

Detection of Cellular Kinases Involved in HCMV Assembly

البحث عن المفسفات الخلوية التي لها علاقة بتجميع فيروس HCMV داخل الخلية

By

Mohammed Qawasmi

Advisors

Dr. Maysa Azzeh

Dr. Mohammad Farraj

November, 2011

Detection of Cellular Kinases Involved in HCMV Assembly

البحث عن المفسفات الخلوية التي لها علاقة بتجميع فيروس HCMV داخل الخلية

By

Mohammed Qawasmi

Advisors

Dr. Maysa Azzeh

Dr. Mohammad Farraj

**This Thesis was submitted in partial fulfillment of the requirements
for the Master's Degree in Clinical Laboratory Science From the
Faculty of Graduate Studies at Birzeit University, Palestine**

November, 2011

I dedicate this work to my father Walid, my mother Nawal, my wife Suzan and my daughter Mira.

Acknowledgments

I would like to thank Dr. Maysa Azzeh for providing me with the opportunity to perform my experiments in the virology laboratory under her guidance. I would like to thank Dr. Mohammad Farraj for his continuous support. Thanks also go to Dr Zaidon Salah for his scientific exchange , and not to forget the members of medical research center who gave me a great support to perform this work: Lina Qrei , Maysa Salah , Dina Ali, Dima Abu Humous , Niveen Saleh , Fatima Hamadah , Mohammed Kurdi and Ahlam Mujahed.

Finally I would like to thank both the internal examiner Dr. Gabi Abu Sada and the external examiner Dr. Kamil Adwan for their efforts in reading and evaluating this work.

MQ

Table of Contents

<u>1. Introduction</u>		1
1.1	<i>Historical background</i>	1
1.2	<i>classifications of Herpesviruses</i>	2
1.2.1	<i>Alphaherpesvirinae</i>	2
1.2.1.1	<i>Genus Simplexvirus</i>	2
1.2.1.2	<i>Genus Varicellovirus</i>	4
1.2.2	<i>Betaherpesvirinae</i>	4
1.2.3	<i>Gammaherpesvirinae</i>	4
1.2.3.1	<i>Genus Lymphocryptovirus</i>	5
1.2.3.2	<i>Genus Rhadinovirus</i>	5
1.3	<i>Human cytomegalovirus</i>	6
1.3.1	<i>Structure of human cytomegalovirus</i>	6
1.3.2	<i>Glycoproteins</i>	8
1.3.2.1	<i>Major glycoprotein (gB; pUL55)</i>	8
1.3.2.2	<i>Glycoprotein H (gH, pUL75)</i>	9
1.3.2.3	<i>Glycoprotein L (gL, UL115)</i>	9
1.3.2.4	<i>UL16 glycoprotein</i>	9
1.3.3	<i>Tegument proteins</i>	10
1.3.3.1	<i>pp65</i>	11
1.3.3.2	<i>UL97 kinase</i>	11
1.3.3.3	<i>pp28 (ppUL99)</i>	13
1.3.3.4	<i>pp150 (ppUL32)</i>	13
1.3.4	<i>Capsid</i>	14
1.4	<i>Life cycle of HCMV</i>	15
1.5	<i>HCMV egress and assembly</i>	18
1.6	<i>Interaction of cellular kinases with HCMV</i>	19
1.6.1	<i>Serine threonine kinases</i>	20
1.6.1.1	<i>Staurosporine</i>	21
1.7	<i>Epidemiology of Human CMV</i>	22

	<i>Infection</i>	
1.8	<i>Diseases caused by HCMV</i>	23
1.8.1	<i>Cytomegalic inclusion disease (CID)</i>	24
1.8.2	<i>Asymptomatic congenital human cytomegalovirus</i>	24
1.8.3	<i>Perinatal infection</i>	25
1.8.4	<i>Human cytomegalovirus mononucleosis</i>	25
1.8.5	<i>Transfusion-acquired human cytomegalovirus infection</i>	26
1.8.6	<i>Human cytomegalovirus infections in immunocompromised patients</i>	26
1.8.7	<i>Other diseases caused by HCMV</i>	26
1.9	<i>HCMV antiviral drugs</i>	27
1.10	<i>Research significance</i>	29
1.11	<i>Objectives</i>	30

