasmic reticulum (ER) stress. It is, therefore, not surprising that viruses have evolved various mechanisms to cope with these responses that limit or inhibit viral replication (Alwine, 2008).

1.8.2 ER stress and HCMV

Human cytomegalovirus (HCMV) infection induces cellular stress responses due to nutrient depletion, energy depletion, hypoxia and synthetic stress, e.g., endoplasmic reticulum (ER) stress. Cellular stress responses initiate processes that allow the cell to survive the stress, where some of these responses may be beneficial to HCMV replication while others are not. Several studies show that HCMV manipulates stress response signaling in order to maintain beneficial effects while inhibiting detrimental effects (Isler et al., 2005b; Hakki et al., 2006). Since inhibition of translation as a result of ER stress would be harmful to HCMV infection, HCMV has to find ways to maintain the cell in the metabolically and translationally active state until the end of its replication cycle. Different studies have illuminated certain mechanisms, by which HCMV overcomes the harmful effects of ER stress to mainatain its life cycle and cellular translation (Child et al. 2004; Kudchodkar et al. 2004; Hakki and Geballe 2005; Isler et al.

2005a, 2005b; Walsh et al. 2005; Hakki et al. 2006; Kudchodkar et al., 2006; Kudchodkar et al. 2007). HCMV pUL38 protein was shown to be required to maintain the viability of infected cells, through blocking cell death induced by Thapsigargin (Xuan et al., 2009). In order to do so, UL38 induces ATF4 expression, that inhibits persistent JNK phosphorylation, and suppresses endoplasmic reticulum stress-induced cell death.

1.9 ER stress drugs

1.9.1 Thapsigargin

The UPR can be induced using drugs such as tunicamycin, which inhibits N-linked glycosylation in the ER, and thapsigargin, which disrupts calcium homeostasis in the ER.

Thapsigargin is a non-competitive inhibitor of a class of enzymes known by the acronym SERCA, which stands for sarco / endoplasmic reticulum Ca^{2+} ATPase. Structurally, thapsigargin (TH) is classified as a sesquiterpene lactone, extracted from a plant; *Thapsia garganica*. Thapsigargin raises cytosolic calcium concentration by blocking the ability of the cell to pump calcium into the sarcoplasmic and endoplasmic reticula which causes these stores to become depleted. Store-depletion can secondarily activate plasma membrane calcium channels, allowing an influx of calcium into the cytosol (Begum et al., 1993).

TH is useful in experimentation examining the impacts of increasing cytosolic calcium concentrations. TH is an effective inhibitor of the Ca2+ ion pump proteins of intracellular membranes located in sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER) of skeletal muscles, cardiac muscles and brainmicrosomes. TH reduced insulin-stimulated glucose transport by about 40% by (Begum et al., 1993) and Protein synthesis in cells was inhibited (Wong et al., 1993).

1.10 Project significance

HCMV has a slow life cycle, which demands the interaction of various cellular and viral gene products. The virus has only one single kinase of it own, which should guarantee the phosphorylation of different cellular and viral proteins achieving different targets in the viral replication cycle including maintaining cell activities to avoid apoptosis. This work focuses on studying one aspect of the viral life cycle in respect to cellular ER stress, namely viral assembly complex. Viral assembly complex (AC) is the site, where variable viral proteins are assembled to start the journey of building progeny viruses. Once the virus assembly complex is built and maintained, successful infectious progeny virions can be produced.

Our work investigates the influence of ER stress on HCMV assembly complex by studying different viral and cellular proteins involved in building and maintaining the assembly complex. The results shed some light on unknown viral proteins involved in modulating ER stress for the benefit of the virus.

1.11 Objectives

1- Investigate the HCMV assembly site and its biological properties by "*in vitro*" activation of cellular ER stress
2- Detect the influence of induced ER stress on HCMV viral replication
3- Study the mechanism, by which ER stress affects the assembly site
4- Determine the time point, at which ER stress affects the viral replication generally
5- Illuminate viral proteins involved in modulating ER stress

Chapter Tow

2. Materials and Methods

2.1. Culturing of Cells

Human foreskin fibroblasts (HFF) (kindly provided by Dr. Nina Mayorek, Hebrew University) is a primary cell line isolated from newborn male's foreskin. The 293T cells (kindly provided by Prof. Chakraborty, University of Giessen, Germany) are Human embryonic kidney cells, that have been transformed by exposing cells to sheared fragments of adenovirus type 5 DNA and contain the SV40 large T-antigen that allows episomal replication of transfected plasmids containing the SV40 origin of replication (Graham and Smiley, 1977). HFF and 293T cells were cultured in flasks (Easy Flask, 25 cm^2, 75 cm^2, 175 cm^2, Nunc, Denmark) or well plates in complete DMEM medium (DMEM; 01-055-1A, Beit Haemek, 1:1000 dilution of Pen/Strep; 03-031-5C, Beit Haemek, 20 mM 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). The amount of complete DMEM medium added to the cells for culturing depends on the type of cell culture unit used as mentioned in Table 2.1. Cells were cultivated in 96% humid CO_2 incubator (5% CO_2) at 37°C (Hera cell incubator). The 293T cells were mainly used for transfection assays (see 2.12). All cell culture work was performed in laminar flow purifier safety cabinet (purifier class II biosafety cabinet, Labconco, USA).

2.1.1 Propagation and Passage Cells

Passaging (also known as subculture or splitting cells) involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly. For this, cells need to be detached; commonly made with a mixture of trypsin-EDTA. Cells were propagated by splitting into two or more culture flasks, or into different well plates, using 0.25% Trypsin-EDTA (03-052-1A, Beit Haemek) was added to the confluent cells in flask for 1-2 minutes. Cells were loosened by gently up and down pipetting using sterile disposable plastic pipettes. Complete medium was added to the non adhering trypsinized cells, gentle mixed and distributed to the new flasks. The new cells are one passage higher than the one they were split from. Since HFF is a primary cell line, it was propagated up to passage 18 in our lab. Experiments were performed on passages 10-18, while the passage number for 293T cells was unknown.

2.1.2 Freezing and Thawing of HF Cell

Cells can be kept for years if frozen in liquid nitrogen. HFF cells were frozen at low passages (6-12). Freezing proceeded as follows for a medium flask ($75cm^2$):

1. Cells were trypsinized as above
2. Cells were collected by centrifugation at 1500 rpm for 10 minutes.
3. Supernatant was discarded and pellet was re-suspended in (50% complete medium, 40% serum, 10% DMSO, dimethylsulphoxide, Sigma-Aldrich, Germany).
4. Suspension was transferred into sterile labeled freezing cryotube (cat#363401, Nunc, Denmark)
5. Cells were frozen overnight at -80˚C, then transferred to the liquid nitrogen container

Thawing Cells:

1. Cryotube containing the required cells was removed from the liquid nitrogen container to room temperature
2. Thawn cells were directly transferred to a small flask containing 4ml DMEM complete medium and mixed

3. Flask was transferred to the 96% humid CO_2 incubator

2.2 Viruses

In this work, HCMV viral strains, either as wild type (wt) or deletion mutant, were used. HCMV strain AD169 (wt-HCMV; American Type Culture Collection) and pp65 deletion mutant (Δpp65) were a kind gift from Prof. Bodo Plachter, University of Mainz-Germany. The UL97 deletion mutant, ΔUL97, was kindly provided by Dr. Mark Prichard (University of Alabama, USA). All viruses' related techniques were performed in laminar flow purifier safety cabinet (purifier class II biosafety cabinet, Labconco, USA).

2.2.1 Propagation of Viruses

HCMV is typically propagated in primary cultures of human fibroblasts that yield relatively high titer stocks and produce plaques when infected with HCMV.

Infection assay

1. Medium was discarded from HFF cells
2. AD169, Δpp65 or ΔUL97 was added at an MOI of 0.05 PFU/ml (diluted in complete DMEM) with the minimal amount of medium volume to cover the cells, i.e. 4 ml for a 175 cm^2 flask
3. Infected cells were incubate in 96% humid CO_2 for 2 h
4. Inoculum was removed and replaced with complete DMEM medium

Moi represents the number of viruses divided by the number of cells. In this work viruses were propagated in 175 cm^2 flasks, which contain $3x10^7$ HFF cells. For this, 4 ml viral suspension (virus stock diluted in complete medium) at moi of 0.001 were added to the cells and incubated in 96% humid CO_2 incubator (5% CO_2) at 37°C. At 10 days post infection (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 to perform a second viral harvest. Viral harvest was centrifuged for 10 min at 1500 rpm to get rid of cell debris; supernatant was transferred to a new

50 ml tube. Finally, 1% DMSO was added to the viral supernatant and the mixture was aliquoted in cryotubes, and frozen at -70^{o}C and in Liquid nitrogen. At 14 days pi, the second viral harvest is performed as above and the highly infected cells were discarded.

2.2.2 Viral Titration and Plaque Assay

Plaque-based assays are the standard method used to determine virus concentration in terms of infectious dose. Viral plaque assays determine the number of plaque forming units (pfu) in a virus sample, which is one measure of virus quantity in our works, based on a microbiological method conducted in petri dishes or multi-well plates. A viral plaque is formed when a virus infects a cell within the fixed cell monolayer. The virus infected cell will lyse and spread the infection to adjacent cells where the infection-to-lysis cycle is repeated. The infected cell area will create a plaque (an area of infection surrounded by uninfected cells) which can be seen visually or with an optical microscope. Plaque formation can take 14 days in the case of HCMV.

In this regards, an aliquot of frozen viral harvest was thawed and viral dilutions of 10^{-1}-10^{-8} (titrations) were prepared step wise (1:10 dilution steps) in complete medium. 90% confluent HF cells cultured in 24 well plates (Nunc, Denmark) were infected with 0.1 ml of either the undiluted viral stock, or one of the 10^{-1}-10^{-8} viral dilutions. Four wells (one column) of the 24 well plates were infected with the same viral dilution to guarantee 4 controls of each dilution. Infected 24 well plates were incubated in 96% humid CO_2 incubator (5% CO_2) at 37°C.

During this incubation period, agarose was prepared. Hereby, 2.5% agarose was dissolved in dH_2O (distilled water) and kept at 50°C. Complete DMEM was also pre-warmed up to 50°C. A 0.25% agarose was prepared in the pre-warmed complete medium at 2 h post infection. At this time, media containing virus was removed from each of the infected well (one column in row) and replaced with 1 ml 0.25% agarose containing complete medium. This procedure has to be done pretty fast to avoid polymerization of the agarose. Plates were then left at room temperature (RT) for 30 min to allow agarose to polymerize and then transferred to the cell culture incubator (96% humid CO_2 incubator (5% CO_2) at 37°C). One week later, another 1ml of agarose-containing DMEM complete 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 infection independently in different cell groups. At 2 weeks pi, viral plaques were counted in wells with separate plaques. The viral titer, also referred to as plaque forming unit (pfu) was calculated as following:

pfu/ml=

#plaques/dxV

d= dilution factor

V = volume of diluted virus added to the well

2.2.3 Viral infection assay

In most experiments, HFF cells were infected with HCMV viral type at moi of 0.5. Cells were counted after trypsinization using the Neubauer chamber. The number of cells is important to determine the virus concentration to be used for a specific moi. The volume of virus containing medium is important as the minimal amount should be used to allow viral infection of the cell. Table 2.1 summarizes the different growth medium and virus containing medium used for each different cell culture unit used in this work.

Table 2.1: Amount of complete DMEM medium used in cell culture and infection experiments.

Cell culture unit	Growth medium (ml)	virus containing medium (ml)
175 cm^2 (big flask)	20	4
75 cm^2 (medium flask)	10	2
25 cm^2 (small flask)	4	1
6 well plate (per well)	3	1
12 well plate (per well)	1	0.4

24 well plate (per well)	1	0.2
Chamber slide (per well)	0.5	0.15

The appropriate amount of virus was always diluted in complete medium to give the desired moi. Complete medium was warmed up to room temperature, while virus stock was used as soon as it was thawed. The virus containing medium was always prepared directly before infection. For infection, medium was removed from cells and virus containing medium at the specific moi was added instead. In most cases infection lasted for 2h, when virus containing medium was removed and replaced with fresh medium or drug containing medium in drug inhibition assays (see 2.7). The hours of post infection (hpi) varied depending on the experiment between 2 hpi and 96 hpi. In mock infections, no virus was added at any time point of the experiment.

2.3 Drug inhibition assay and Inducing ER stress

To investigate the influence of ER stress on HCMV infected cells, BAPTA-AM (cat# 11696, Sigma-Aldrich, Germany) and Thapsigargin (cat# 9033, Sigma-Aldrich, Germany) were utilized. Stock solutions of both drugs were prepared in DMSO and therefore the same concentration of DMSO was used in drug control experiments. Initially, these drugs were tittered before calibration experiments to identify the concentration, which does not harm the cells, but induce ER stress. Starting with 2 µM, a concentration of 0.5 µM was found to be the optimal concentration, which was not toxic to the cells, but induced ER stress.