2. Materials and Methods **31**

2.1	<i>Cell culture</i>	31
2.1.1	<i>Cell lines and media</i>	31
2.1.2	<i>Propagation and Passage of HFF Cell</i>	32
2.1.3	<i>Freezing and Thawing of HFF Cell</i>	32
2.2	<i>Viruses</i>	33
2.2.1	<i>Propagation of Viruses</i>	34
2.2.2	<i>Viral Titration and Plaque Assay</i>	35
2.2.3	<i>Viral infection assay</i>	36
2.3	<i>Immunofluorescence (IF)</i>	38
2.4	<i>Monoclonal, polyclonal antibodies and fluorescing markers</i>	39
2.5	<i>Fluorescence Image capturing</i>	40
2.6	<i>Cell lysis for protein extraction</i>	41
2.7	<i>Western Blot analysis (WB)</i>	42
2.7.1	<i>Preparation of the polyacrylamide</i>	42

	<i>Gels</i>	
2.7.2	<i>Sample Preparation</i>	44
2.7.3	<i>Blotting</i>	44
2.7.4	<i>ECL Reaction</i>	45
2.7.5	<i>Membrane Stripping</i>	46
2.7.6	<i>Gel and membrane Staining</i>	47
2.7.7	<i>Co-Immunoprecipitation (Co-IP)</i>	47
2.8	<i>Drug inhibition assay; serine/threonine inhibitor set</i>	48
2.8.1	<i>Golgi disruption assay</i>	51
2.8.2	<i>Recording structures of subcellular distribution</i>	52
2.9	<i>Kinetic experiments</i>	52
2.10	<i>DNA extraction</i>	53
2.11	<i>Viral load; Real time PCR method</i>	54
3. <u>Results</u>		6
3.1	<i>Subcellular distribution of pp28 in AD169 infected cells</i>	56
3.2	<i>Influence of Bisindolylmaleimide I on subcellular distribution of pp28</i>	57
3.3	<i>Influence of H-89 on subcellular distribution of pp28</i>	58
3.4	<i>Influence of KN-93, ML-7 and protein kinase G on subcellular distribution of pp28</i>	59
3.5	<i>Influence of staurosporine on subcellular distribution of pp28</i>	60
3.6	<i>Staurosporine does not affect early stages of infection, nor does it affect AC if added to cells before infection</i>	64
3.7	<i>Influence of staurosporine on subcellular distribution of Golgi in uninfected cells</i>	65

3.7.1	<i>Co-localization of Golgi marker WGA with tegument proteins in staurosporine infected cells</i>	67
3.8	<i>The reversibility of staurosporine inhibition in AD169 infected cells</i>	69
3.9	<i>Subcellular distribution of trans Golgi and distal Golgi in AD169 infected HFF cells</i>	70
3.10	<i>The nucleus of AD169 infected cell is also affected by staurosporine</i>	74
3.11	<i>The role of Golgi in building the assembly complex</i>	75
3.12	<i>The influence of staurosporine on viral load</i>	76
3.13	<i>Influence of staurosporine on viral titer</i>	78
3.14	<i>Staurosporine does not affect viral entry or viral exit</i>	80
3.15	<i>Kinetic development of the vacuoles in staurosporine inhibited AD169 infected cells</i>	81
3.16	<i>The influence of staurosporine on tegument protein expression</i>	82
3.17	<i>The influence of staurosporine on cellular proteins in HCMV infected cells</i>	84
3.18	<i>The influence of staurosporine on cellular serine/threonine in HCMV infected cells</i>	85
4. <u>Discussion</u>		87
4.1	<i>The influence of wide range serine/threonine kinase inhibitors on subcellular distribution of HCMV tegument protein and AC marker pp28</i>	88
4.2	<i>The function of staurosporine and its effect on HCMV assembly</i>	89
4.3	<i>Subcellular distribution of AC and other effects during staurosporine</i>	90

inhibition

4.4	<i>Titer of viruses in staurosporine inhibited cells</i>	92
4.5	<i>Staurosporine modulated the Golgi structure in infected cells</i>	94
4.6	<i>The AC is Golgi derived and very robust structure</i>	94
4.7	<i>Expression of viral teguments protein during staurosporine inhibition</i>	95
4.8	<i>Cellular Serine/threonine kinase is affected in infected staurosporine inhibited cells</i>	96
5. References		97