Drugs were added to HFF cells pre-infection or at post infection with HCMV virus. In the first case, cells were incubated for 30 minutes with medium containing either drug or DMSO control. Cells were then either infected or mock infected at moi of 0.5, 2 h later virus containing medium was removed and replaced with fresh medium. In the second case, cells were either infected or mock infected at moi of 0.5, 2 h later virus containing medium was removed. Cells were washed twice with complete medium before adding complete medium containing either the drug or DMSO control. With the exception of kinetic experiments, the effect of ER stress was detected

at 96 hpi. At 96hpi supernatant was frozen to perform plaque assay and viral load, while cells were subjected to IF (see 2.5) or western blot (WB, see 2.9). Frozen supernatants of drug inhibition versus no inhibition were thawed and subjected to plaque assay and viral load analysis.

2.4 Kinetic experiments

These experiments were performed to detect the influence of ER stress inducer thapsigargin part during HCMV infection. Infection was performed as detailed in 2.2.3 or mock infected on 12 well plates for immunofluorescence (IF) analysis and 6 well plates for protein analysis. Testing was performed every 2 hpi or in 24 hpi intervals (24 hpi, 48 hpi, 72 hpi and 96 hpi) and subjected to IF analysis, while 200 µl aliquoted supernatants at these exact time points were frozen at -70 ºC and later subjected to viral load analysis. For Western blot kinetic analysis, infected versus mock infected cells were lysed every 2 hpi or in 24 hpi intervals (24 hpi, 48 hpi, 72 hpi and 96 hpi) and subjected to WB analysis as detailed in 2.9. In some cases the drug was added before infection and then infected as in 2.2.3 and subjected to kinetic experiment.

2.5 Immunofluorescence (IF)

Immunofluorescence (IF) is widely used for the rapid diagnosis of virus infections by the detection of virus antigen in clinical specimens, as well as the detection of virus-specific IgG or IgA or IgM antibody. Cells were grown directly on pre-sterilized coverslips (18 mm) in 12 well plates or on 8-well glass slides (Labtek chamber slid, cat#177402, Nalge Nunc International, Naperville, IL 60563). The cells were infected at moi of 0.5, once cells were 90% confluent. Two hours after infection, virus containing complete DMEM was removed and replaced with virus free complete media for 96h (=96hpi). At 96 hpi, 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, washed with PBS (3X), permeablized with 0.1% Triton X-100 (T8787, Sigma-Aldrich, diluted in PBS) for 1-2 min at RT. After another was (5X with PBS), blocking with 1% bovine serum albumin (BSA, 0175,

AMRESCO Inc., USA, prepared in PBS) was performed. Blocking was performed for 30-60 minutes or overnight at 4°C.

First antibody was diluted in 0.5% BSA and incubated with cells for 2 hrs at RT or over night at 4^{o}C. In case of co-localization experiments, the first two antibodies of the different antigens were pre-mixed before adding them to the cells. After sucking out the antibody solution, cells were washed five times intensively and carefully.

The secondary antibody was 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 the dark. In case of co-localization experiments of IF, secondary antibodies were pre-mixed before adding them to the cells. Before the last wash, the nucleus was stained with 1µg/ml 4′, 6-diamidino-2-phenylindole, 2HCl (DAPI, 268298, Calbiochem, Germany) for 10 min at RT. Cells were washed 5 times with PBS. Finally, Mounting medium was done to helps preserve your sample and raises the refractive index to give good performance with oil objectives. cells were covered with mounting anti-fading solution (0.5% g n-porpyl gallate; P3130, Sigma-Aldrich, Germany; 100mM Tris pH 9 , 70% glycerol).

In case of 8-well chamber slides, the wells were carefully peeled off, covered with a cover slip and gently pressed using kimwipes to get rid of excessive mounting solution. The edges of both surfaces of the slide and cover slip were carefully glued with colorless nail polish. In case of glasses in 12 well plates, glasses were inverted on objective slide, gently pressed using kimwipes to get rid of excessive mounting solution and carefully glued with colorless nail polish to the objective slide.

2.6 Monoclonal, Polyclonal Antibodies and Fluorescing Markers

Variable monoclonal and polyclonal antibodies used in this work were to perform either IF or Western blot (WB) experiments.

Primary mouse monoclonal antibodies (mAbs) against HCMV: pp28 (CA004-100), pp65 (CA003-100), ICP22 Delayed Early Protein, against CMV ICP36 DNA Binding Protein , CMV gb-CH28, and glycoprotein B (gB, CA005-100) and IE1/IE2 (CH160) were purchased from Virusys Corporation (Sykesville, MD, USA).

The cellular antibodies were mainly rabbit and polyclonal, so co-localization with viral antibodies can be performed. GRP78 Bip (ab21685), beta-Actin-Loading control (ab8227) and eIf-2α (ab32157) were purchased from Abcam (Abcam, United Kingdom). CREB-2 (C-20) was purchased from Santa Cruz Biotechnology inc. (USA). Secondary antibodies, Goat anti Rabbit-Cy2 (111-225-144), Goat anti Rabbit-Cy5 (111-175-144), Goat anti-Rabbit-HRP (111-035-003), Goat anti-Mouse-HRP, Donkey anti-Mouse-Cy2 (715-225-150), Donkey anti-Mouse-Cy5 (715-175-150) were purchased from Jackson Immunoresearch (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). 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).

2.7 Fluorescence Image capturing

Visualizing immunofluorescence staining and capturing was done using an Olympus BX60 and Olympus digital camera DP71 (Olympus, Japan). DAPI staining of the nucleus was visualized via U-MWU mirror, Cy2 via U-MWIB and Cy5 via U-MWIY2 (all Olympus mirrors, Olympus, Japan) respectively. Image of each fluorescence color was captured in single mode using analySIS LS report program (Olympus, Japan). In co-localization experiments, the same image was captured via the different mirrors in single mode and merged via Picture Merge Genius program (EasyTools Software, Inc., USA).

2.7.1 Recording structures of subcellular distribution

Once, a change in nuclear shape or in subcellular distribution was detected via IF, a statistical analysis was performed, to decide, whether this change is statisticaly relevant or not. For this, structures were recorded in 10 fields of at least 100 cells from 3 different experiments. Alternatively, structures were recorded from captured images.

2.8 Cell Lysis for protein extraction

For lysis, DMEM was removed at 96 hpi or from mock infected cells and washed with ice cold PBS. A minimum amount of cold PBS was used in the last wash, in which cells were scraped down into ice cold eppendorf tubes using sterile cell scraper (cat#70-1250, Biologix, KS, USA). Cells were down centrifuged for 10 minutes at 3000 rpm and 4ºC and re-suspended in ice cold cell lysis buffer. Cell lysis buffer was partially freshly prepared prior to the experiment and kept on ice as explained below.

Stock buffer for cell lysis (50mM Tris pH 7.4, 150mM NaCl, and 10% glycerol) was prepared, filtered and stored at 4ºC. Just before use 0.5% NP40 was added as well as protein kinase inhibitor mix (Leupeptin, 1 µg/ml, L2884, Aprotinin, 2.5 µg/ml, A4529, PMSF, 1 mM, P7626 and sodium vanadate, 1mM, S6508, Sigma-Aldrich, Germany). The total amount of lysis buffer depended on the amount of cells; 0.5 ml for medium flask, 0.3 ml for small flask and 0.2 ml for 6 well plate respectively. After 30 minutes on ice with gently mixing in 10 minutes intervals, cell debris was down centrifuged for 10 minutes at 13000 rpm and 4ºC by. Supernatant was kept in 30 µl aliquots frozen at -70 ºC.

2.9 Western Blot analysis (WB)

2.9.1 Preparation of the polyacrylamide Gels

BioRad vertical gel electrophoresis system was used (165-3302 Mini-PROTEAN 3 Electrophoresis Module, BioRad, CA, USA). We was used two types of gels to complete WB assay the first one was called staking gel which is must be used to be sure all the samples loaded

on the gel and run on the same line. But these gel must be prepared after the resolving gel was polymerized.

5% stacking solution was prepared:

- 1.4 ml dH_2O
- 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
- 10% resolving gel for 2 gels:
- 7.9 ml dH_2O
- 6.7 ml 40% Polyacrylamide (PAA, Beit Haemek)
- 5 ml 1.5 M Tris pH 8.8.
- 0.2 ml 10% SDS (in dH_2O)
- 0.2 ml 10% ammonium per sulfate (APS, prepared in dH_2O and frozen until use)
- 0.008 ml TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine, T9281, Sigma, Germany)

Solution was loaded bubble free in BioRad casting system (165-3302 Mini-PROTEAN 3 Electrophoresis Module, BioRad, CA, USA) using 1.5 mm comb.

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 if found.

2.9.2 Sample Preparation

1- 10 µl of 4x Lammli buffer (20mM Tris, pH 6.9, 4% SDS, 40% Glycerol, 0.004% Bromphenol blue+ freshly added 8.4% β-Mercaptoethanol) was added to the 30 µl of protein aliquot (see 2.5).
2- Samples were denaturated at 95 °C for 10 min and kept on ice.

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

4- Proteins were run at 80 V (constant Ampere) for 30 min initially, then at 130 V along with a protein ladder (SM1841, Fermentas).

2.9.3 Blotting (Transfer)

1- 8.5 X 6.5 cm PVDF membrane (1 per gel, BioTraceTMPVDF, Pall corporation, FL, USA) and 8.5 X 6.5 cm Whatmann paper (4 per gel, 580x580 mm thick Whatmann) were prepared

2- PVDF membrane was activated: 5 min incubation in methanol followed by washing in dH_2O and finally well covered in transfer buffer (3g Tris base, 14.4g Glycin, 800ml dH_2O 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 ⇨ 3whatmann

5- Blotting for 1hr, at 25V, constant Ampere in semi dry blotter (this may be subjected to change based on the size of the protein)

6- 1% dry skim milk (Fluka 70166, Sigma-Aldrich, Germany) prepared in PBS containing 1% Tween was used for blocking for 30 minutes

8- An appropriate dilution of first antibody diluted in PBS containing 1% Tween was added to the membrane under gentle agitation for 2h

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

10-Secondary antibody was diluted in PBS containing 1% Tween and added to membrane for 30 min at RT with shaking

11-Membranes were washed 3 times (15 min each) with PBS, last wash was kept until ECL reaction was performed on the same day.

2.9.4 ECL Reaction

2 tube were equally filled with 6 ml 100 mM Tris pH 8.5, 3.3 µl 30% H_2O_2 was added to one tube and 60 µl Luminol (250 mM in DMSO, A8511, Sigma-Aldrich, Germany) +26.6 µl p-coumaric acid (90 mM in DMSO, C9008, Sigma-Aldrich, Germany) were added to the other tube. Solutions in the two tubes were added simultaneously to the membranes and hand shaked with membranes for 1 min. In a dark room, membrane was placed in a Nylon file and covered with medical X-ray film for few seconds or minutes (based on the experience with the antibody to be checked). Finally, negative was developed using a developing machine. If signals were strong, a further shorter incubation of the membrane with negative was performed, if signal was week, a longer incubation was performed before re-developing. Membranes were kept at -20°C, stripped and blotted with another antibody if needed.

2.9.5 Membrane Stripping

Stripping membranes is a method used to denaturate already bound antibodies to allow the access and binding of new antibodies. For this, membrane was treated for 5 min with 300 mM NaOH (in dH_2O) shaking, washed 5 times (15 min each) with PBS before a new block with 1% milk or in 1% skim milk (see 2.9.3) followed and continued as mentioned in 2.9.3.

2.10 Bacterial Transformation

Transformation is the method by which naked DNA is inserted into bacterial cell. In most cases, transformation is performed using competent *E. coli* strains BL21 and DH5α (both strains were kindly provided by Dr. Tchatalbachev, University of Giessen, Germany). This method is essential to amplify plasmid DNA.

2.10.1 Preparation of competent bacterial cells

Competent bacterial cells were prepared according to the classical method of Cohen et al (Cohen et al, 1972). All bacteria were grown and cultured in Luria-Bertani medium (LB; 10g Bacto-

tryptone, 5 g Yeast extract, and 10 g NaCl dissolved in 1 L dH_2O and autoclaved). An overnight culture from frozen stock (mix of 800 µl bacterial culture and 200 µl Glycerol frozen at -70 ˚C) of either BL21 or DH5α was prepared. A 1:100 dilution (1500 µl bacteria to 150 ml fresh LB medium) was made regularly on the day of the experiment in Erlenmeyer flask and left to grow by shaking at 37 ˚C (shaker incubator; Barnstead Laboratory apparatus, USA). Once the OD_{578} reached 0.2-0.3 (normally within 1-2 h), bacterial cells were centrifuged for 10 min at 4000 rpm and 4 ˚C. The supernatant was discarded completely but carefully, replaced by 30 ml ice cold 100 mM $CaCl_2$ and incubated on ice for 30 min. Competent cells were re-centrifuged by refrigerated centrifuge for 10 min at 4000 rpm. Finally, bacterial cells were re-suspended in 3 ml $CaCl_2$ and kept on ice in 50 µl until transformation experiment or frozen at -70 ˚C after adding 1% DMSO.

2.10.2 Transformation of UL97 plasmid DNA

1-5 µl (10-9 ng) plasmid DNA encoding Ampicillin or Kanamycin resistance gene were added to 50 µl competent cells (see 2.10.1), mixed gently and incubated on ice for 15 min followed by 5 min incubation at 37˚C. This step was repeated. 400 µl LB medium was added to the mixture and incubated for 1 h shaking at 37˚C. This is the step, in which the transformed cells are propagating. Finally, the bacterial mixture is spread on LB-Agar containing Ampicillin (100 µg/ml) or Kanamycin (30 µg/ml) and grown overnight at 37˚C.

Bacterial colonies were picked on the next day and analyzed directly per PCR or grown in LB containing the desired antibiotic, subjected to DNA analysis followed by restriction enzyme analysis.

2.11 DNA extraction from eukaryotic cells

Viral supernatants or lysed cells were thawed and subjected to total DNA extraction using QIAamp DNA Mini Kit (cat#51304, Qiagen, Germany). In most cases 200 µl of viral supernatant were aliquoted and subjected directly to DNA extraction, while lysed cells were

adjusted to 200 µl using PBS. The extraction steps were performed according to the manufacturer's instructions as followed:

1. 20 µl Qiagen Proteinase K were pipetted into the bottom of a 1.5 ml microcentrifuge tube.
2. 200 µl samples were added to the microcentrifuge tube. If the sample volume is less than 200 µl, PBS was added to adjust the amount.
3. 200 µl Buffer AL were added to the sample, mixed by pulse-vortexing for 15 s.
4. Mixture was incubated at 56 ºC for 10 min followed by a short spin down
5. 200 µl ethanol (96-100 %) were added to the sample, mixed by vortexing and briefly centrifuged to remove drops from the inside of the tube lid.
6. Mixture from step 5 was applied carefully to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and column in the collection tube was centrifuged at 8000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2ml collection tube, and discard the tube containing the filtrate.
7. 500 µl Buffer AW1 were added to the QIAamp Mini spin column and add without wetting the rim and centrifuged at 8000 rpm for 1 min. Filtrate was discarded.
8. The QIAamp Mini spin column was replaced in a clean 2 ml collection tube, 500 µl Buffer AW2 were added and. Filtrate was discarded and centrifuging step was repeated.
9. Finally, the QIAamp Mini spin column was placed in a clean microcentrifuge tube, 200 µl elution buffers was added to the column and centrifuged at 14000 rpm for 3 min. The filtrate in this step resembles the isolated DNA and was kept at -20 ºC.

2.11.1 UL97 Plasmid DNA isolation (“Maxi prep”)

For plasmid DNA isolation, transformed bacterial cells containing the desired plasmid were grown overnight in LB medium containing the suitable antibiotics. For maximal preparation of plasmid DNA, Maxi prep was performed which requires 300 ml fresh overnight bacterial culture. The maxi plasmid extraction procedure was performed using Fast Ion™ Plasmid Kit (YPI25,

RBC Biosciences Corp., Taiwan) according to manufacturer's instructions. A 300 ml of overnight bacterial culture grown in LB medium at 37 ^{0}C were centrifuged at 6000 rpm for 15 minutes. Bacterial pellet was re-suspended in 10 ml PM1 buffer (supplemented with RNase A), vortexed well and re-suspended in 10 ml PM2 and incubated for few minutes at RT. The last steps allow the suspension to show some clearance due to the lysing action of the buffer, which is neutralized by adding 10 ml PM3 buffer. The mixture is inverted 10 times and centrifuged at 15000 rpm for 20 minutes at RT.

Meanwhile, the provided column (PI Column) was equilibrated by applying 5 ml PEQ buffer, flow through was discarded and the supernatant from the neutralized bacterial solution was applied to the equilibrated PI Column. The flow through was discarded and bound DNA was washed by 12 ml PW buffer. Bound DNA was eluted by adding 8 ml PEL buffer, and the flow through DNA was precipitated by Isopropanol (75% of the eluted volume) and vortexing well. DNA was precipitated at 20000 rpm for 30 minutes at 4 ^{0}C, the supernatant was discarded, and the precipitate was washed by 75% ethanol, re-centrifuged (20000 rpm for 30 minutes at 4 ^{0}C). The supernatant was discarded and the precipitated DNA was allowed to air dry. Finally, the DNA was re-suspended in 500 µl ultra pure water.

2.12 Transfection of 293 cells

Transfection is a method, by which naked DNA is inserted into eukaryotic cells. Expression of this DNA should be detectable by different biochemical assays including WB and IF. In this 293T cells were subjected to transfection either in 12 well plates (for IF analysis) or in 6 well plates (for WB protein analysis). Transfection was performed using the PolyFect Transfection reagent kit (cat#301107, Qiagen, Germany) according to the manufacturer's instruction as followed:

1. Cells are seeded a day before transfection, 1x 10^5 cells per well (6 well plate) and 1X10^4 per well (12 well plate containing glass slides).
2. Cells were incubated at 37°C and 5% CO_2.

3. Meanwhile, 2 µg/1 µg DNA dissolved in 100 µl/50 µl serum and antibiotic free DMEM medium, and mixed with 20 µl/10 µl Polyfect transfection reagent at RT per well for 6 well plate/12 well plate. The mixture was incubated for 5–10 min at RT to allow for complex formation.

6. The complex was gently pipetted into the well on the freshly seeded cells and incubated at 37°C and 5% CO_2 for 48 h.

A GFP labeled plasmid was used a positive control for transfection efficacy.

2.13 Viral load; Real time PCR method

Viral supernatant was thawed and total viral DNA was extracted from 200 µl sampels (see 2.10). DNA was either frozen at -20 °C or directly subjected to viral load analysis. Real-time PCR for viral load detection was performed using an ABI Real Time PCR 7500 (applied biosystems, USA). Positive controls, negative controls and samples were tested in duplicate manner. A total of 20 µl reaction mixture consisted of 5µl control (positive control; standard, negative control, ultra pure water) or sample's DNA, 10 µl PCR master mix (Power SYBER Green, cat# 4367659, Applied Biosystems, United Kingdom), 1 µl of each primer (forward and reverse, each at 10 pmol/µl, Metabion, Germany), and 3 µl ultra pure water.

A validated viral DNA (CMV_{AD169}, quantitated DNA PCR control, cat# 08-925-000, Abionline, MD, USA) was used as standard in all experiments. The standard was serially diluted in ultra pure water from 10^6 down to 10^1 copies/µl.

The forward and reverse primers were chosen at the Virology laboratory using the major glycoprotein DNA of HCMV (NC_006273.2, http://www.ncbi.nlm.nih.gov/nuccore) as a template after downloading it into CloneManager program.

CMV –gb-F: 5'-CTA TCG CGT GTG TTC TAT GGC-3'

CMV –gb-R: 5'-CAG GTG ACA TTC TTC TCG TCC-3'

The amplification reaction started with 2 min at 50 °C, followed by 10 min at 95°C and final 45 cycles as following: 95°C for 15s and 60°C for 1 min followed by 15 sec at 95˚C, 1min at 60˚C, 30 sec at 95˚C and finally 15sec at 60˚C.

2.14 Amplification of UL97 gene

UL97 gene was amplified by PCR using of 25 µl total PCR reaction which included 5.25 µl extracted DNA (see 2.11.1), 1µl of each primer (each 10 pmol/µl)
5'- TGG CCG ACG CTA TCA AAT TT-3'
5'-CGA CAC GAG GAC ATC TTG-3' (Jared et al., 2007), and 12.5 µl PCR master mix (Ready Mix PCR Master Mix of Thermo scientific, Cat. No. AB-0575\Dc\LD). The reaction was carried out in PCR tube (0.2 ml Axygen INC, USA) using Swift™ MaxPro Thermal Cyclers (ESCO Global, USA). The PCR reaction started with a single hot start step for 5min at 95ºC followed by 45 cycles in the following order, 20 s at 95ºC for denaturation, 1 min at 45 ºC for annealing and 2 min at 72 ºC for extension respectively. A final elongation step was performed for a further 10 min at 72 ºC, assuring the complete extension of the amplified product.

Chapter Three

3. Results

3.1 Induction of ER stress using Thapsigargin (TH)

In order to detect the ability of Thapsigargin (TH) to induce ER stress, cells were incubated with TH containing medium for 96 h. The induction of ER stress was detected using BIP antibody. BIP is a good marker for ER stress detection(Buchkovich et al., 2008). Using TH, ER stress was induced as clearly detectable via IF results. The expression of BIP in TH treated cells compared with those untreated reflects the induction of ER stress.

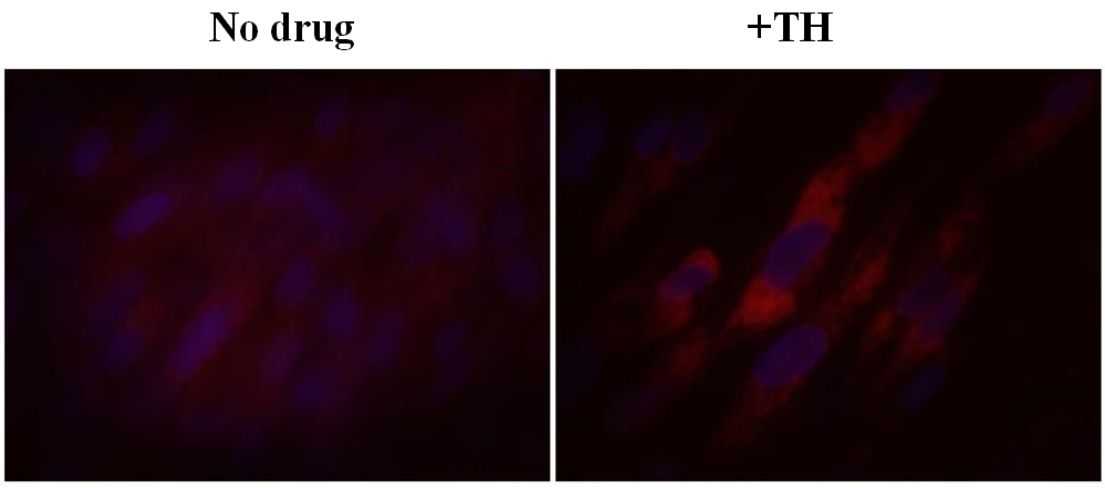


Figure 3.1: Induction of ER stress in HFF cells using TH. HFF cells were incubated with TH containing media for 96 h. Cells were then subjected to IF staining (see also 2.2.3) using rabbit anti BIP as first antibody diluted in 0.5% BSA and added to the blocked cells for 2h at RT. After intensive washes, the 2nd antibody was added for 30'; Cy5 anti-Mouse (red). The nucleus was counter-stained with DAPI

(blue). Staining was visualized with Olympus BX60 and Olympus DP71 digital camera.

3.2 Induction of ER stress using TH in HCMV infected HFF cells

In order to investigate the effect of TH in HCMV infected HFF, cells were first infected with AD169 for 2 h as detailed in 2.2.3. TH containing medium was added at 0 hpi. Initially the effect of TH was detected only at 72hpi and 96hpi using BIP as ER stress marker.

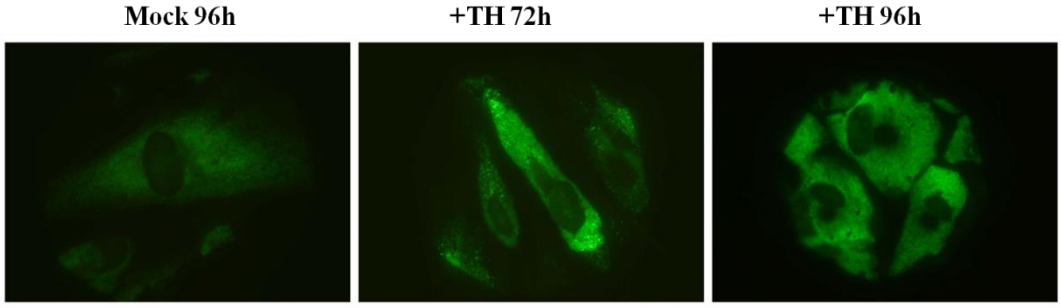


Figure 3.2A: Kinetic induction of ER stress in AD169 infected HFF. HFF cells were infected with AD169 at moi of 0.5; virus containing media was removed after 2h and replaced with media containing TH (TH, see drug inhibition assay, 2.3). At 72 hpi and 96 hpi cells were subjected to IF analysis as detailed in 2.3. BIP antibody was diluted in 0.5% BSA and added to the blocked cells for 2h at RT. After intensive washes, the 2nd antibody was added for 30'; Cy2 anti-Rabbit (green). Staining was visualized with Olympus BX60 and Olympus DP71 digital camera.