List of Tables

<u>Table</u>		<u>Page</u>
2.1	<i>Amount of complete DMEM medium used in cell culture and infection experiments.</i>	37
2.2	<i>Stock and working solution of the serine/threonine inhibitor set</i>	50

List of Figures

<u>Figure</u>		<u>Page</u>
1.1	<i>An illustration that shows HCMV structure</i>	7
1.2	<i>Summary of the HCMV life cycle.</i>	17
1.3	<i>Molecular structure of Staurosporine</i>	22
3.1	<i>Subcellular distribution of pp28 in AD169 infected cells.</i>	57
3.2	<i>Influence of Bisindolylmaleimide I on subcellular distribution of pp28.</i>	58
3.3	<i>Influence of H-89 on subcellular distribution of pp28.</i>	59
3.4	<i>Subcellular distribution of pp28 in either KN-93, ML-7 or protein kinase G treated AD169 infected HFF.</i>	60
3.5 A	<i>Subcellular distribution of pp28 in staurosporine inhibited AD169 infected HFF.</i>	61
3.5 B	<i>distribution of pp28 in staurosporine inhibited ΔUL97 infected HFF.</i>	62
3.6 A-B	<i>Subcellular distribution of pp28 (A) and pp65 (B) in non-inhibited AD169 infected cells versus staurosporine inhibited infected cells.</i>	63
3.7 A	<i>Staurosporine does not affect the subcellular distribution of IE1.</i>	64
3.7 B	<i>Subcellular distribution of pp28 in HFF cells pre-treated with staurosporine.</i>	65
3.8	<i>Influence of staurosporine on uninfected cells.</i>	66
3.9 A-B	<i>A. Co-localization of pp28 with WGA Golgi marker in MOCK inhibited HCMV infected cells versus staurosporine inhibited infected cells. B. Co-localization of pp65 with WGA Golgi marker in MOCK inhibited HCMV infected cells versus staurosporine inhibited infected cells.</i>	68
3.10	<i>Block-Release of staurosporine inhibition.</i>	69
3.11	<i>Subcellular distribution of trans Golgi marker TGN46 in AD169 infected cells.</i>	71
3.12	<i>Co-localization of TGN46 with tegument protein pp28.</i>	72
3.13	<i>Subcellular distribution of cis Golgi marker GOLPH4 in AD169 infected cells.</i>	73
3.14	<i>Co-localization of GOLPH4 with tegument protein pp28.</i>	73
3.15	<i>Correlation between nuclear shape and structure of assembly complex.</i>	74

3.16	<i>The role of Golgi in building the assembly complex.</i>	76
3.17	<i>Influence of moi and drugs on HCMV viral load.</i>	77
3.18	<i>Influence of staurosporine on Viral Titration.</i>	79
3.19	<i>State of staurosporine inhibition in HCMV life cycle</i>	80
3.20	<i>Vacuole building in staurosporine treated AD169 infected cells.</i>	82
3.21 A-B	<i>Protein expression of tegument proteins pp65 (A) and pp28 (B) with or without staurosporine inhibition.</i>	83
3.22	<i>TGN46 expression in HCMV infected and HCMV infected, staurosporine inhibited cells.</i>	84
3.23	<i>IP of serine/threonine kinase in HCMV infected and HCMV infected, staurosporine inhibited cells.</i>	86
4.1	<i>Illustration of vacuoles in assembly complex (AC).</i>	92