As clearly illustrated in figure 3.2A, ER stress was induced in infected cells in first picture infected but non treated with TH , but remarkably induced in TH treated infected cells in time dependent manner by the kinetic experiment we was do it we noticed theses results. The effect of TH was well reflected by the strong intensity of the Cy2 marker. This clear intensity was visible in all experiments performed using TH in this work. At least 100 experiments done in this work for the different co-localizations and investigations (see below) confirmed this result constantly.

As clearly demonstrated via IF analysis, ER stress induction resulted in the induction of ER stress marker BIP. This induction was confirmed using WB analysis in order to illuminate the BIP protein expression in quantitative manner.

Hereby, HFF cells were infected with either AD169 or pUL97 deletion mutant (ΔUL97) or infected and treated with TH. At 96 hpi, cell lysates were subjected to WB analysis using equal amount of protein as indicated via β-actin immunobloting (see lower panel, figure 3.2B).

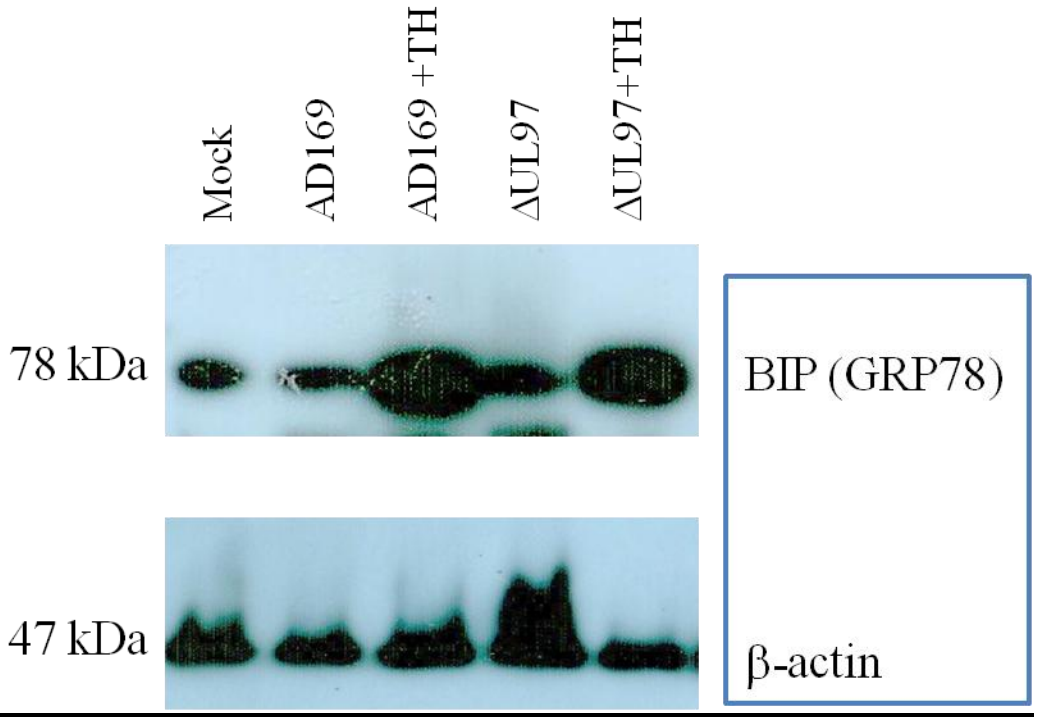


Figure 3.2B: BIP protein expression in HCMV infected cells. HFF cells were infected with either AD169 or ΔUL97 or infected and treated with TH. At 96 hpi, cell lysates were subjected to WB analysis. BIP was detected with anti Rabbit BIP antibody (upper panel), β-actin with anti rabbit β-actin antibody (lower panel), followed by HRP conjugated anti rabbit respectively.

Figure 3.2B emphasized the results of IF analysis, showing a clear quantitative induction of BIP amount via immunoblot in TH treated infected cells. Interestingly,

in case of ΔUL97 infection, the BIP amount was dramatically reduced if compared with ΔUL97 infected TH treated cells. There is no clear difference between AD169 and ΔUL97 on infected cells regarding BIP protein expression level.

3.3 HFF cells during HCMV infection and TH treatment

HFF cells are known to be the only well infected cells for in-vitro experiments of HCMV infection assays. In order to test whether the cells are affected during infection and treatment, cells were infected with either wt-HCMV; AD169 or pUL97 deletion mutant (ΔUL97) as detailed in 2.2.3 and mock or drug treated using TH.

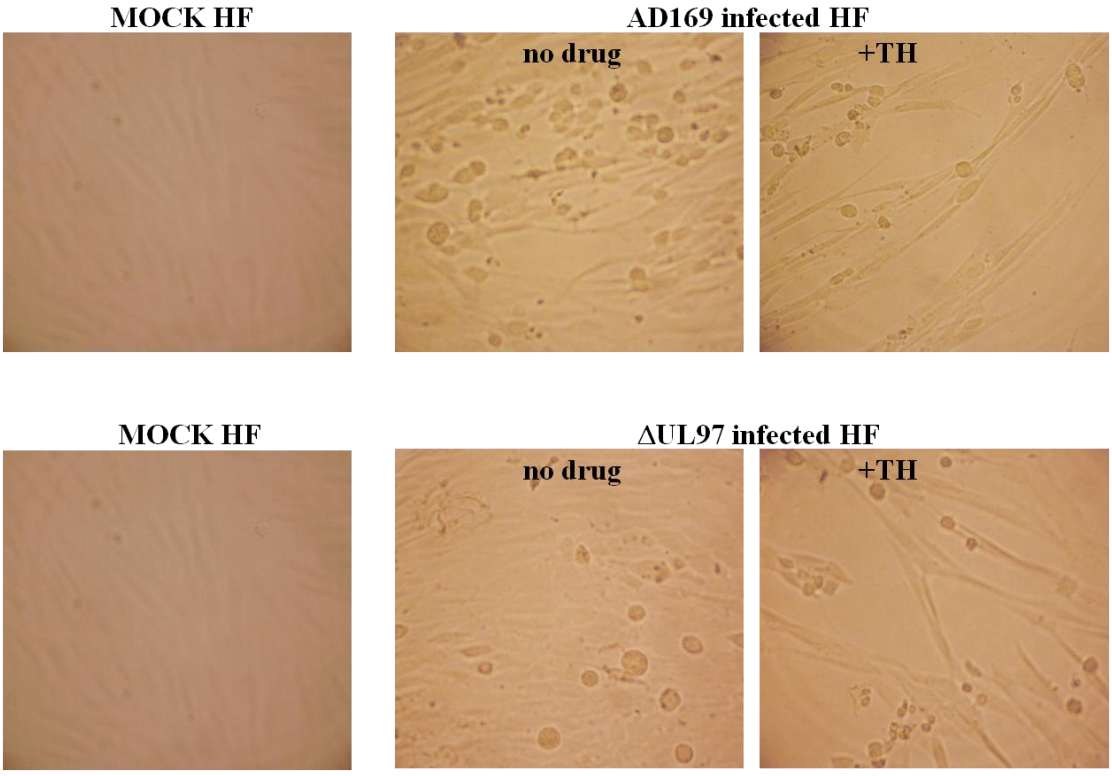


Figure 3.3A. HFF cells during HCMV infection and TH treatment. HFF cells were either mock infected or infected with AD169 or ΔUL97 at 0.5 moi. Infected cells were either mock treated or TH treated at 0 hpi. At 96 hpi, images were

captured through Olympus CKX41 inverted microscope using a regular digital camera (Olympus FE-46, 12 Megapixel).

As clearly shown in figure 3.3A, many cells died due to infection and TH treatment. Although infection by itself did not reduce, cell number while HFF cells clearly suffered from TH treatment.

In order to show that the cells surviving the treatment were not structurally affected, a known cellular marker was used (see figure 3.3B). Cellular AC markers are modified accordingly with HCMV infection (Sanchez et al 2000a, 2000b; Azzeh et al, 2006; Das et al, 2009). Therefore, it will be very important to examine, whether the ER stress effect of TH affects Golgi in uninfected cells. Hereby HFF cells were either mock treated or TH treated for 96 h. Cells were then subjected to IF analysis using rabbit anti TGN (Trans Golgi network) marker (green) and counterstained with DAPI (nuclear staining; blue).

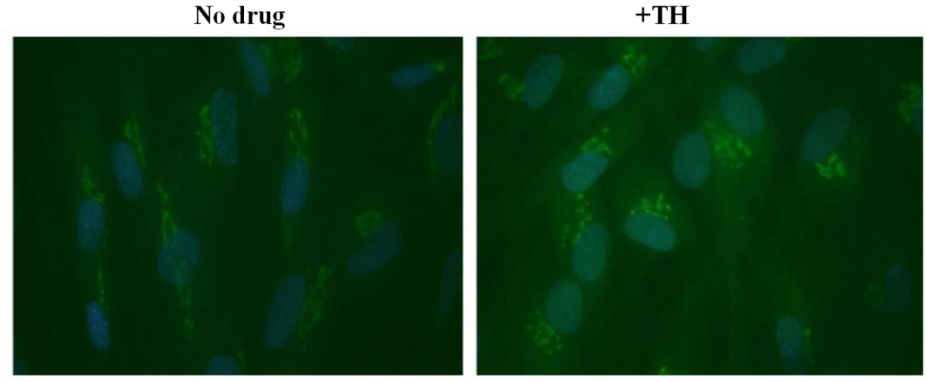


Figure 3.3B: Subcellular distribution of TGN in mock treated versus TH treated cells. HFF cells were either mock treated or subjected to drug inhibition using TH for 96 h. Treated cells, versus mock treated cells were subjected to IF analysis using rabbit anti TGN marker (green) and blue DAPI stain for nucleus.

TGN distributed in mock non treated cells as thread like shape around the nucleus as expected. In cells treated with TH, TGN was more compact but distributed similarly

as in mock non treated cells. This experiment indicated that ER stress does affect or damage the machinery (Golgi) involved in the AC building.

3.4 The role of ER stress in subcellular distribution of viral assembly complex (AC) marker pp28

Figure 3.2 revealed that ER stress is induced using TH in HCMV infected cells. Here, the effect of ER stress induction on HCMV viral assembly complex (AC) was detected using pp28 AC marker. For this cells were infected with either wt-HCMV; AD169 or pUL97 deletion mutant (ΔUL97) as detailed in 2.2.3 and mock or drug treated using TH. Cells were stained for pp28 antibody and visualized using anti mouse Cy2.

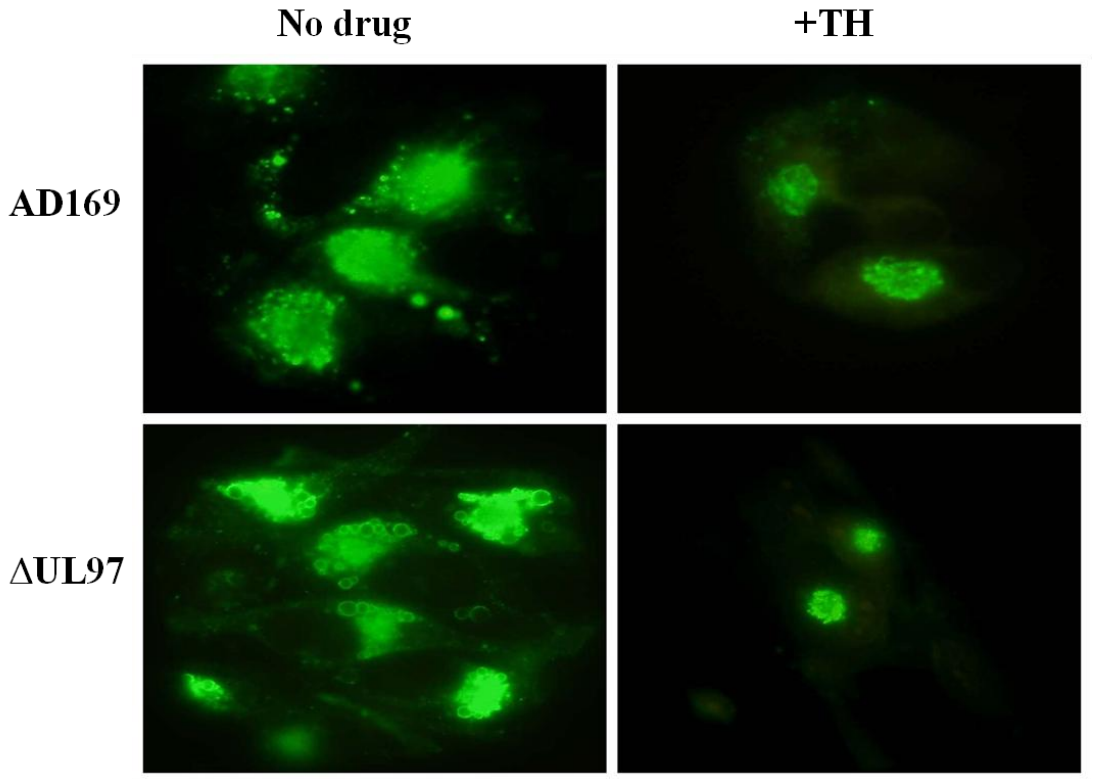


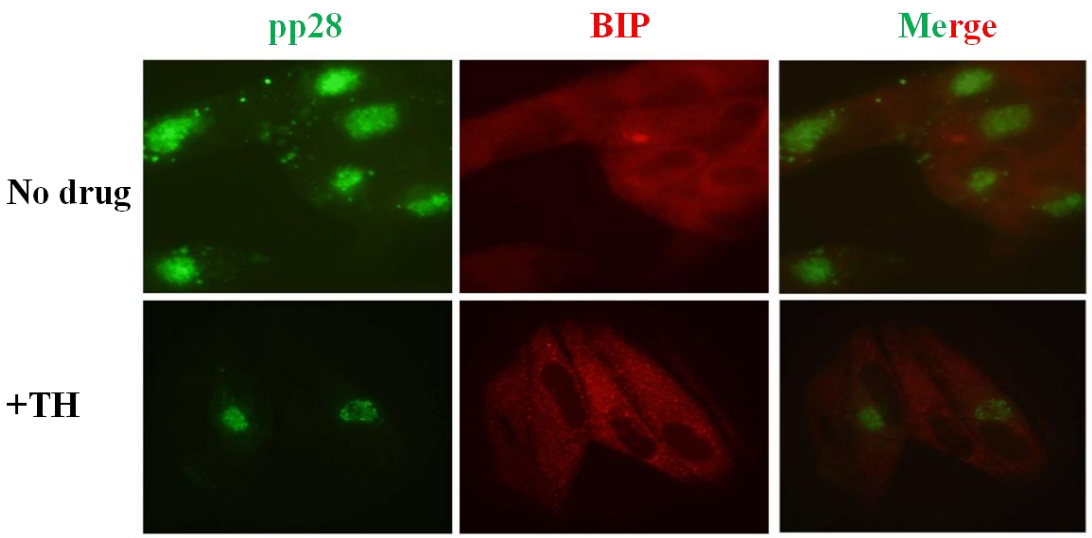
Figure 3.4: Subcellular distribution of pp28 viral tegument protein in AD169 or ΔUL97 infected cells. HFF cells were infected with AD169 (upper row) or ΔUL97 (lower row) and subjected to drug inhibition using TH at 0 hpi. Infected treated cells, versus infected mock treated cells were subjected to IF analysis using mouse anti pp28 and Cy2 anti mouse as secondary antibody (green).

As clearly observed in 3.4, in the absence of TH, pp28 assumed its "bulb"-like subcellular distribution in AD169 infected cells and the crown"-like vacuole rich juxtanuclear structure in ΔUL97 infected cells (Azzeh et al., 2006). Treating infected cells with TH did not alter the subcellular distribution of pp28 in AD169 infected cells. However, TH affected the pp28 subcellular distribution in ΔUL97 infected cells. Hereby, pp28 assumed the original "bulb"-like structure known from wt-HCMV infected cells. Interestingly, also the "kidney"-like shaped nucleus, typical for AD169 infection (Azzeh et al, 2006) was also observed in TH treated ΔUL97 infected cells. This result is an initial indication that pUL97 (gene product of UL97) may be important in HCMV induced ER stress. It is also clear that the PP28 fluorescence signal was less intensive in both infection modes, once cells were treated with TH.

3.5 Co-localization of BIP ER marker with pp28 viral AC marker

In this experiment, the localization of BIP relatively to pp28 HCMV tegument protein was detected. Co-localization experiments of cellular ER marker BIP with viral tegument protein pp28 were performed to see whether BIP is involved in AC building. For this, HFF cells were infected with either AD169 or ΔUL97 and mock treated or TH treated at 0 hpi. Antibodies against both proteins; BIP and pp28 were used simultaneously in the IF analysis.

A



B

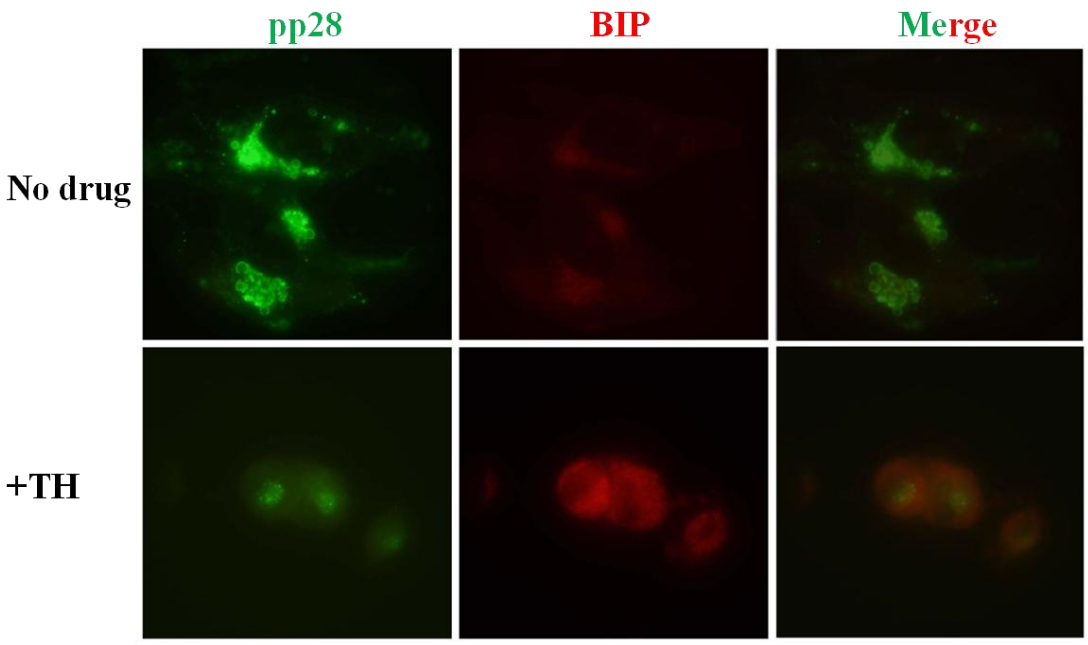


Figure 3.5: Co-localization of ER stress marker; BIP with pp28 viral tegument protein in AD169 (A)/ΔUL97 (B) infected cells. HFF cells were infected with AD169 (A) or ΔUL97 (B) and subjected to drug inhibition using TH at 0 hpi. Infected treated cells, versus infected mock treated cells were subjected to IF analysis using rabbit anti BIP as first antibody and anti rabbit Cy5 as secondary antibody (red) or mouse anti pp28 and Cy2 anti mouse as secondary antibody (green).

In respect to co-localization, pp28 does not co-localize with BIP in either virus infected cells. This fact was constant when cells were treated with TH. A partial co-localization was observed only in some cells.

3.6 Co-localization of other ER marker Calnexin with pp28

Further ER markers were tested for their possible involvement in the AC building. Calnexin is one of these candidates, since it is an integral protein of the ER. As the case for other ER resident proteins, Calnexin acts as a molecular chaperone and participate in the proper folding of polypeptides. For co-localization experiments of Calnexin with pp28, HFF cells were infected with either AD169 or ΔUL97 and mock treated or TH treated at 0 hpi. Antibodies against both proteins; Calnexin and pp28 were used simultaneously in the IF analysis.

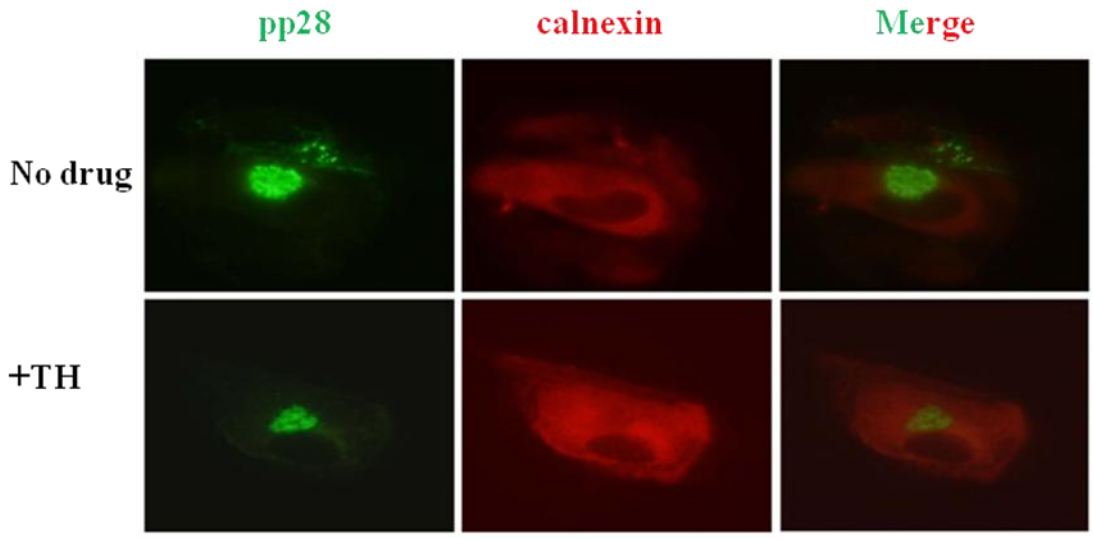


Figure 3.6A: Co-localization of Calnexin with pp28 viral tegument protein in AD169 infected cells. HFF cells were infected with AD169 and subjected to drug inhibition using TH at 0 hpi. Infected treated cells, versus infected mock treated cells were subjected to IF analysis using rabbit anti Calnexin as first antibody and anti rabbit Cy5 as secondary antibody (red) or mouse anti pp28 and Cy2 anti mouse as secondary antibody (green).

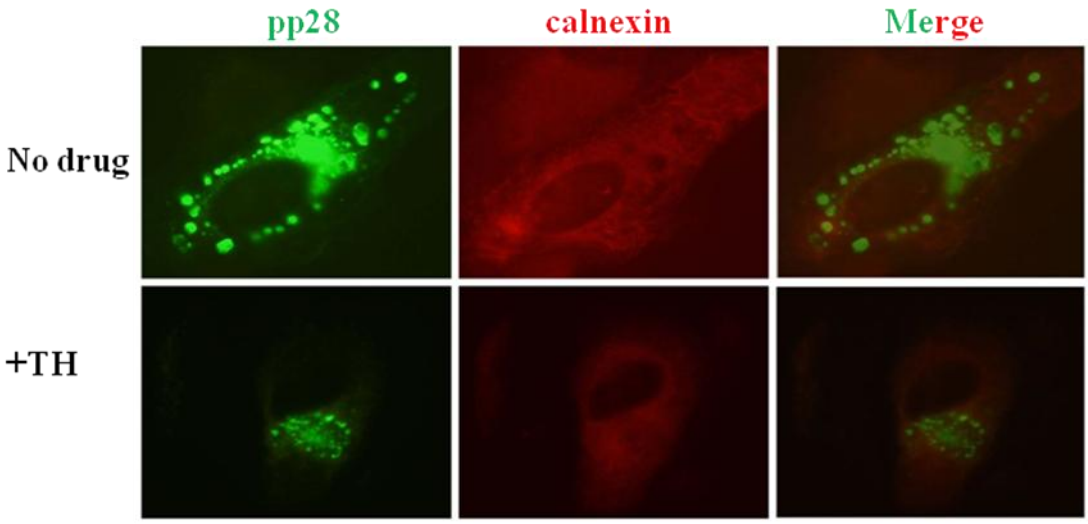


Figure 3.6B: Co-localization of Calnexin with pp28 viral tegument protein in ΔUL97 infected cells. HFF cells were infected with ΔUL97 and subjected to drug inhibition using TH at 0 hpi. Infected treated cells, versus infected mock treated cells were subjected to IF analysis using rabbit anti Calnexin as first antibody and anti rabbit Cy5 as secondary antibody (red) or mouse anti pp28 and Cy2 anti mouse as secondary antibody (green).

The co-localization experiments illuminate clearly, that ER marker Calnexin does not co-localize with pp28 at the AC. Furthermore, the experiments showed that the subcellular distribution of Calnexin was not clearly affected by ER stress induction using TH in either infection cases (AD169 or ΔUL97).

3.7 Co-localization of other ER marker EEA1 with pp28

ER markers EEA1 was reported earlier to co-localize to the center of the AC (Das et al., 2007). Here, the co-localization of EEA1 to the AC using pp28 AC marker was detected under ER stress condition in wt and in ΔUL97 deletion mutant infected cells. EEA1 (early endosomal antigen 1) is specific to early endosome and essential for fusion between early endocytic vesicles. Early endosomes are cytoplasmic compartments which fuse with endocytic vesicles for redistribution of extracellular compounds to alternate destinations.

For this co-localization experiments of EEA1 with pp28, HFF cells were infected with either AD169 or ΔUL97 and mock treated or TH treated at 0 hpi. Antibodies against both proteins; EEA1 and pp28 were used simultaneously in the IF analysis.

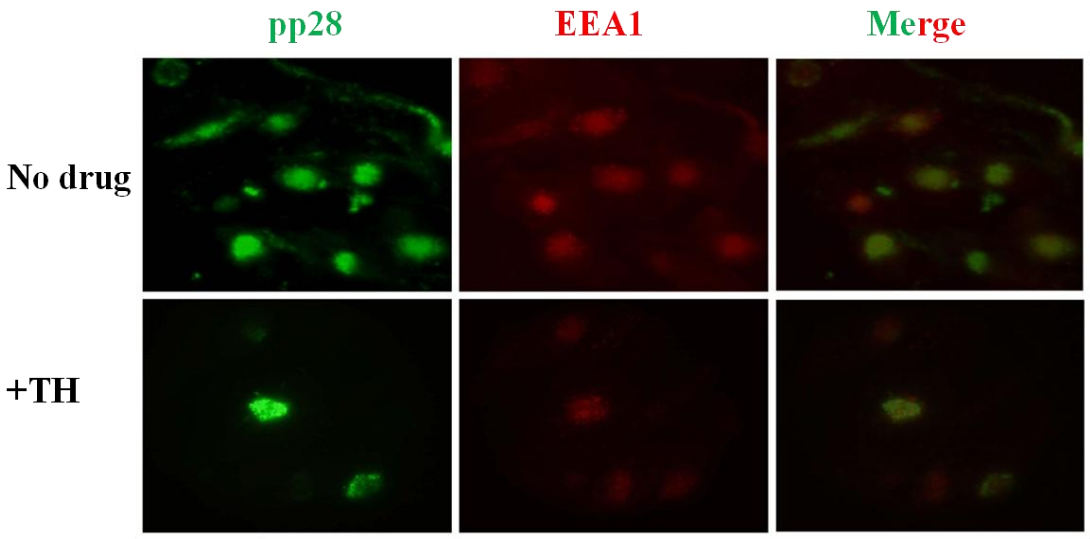


Figure 3.7A: Co-localization of ER stress marker; EEA1 with pp28 viral tegument protein in AD169 infected cells. HFF cells were infected with AD169 and subjected to drug inhibition using TH at 0 hpi. Infected treated cells, versus infected mock treated cells were subjected to IF analysis using rabbit anti BIP as first antibody and anti rabbit Cy5 as secondary antibody (red) or mouse anti pp28 and Cy2 anti mouse as secondary antibody (green).

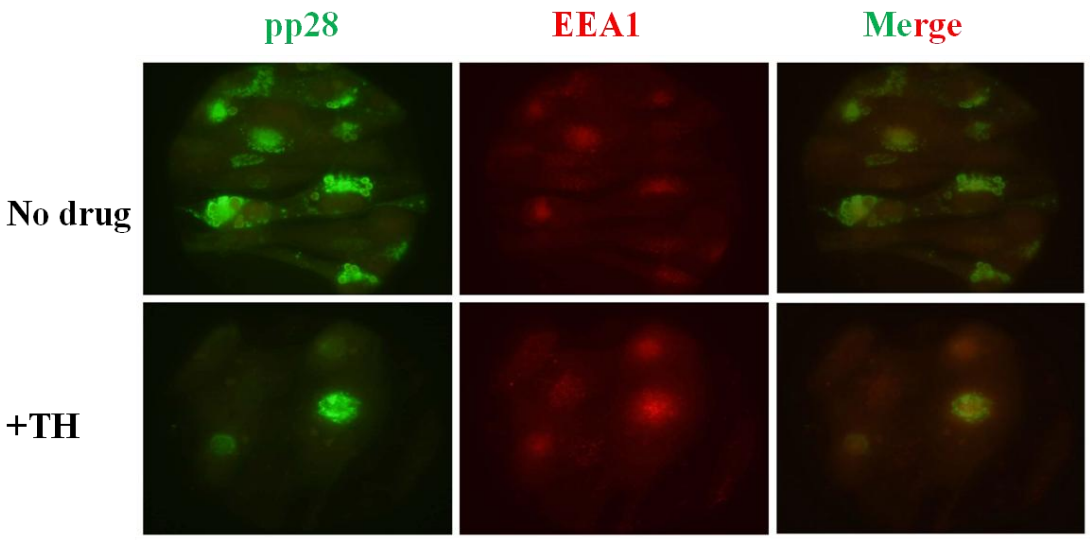


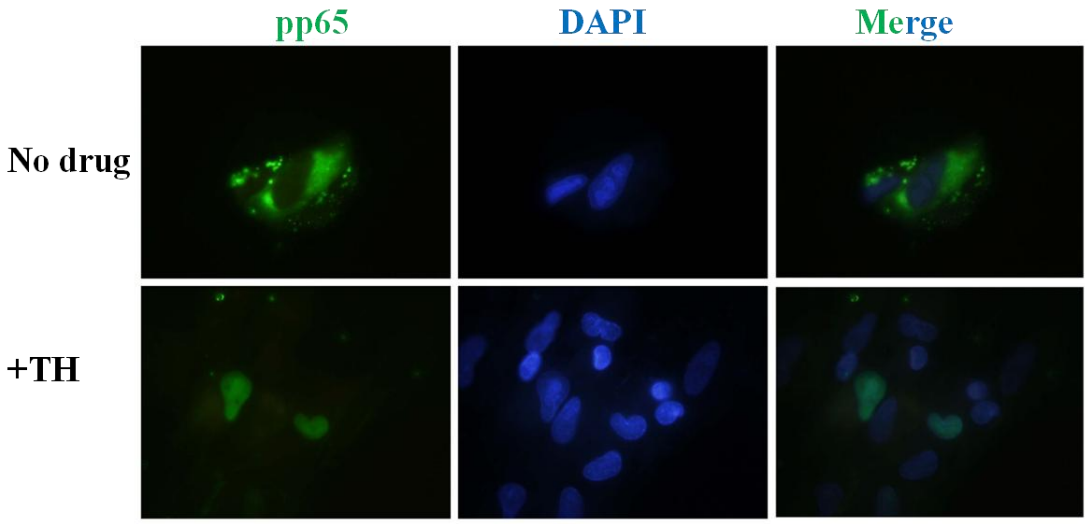
Figure 3.7B: Co-localization of ER stress marker; EEA1 with pp28 viral tegument protein in ΔUL97 infected cells. HFF cells were infected with ΔUL97 and subjected to drug inhibition using TH at 0 hpi. Infected treated cells, versus infected mock treated cells were subjected to IF analysis using rabbit anti BIP as first antibody and anti rabbit Cy5 as secondary antibody (red) or mouse anti pp28 and Cy2 anti mouse as secondary antibody (green).

The results presented here demonstrated that EEA1 did indeed co-localize to the AC and mainly to the center of the AC in wt infected cells. Interestingly, the co-localization in UL97 infected cells was very limited in untreated cells, but obvious in UL97 infected treated cells. This is further evidence that strengthen the point that UL97 plays a role in ER stress.

3.8 The role of ER stress in subcellular distribution of pp65

pp65 is an early late protein and the major tegument protein of HCMV (see 1.3.3.1) and co-localizes to the AC. Here, we were interested in investigating the effect of ER stress induction on pp65 in AD169, as well as in ΔUL97 deletion mutant. HFF cells were infected by either AD169 or ΔUL976 and mock treated or treated by TH at 0 hpi. Infected cells were subjected to IF analysis at 96 hpi using anti mouse pp65 followed by anti mouse Cy2.

A



B

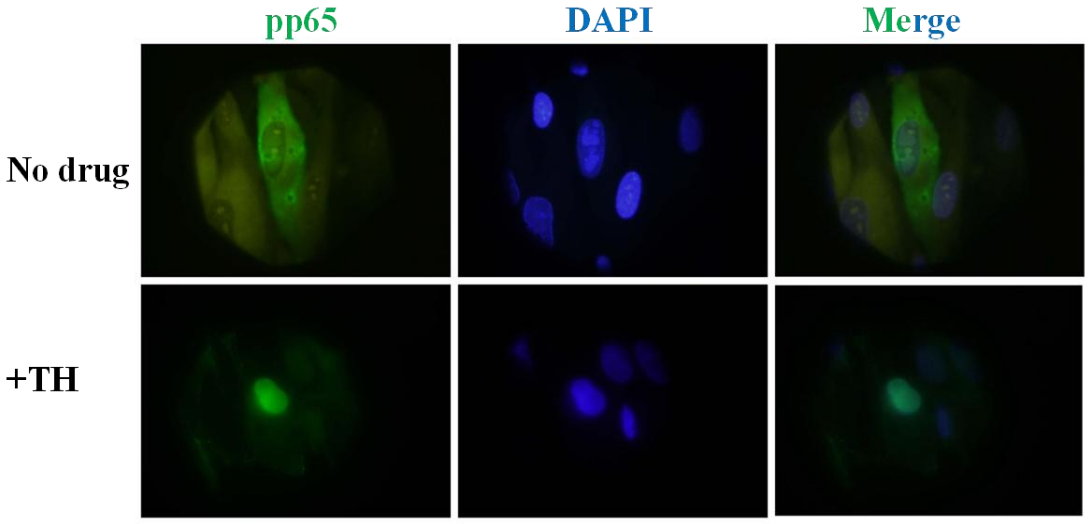


Figure 3.8: Subcellular distribution of pp65 in AD169 (A) or ΔUL97 (B) infected mock treated versus TH treated cells. HFF cells were infected with AD169 (A) or ΔUL97 (B) and subjected to drug inhibition using TH at 0 hpi. Infected treated cells, versus infected mock treated cells were subjected to IF analysis using mouse anti pp65 and Cy2 anti mouse as secondary antibody (green). Nucleus was counterstained with DAPI.

pp65 is known to distribute within the nucleus in the first 48 hpi in cells infected with either AD169 or ΔUL97, however it localizes clearly to the cytoplasm after 72 hpi. Interestingly, inducing ER stress using TH retarded pp65 clearly in the nucleus at 96 hpi in most infected cells. This result indicated that ER stress delays nuclear egress of viral early late gene proteins.

So far, we were able to detect different viral proteins using IF analysis, however, these results did not reflect the quantitative status of these proteins. pp65 is the major tegument protein and involved in AC building (Prichard et al, 2005, Azzeh et al., 2006). Therefore, pp65 was quantitatively detected using WB analysis in either mock treated or TH treated AD169 or ΔUL97 infected HFF cells.

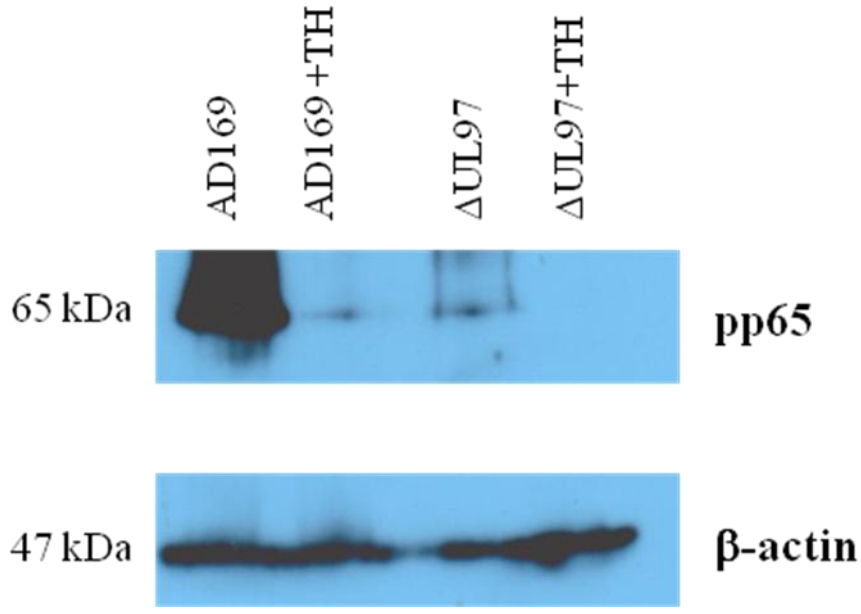


Figure 3.8C: Tegument protein pp65 expression during ER stress. HFF cells were infected with either AD169 or ΔUL97 or infected and treated with TH. Equal amounts of total protein lysates were subjected to western blot analysis at 96 hpi. pp65 was detected with anti mouse pp65 (upper panel), β-actin with anti rabbit β-actin (lower panel), followed by either anti rabbit or anti mouse HRP respectively.

Figure 3.8C showed surprisingly that a clear reduction in pp65 expression occurred in TH treated cells (see pp65 immunoblot, upper panel). Furthermore, pp65 expression was also reduced in ΔUL97 infected cells if compared to AD169 infected cells. β-actin was used to ensure that equal amounts were loaded on gel are equal (protein loading control).

3.9 The influence of ER stress on early genes

Our results revealed that HCMV assembly proteins were clearly affected by inducing ER stress, therefore it was essential to check, whether ER stress may also affect early genes. Early genes are those HCMV viral genes, which are expressed within the first hours of infection, i.e. 24 hpi and do not redistribute to the cytoplasm. An example of these genes is IE1/2 (immediate early protein 1 and 2). HFF cells were infected as above and subjected to IF analysis using mouse anti IE1/2.

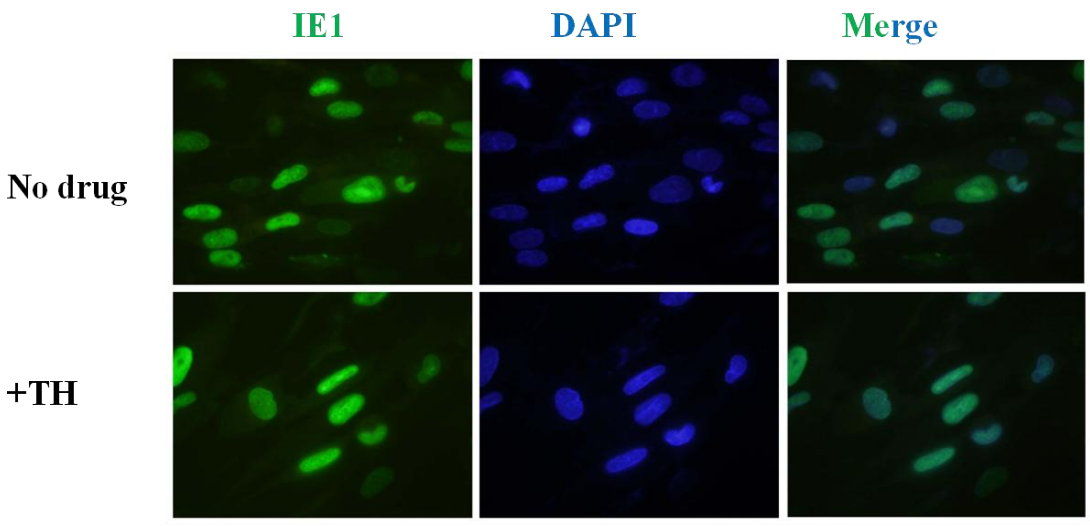


Figure 3.9A: Subcellular distribution of IE1/2 in AD169 infected mock treated versus TH treated cells. HFF cells were infected with AD169 and subjected to drug inhibition using TH at 0 hpi. At 96hpi infected treated cells, versus infected mock treated cells were subjected to IF analysis using mouse anti IE1/2 and Cy2 anti mouse as secondary antibody (green). Nucleus was counterstained with DAPI.

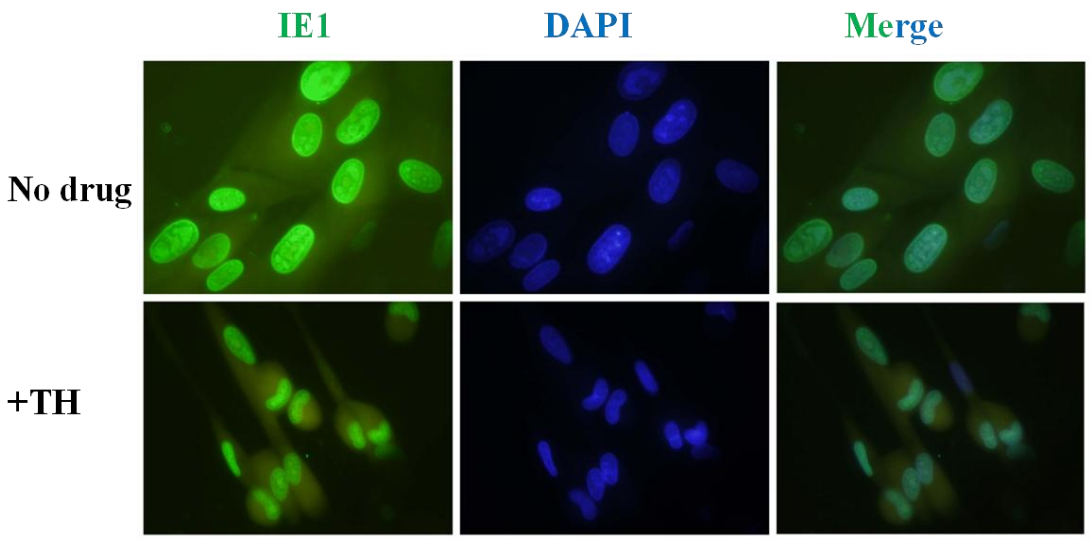


Figure 3.9B: Subcellular distribution of IE1/2 in ΔUL97 infected mock treated versus TH treated cells. HFF cells were infected with ΔUL97 and subjected to drug inhibition using TH at 0 hpi. At 24hpi infected treated cells, versus infected mock treated cells were subjected to IF analysis using mouse anti IE1/2 and Cy2 anti mouse as secondary antibody (green). Nucleus was counterstained with DAPI.

Detecting early gene IE1/2 in infected cells revealed that its subcellular distribution was not affected in TH treated cells, if compared to mock treated cells. These results indicated that viral immediate early genes are not targeted by ER stress induction.

3.10 ER stress effect on viral titer and viral DNA load

Viral titration assays were performed, in order to assess the influence of TH on viable progeny viruses using the traditional plaque assay method (see 2.2.2). Cells were hereby subjected to infection assay using AD169 or ΔUL97, mock inhibited or TH

inhibited in drug inhibition assay. Supernatants were subjected to plaque assay analysis at 96 hpi. The viral titer was strikingly reduced in the TH treated AD169 infected cells compared to TH inhibited ΔUL97 infected cells (figure 3.10). In the first case the viral titer was reduced almost 3 log, but only less than 0.5 log in the second case (figure 3.10).

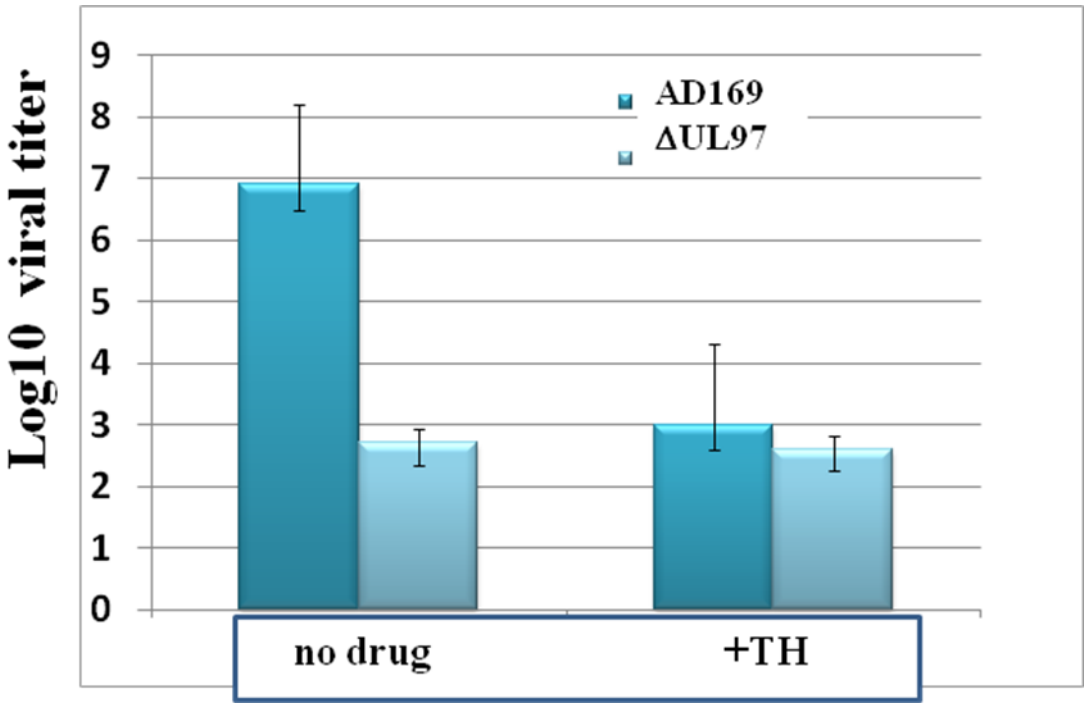


Figure 3.10A: Influence of TH on Viral Titration. HFF cells were infected with either AD169 or ΔUL97 and either subjected to drug treatment or mock treated. At 96 hpi viral supernatants of treated versus mock treated infected cells were subjected to plaque assay. Plaques were counted from 3 different experiments; Log 10 of viral titers is given on the Y axis and infection condition on the X axis.

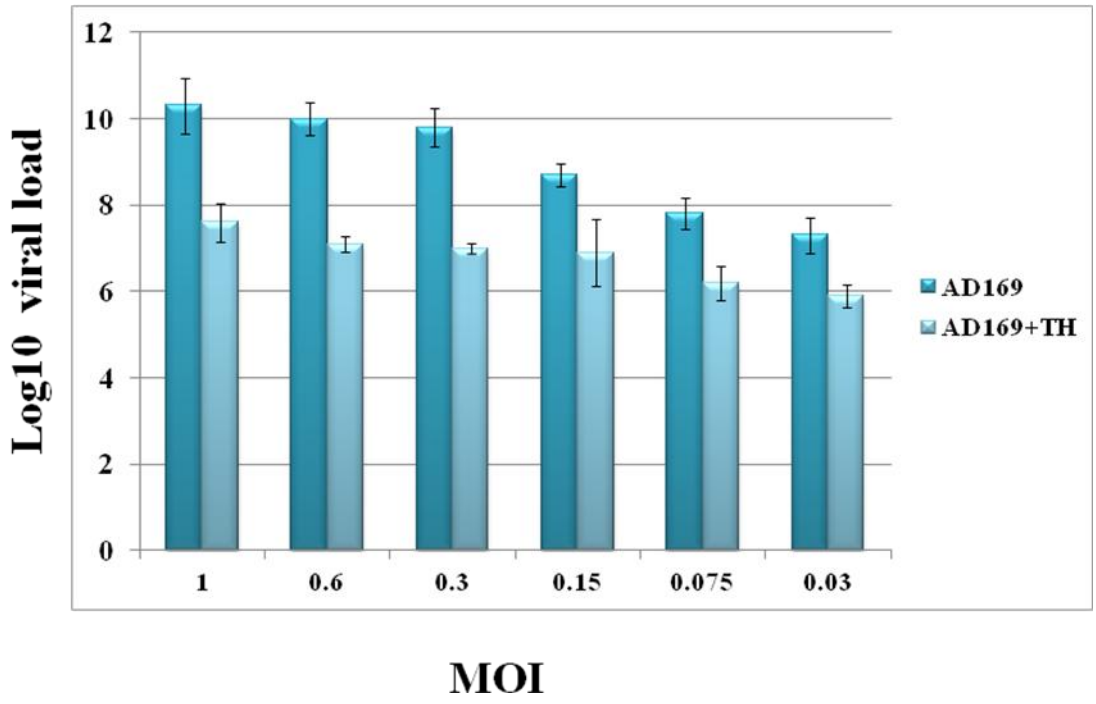


Figure 3.10B: Influence of moi and TH on HCMV viral load. HFF cells were infected with different moi of AD169 and mock treated or TH treated using drug inhibition assay. Supernatants were collected at 96 hpi and subjected to DNA extraction followed by RT-PCR. Calculated Log10 of quantities is given on the y axis and moi on the x axis.

The viral load was determined in infection or drug "inhibition" assays using different viral moi, to see whether the TH affects the cells or the virus directly.

As clearly indicated in figure 3.10B, TH reduced the viral load overall, however, this reduction was lower when less moi was used. While the viral load was reduced 1.5-2 log at moi 0.3-1, it was only reduced 0.5-1 log at moi 0.03-0.15 respectively.

Chapter Four

4. Discussion

In the struggle between viruses and host cells, taking control over the cell's death machinery is very crucial for survival. Several viruses, including human cytomegalovirus (HCMV) induce ER stress and activate UPR signaling (Goodrum et al., 2002; Su et al., 2002; Tardif et al., 2002; Isler et al., 2005a). Due to the 96 hours long life cycle of HCMV, the virus needs to keep the cell functioning at least for its life cycle period and avoid any detrimental effects of cellular stress. The role of the viral UL97 kinase activity and assembly complex in HCMV induced ER stress was not elucidated yet. Our data provides evidence for the influence of induced ER stress on HCMV assembly complex and a role of the viral UL97 kinase in modulating this HCMV induced ER stress.

4.1 Influence of TH induction on BIP and HFF cells

In order to verify the role of ER stress and UL97 on the HCMV life cycle, we used Thapsigargin as ER stress inducer. Initially we demonstrated that treating HFF cells with Thapsigargin indeed induced ER stress using BIP as ER marker. It is known that BIP is induced by HCMV at early phase of infection within the first 48 postinfection (Isler et al, 2005a), in order to prevent UPR activation (Isler et al., 2005a). At late phase of infection, HCMV was reported to target BIP degradation to allow the UPR activation (Isler et al., 2005a). Furthermore, the increase in BIP levels was also shown to play a role in building HCMV assembly complex and egress (Buchkovich et al., 2008; 2009).

4.2 ER stress response and continuous BIP induction

In order to test the effect of continuous BIP induction and thus UPR activation, we treated HCMV infected cells with Thapsigargin. At 96hpi, BIP expression was still induced in Thapsigargin treated infected cells, indicating successful continuous ER stress. This was demonstrated by both, IF and Western blot analyses using BIP specific antibody. The induction of ER stress using TH was further investigated in respect to HFF viability. Direct capture of cells infected and subjected to TH treatment showed no evidence for changes in monolayer structure. It is essential in these experiments to exclude the direct effect of TH on cells and cell structure. A further evidence was made using a golgi marker in HFF cells versus TH treated HFF cells. The golgi structure was not affected by TH treatment nor was it dispersed or depleted.

4.3 The influence of ER stress induction on assembly complex

The subcellular distribution of HCMV assembly complex had been shown earlier to be a "bulb" like juxtanuclear structure, where viral tegument and glycoproteins as well as cellular Golgi and ER proteins are co-localized (Sanchez et al., 2002a; Sanchez et al., 2000b; Azzeh et al., 2006). The formation of this assembly complex is a prerequisite for production of mature progeny of HCMV virions. Treating cells with Thapsigargin did not affect the assembly complex formation, or the co-localization known pattern of viral and cellular proteins. However, it led to a decrease in the IF intensity signals of the "bulb" like structure (figure 3.4, 3.5 and 3.6). This might be the mechanism caused by which viral load and viral titer were reduced as shown in figures 3.10A and B (discussed below in 4.5).

It is known that inhibition of translation may permit cells to recover from stress, but would not benefit HCMV replication. The slow replicative cycle characteristic for HCMV requires the virus to maintain the host cell in a metabolically and translationally active state for an extended period; thus HCMV is obliged to abrogate this type of cellular response. A number of studies have shown that HCMV infection induces several mechanisms to overcome the negative effects of stress responses and maintain translation (Child et al. 2004; Kudchodkar et al. 2004; Hakki and Geballe 2005; Isler et al. 2005a, 2005b; Walsh et al. 2005; Hakki et al. 2006; Kudchodkar et

al. 2006; Kudchodkar et al. 2007). Showing that Thapsigargin induced ER stress resulted in assembly complex malformation reflects the fact that continuous ER stress inhibited the active metabolically and translationally state for an extended period. Inhibition may be due to the fact that the virus is in need to complete its life cycle. Although inhibition was clearly obvious using pp28 as viral assembly complex and true late protein, inhibition was very drastic for the early pp65 protein as demonstrated by western blot analysis (see below).

Immunofluorescence assays suggested that the site of cytoplasmic envelopment is a juxtanuclear structure that includes viral tegument proteins as well as multiple virus-encoded envelope proteins (Sanchez et al, 2002a,b). Using antibodies to cellular proteins, the cytoplasmic assembly complex has been shown to partially overlap with a component of the trans-Golgi network (Sanchez et al, 2002a, b; Azzeh et al., 2006; Das et al., 2007). Interestingly, although pp28 subcellular localization was not changed, as shown in TH treated cells, we observed a decrease in pp28 fluorescing signal in immunofluorescence assays due to TH treatment. These results evoked the possibility that TH leads to a decrease in pp28 expression levels in HCMV infected HFF. Moreover, these results can explain, at least in part, that the decrease in pp28 after TH is responsible for lowered viral titer (see below 4.4).

A further indication for a possible influence of ER stress on the assembly complex was eliminated in pp65 detection via immunofluorescence as well as via immunoblot. The immunofluorescence experiments detecting pp65 show an unfortunate retardation of pp65 in the nucleus. This could be one of the indirect effects of ER stress on the assembly complex formations as pp65 is one of the AC components (Prichard et al., 2006; Zawahreh et al., 2008; Qawasmi et al, 2011). More drastically was the pp65 protein expression detected under ER stress induced by TH. The levels of pp65 expression decreased more than 100X in AD169 infected HFF treated with TH and was not detectable in ΔUL97 infected HFF after TH treatment. This is a further evidence for the influence of ER stress on the HCMV assembly complex, which has a consequence on replication and the formation of viral particles.

4.4 Influence of ER stress on early genes

Detecting early gene IE1/2 in infected cells revealed that its subcellular distribution was not affected in TH treated cells, compared to mock treated cells. This result indicated that ER stress affected early late (48 hours post infection) to late viral genes only, which are involved in the later phase of HCMV life cycle. In other words, ER stress affected mainly late phase of HCMV infection cycle.

4.5 The influence of ER stress on ER markers in HCMV infected cells

The co-localization experiments illuminated clearly, that ER marker Calnexin does not co-localize with pp28 at the AC of HCMV infected cells. Furthermore, the experiments showed that the subcellular distribution of Calnexin was not clearly affected by ER stress induction using Thapsigargin in either infection cases (AD169 or ΔUL97). Similar results were also observed for BIP, although partial co-localization was observed occasionally. Both calnexin and BIP are chaperone proteins. BIP was actually reported to play a role in building AC in HCMV infected cells (Buchkovich et al., 2008; 2009). However, its co-localization with HCMV AC components was not demonstrated.

On the other hand, our results demonstrated that EEA1 did indeed co-locale to the AC and mainly to the center of the AC in wt infected cells. This is in line with previous a previous study, which revealed that EEA1 is a component of the HCMV AC (Das et al., 2007). The co-localization however, was affected in ΔUL97 infected cells and was one of the factors supporting our proposal in regard to the role of UL97 kinase in ER stress are implicated below.

4.6 The role of UL97 in modulating ER stress

Although the intact UL97 open reading frame is not essential for viral replication, its deficiency reduces virus yield by more than 2 orders of magnitude (Prichard et al., 1999).

Here we provided convincing evidence for the involvement of UL97 in ER stress in HCMV infected cells. Our results show that while BIP protein was induced in AD169 as well as in ΔUL97 infected cells. This result indicated that UL97 is involved in cellular ER stress induction. This could be a critical factor for HCMV replication and life cycle completion. Moreover, our results clearly showed that UL97 affected pp28 subcellular distribution of pp28 AC marker in infected cells, treated with TH. Hereby, pp28 assumed the original "bulb"-like structure known from wt-HCMV infected cells. Interestingly, also the "kidney"-like shaped nucleus, typical for AD169 infection (Azzeh et al, 2006) was also observed in Thapsigargin treated ΔUL97 infected cells. Additionally, the co-localization of EEA1 with AC marker in ΔUL97 infected cells was very limited in TH untreated cells, but obvious in treated cells infected with wt AD169. Furthermore, pp65 protein expression was drastically reduced in ΔUL97 infected cells if compared to AD169 infected cells. Altogether, these experimental facts strengthens the idea that UL97 kinase may play an essential role in HCMV induced ER stress.

4.7 ER stress and viral titration

Viral plaque assays showed clearly that treating infected cells with TH had a critical influence on mature virion production. This indicated that the influence of ER stress on the viral proteins discussed above was only a part of the ER stress consequences on HCMV. Determination of viral titer via plaque assays is based on the detection of mature infectious virion particles, which are able to produce plaques. The decrease in viral titer in TH treated infected cells were in agreement with previous studies (Isler et al., 2005b). Electron microscopic (EM) analysis had shown that TH severely disrupted the maturation of HCMV virions (Isler et al., 2005b).

4.8 HCMV DNA detection by viral load assay

On the contrary, viral load experiments showed less drastic decrease in HCMV DNA detected in TH treated infected cells. The reason is that even DNA of defected viruses can be detected viral Real time PCR and not only mature infectious virions as the case with plaque assays. Nevertheless, viral load detection revealed that the TH effect is clearly dependent on the viral titer used initially for infection. Furthermore, these results are further evidence for the arguments discussed in 4.1 that TH affects infected cells rather than mock infected cells, as the decrease in viral DNA detected in TH treated cells was less prominent in cells infected with low moi.

4.9 Conclusions

The experimental set up presented here provided an insight in the field of viral-cellular interaction research. The basics of measurement of ER stress induction with TH were in line with previously published data, making it a solid platform to peruse the research questions proposed here.

The involvement of viruses with this massive cellular response (ER stress) had shed a light on various aspects of viral/cellular interaction and the potential of infection under such cellular circumstances.

The data presented here provided evidence for the involvement of viral tegument proteins pp28, pp65 and pUL97 in ER stress. ER stress indeed affected the AC complex especially in ΔUL97 infected cells. Building on this initial evidence, the possible role of the only viral kinase UL97 in ER stress was evoked. It became clear that pp28 and pp65 were probably part of the tools for UL97 modulating the ER stress for the best benefit of the viral replication cycle. Finally, identifying its role in ER stress, our results elucidated that the various functions of the only HCMV kinase were not exploited yet, and still a subject for further research.

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العنوان: تأثير أجهاد الشبكة الأندوبلازمية على مركز تجمع الفيروس و دورة حياته

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الملخص

عند الاصابة بفيروس تعد السيطرة على آلية موت الخلية أمراً بالغ الأهمية من أجل بقاء الفيروس. العديد من الفيروسات، بما فيها فيروس (HCMV) تحفز اجهاد الشبكة الاندوبلازمية (Endoplasmic Reticulum – ER) وبروتينات تسمى (UPR). ولقد تم دراسة العلاقة بين إجهاد الشبكة الاندوبلازمية مع هذا الفيروس تحديداً من جوانب عدة ، ولكن تأثير إجهاد هذه الشبكة على مركز تجمع الفيروس لم يتم ايضاحه تماما.

يركز هذا العمل على جانب واحد من دورة حياة الفيروس فيما يتعلق بإجهاد الشبكة الاندوبلازمية. إن مركّز تجمع الفيروس هو الموقع الذي يتم فيه تجميع البروتينات الفيروسية لبدء عملية التكاثر. ونظراً لأن دورة حياة فيروس (CMV) في الخلايا البشرية تمتد إلى 96 ساعة، فإن الفيروس بحاجة لأن يحافظ على عمل الخلية طوال دورة حياته على الأقل ويتجنب أي تأثيرات ضارة للإجهاد الخلوي.

في هذه الدراسة تم استخدام Thapsigargin كمحفز لإجهاد الشبكة الاندوبلازمية في الخلايا المصابة بالفيروس في مقابل خلايا أخرى مصابة إصابة وهمية. واستخدمت الخلايا الليفية للقلفة البشرية (HFF) لفحوصات الإصابة باعتبارها نموذج مخبري ناجح لدراسة عدوى هذا الفيروس. استخدمنا سلالات برية من الفيروس تسمى ب AD169 وسلالات أخرى تسمى ب UL97 والتي تملك طفرة جينية تجعلها تفتقر الى الانزيم المفسفر . إن UL97 هو المفسفر الوحيد في هذا الفيروس ، وبالتالي فهو الهدف الرئيسي للأدوية المضادة للفيروسات، وقد أظهرت دراسات سابقة أنه يقوم بفسفرة كل من البروتينات الفيروسية والخلوية وبالتالي تسهيل الإصابة بالعدوى. وأظهرت النتائج التي حصلنا عليها أن إجهاد الشبكة الاندوبلازمية قد أثر على مكان و بروتينات مركز تجمع الفيروس. وكشفت تجارب لاحقة أن اهذا المفسفر الفيروسي (pUL97) كان مشاركاً في تغير إجهاد الشبكة الاندوبلازمية الذي بدوره أثر على مركز تجمع الفيروس ، كمية ومعيار الفيروس بالجسم. وتم التحقق من هذا التأثير عن طريق فحص بروتين pp65 وهو أحد بروتينات مركز تجمع الفيروس ، تم فحص هذا البروتين بتقنية western

blotوالتي كشفت تأثيرات مهمة على هذا البروتين المهم. كما أن الدراسة أخذت بعين الاعتبار أن ال BIP وهو مؤشر لاجهاد الشبكة الاندوبلازمية قد استُحِث بشكل يتناسب مع التأثير الحاصل على مركز الفيروس.

إن إجهاد الشبكة الاندوبلازمية يشكل حقلاً علمياً قائماً بذاته، وقد ساهم التدخل الكبير لهذه الفيروسات في نظام التفاعل والاستجابة في الخلايا الى إلقاء الضوء على العديد من الجوانب في بحوث التفاعل الفيروسي مع النظام الخلوي وفي مجال العدوى واحتماليتها. وتقدم البيانات التي تعرض هنا برهاناً واضحاً على مشاركة بروتينات مركز التجمع الفيروسي (بواسطة المفسفر UL97) في إجهاد الشبكة الاندوبلازمية.