List of Abbreviation

HCMV	Human Cytomegalovirus
MOI	Multiplicity of Infection
hpi	Hours post infection
AC	Assembly Complex
BFA	Brefeldin A
PCR	Polymerase Chain Reaction
HFF	Human foreskin fibroblast
wt	Wild Type
HSV	Herpes simplex virus
HHV	Human Herpes Virus
VZV	Varicella-zoster virus
EBV	Epstein-Barr virus
KSHV	Kaposi's Sarcoma-associated virus
VCA	Viral Capsid Antigen
KS	Kaposi Sarcoma
HIV	Human Immunodeficiency virus
KBP	Kilo Base Pair
NIEPs	Non Infectious enveloped particles
GB	Glycoprotein B
MICB	MHC class I- related Chains
NK	Natural Killers
ORF	Open Reading Frames
kD	Kilo Dalton
Δ UL97	UL97 kinase deletion Mutant
CDK	Cyclin dependent kinase
NLS	Nuclear Localization Sequence
MCP	Major Capsid Protein
mCP	Minor Capsid Protein
AP	Assembly Protein
HHV	Human Herpes Virus
ULBP	UL16-Binding Proteins
RB	retinoblastoma pocket proteins
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
HSPGs	heparin sulfate proteoglycans
EGFR	epidermal growth factor receptor

IE	immediate early
L	Late
NGIC	Non-glycosidic Indolocarbazole I
CaMKK	calmodulin-dependent kinase kinase
CaMKII	calmodulin-dependent kinase II
SRPK1	Serine/threonine protein kinase 1
PKA	Protein kinase A
PKG	protein kinase G
PKC	protein kinase C
MLCK	Myosin light chain kinase
CID	Cytomegalic inclusion disease
AIDS	acquired immunodeficiency syndrome
HAART	highly active antiretroviral therapy
CMV-IGIV	Cytomegalovirus Immune Globulin Intravenous
CML	chronic myelogenous leukemia
pi	post infection
RT	room temperature
pfu	plaque forming unit
IF	Immunofluorescence
WB	Western blot
mAbs	monoclonal antibodies
FITC	fluorescein isothiocyanate
ON	over night
Co-IP	Co-Immunoprecipitation
RT PCR	Real Time PCR
BSA	Bovine Serum Albumin
BIM	Bisindolylmaleimide I
ST	Staurosporine
WGA	Wheat germ agglutinin
TGN	trans-Golgi network

Abstract

The cytoplasmic assembly complex (AC) in HCMV-infected human foreskin fibroblasts (HFF) is a distinguishable “bulb”-like juxtannuclear structure. The morphology of the AC is dependent on the activity of the viral-encoded serine/threonine kinase, pUL97. The morphology of AC also changes when wt-HCMV infected HFF cells were treated with NGIC-I, kinase C inhibitor. Here we employed a set of serine/threonine kinase inhibitors to test whether the HCMV assembly or the AC structure by itself are affected by serine/threonine activity. Our drug inhibition assays indicated that only staurosporine, a broad range serine/threonine kinase inhibitor affected the subcellular distribution of viral tegument protein and Golgi markers residing the AC. Staurosporine inhibition resulted in a "bulb-like structure of the AC highly punctuated with vacuoles on the rims. Regarding the nature of these vacuoles, we observed a damage of the AC structure and vacuoles with Brefeldin A. Our kinetic experiments using staurosporine inhibitors revealed that the vacuoles were clearly detectable at 60hpi. Staurosporine inhibition also remodelled the nuclear shape into a "boat"-like yet unpublished structure. Interestingly, the effect of staurosporine was reversible in block-release assays indicating that its inhibition activity is restricted to infected cells. Another confirmation of this data came from the moi dependent staurosporine drug inhibition assays. Hereby, the inhibition activity of viral load as measured via real time PCR was highly reduced in higher moi infections and less reduced in lower moi infections. Overall, staurosporine reduced the viral load and the viral titer measured via plaque assay. However, the viral titer was remarkably inhibited, since plaques can only emerge from viable viruses, while DNA from even defected viruses can be measured by viral load assay.

While inhibition with staurosporine reduced the wild type infection, the deletion mutant virus (Δ UL97) was hardly affected. Nor the AC structure in Δ UL97 infected cells, viral titer or viral load were affected during inhibition with staurosporine. However, expression of viral protein pp65 was dramatically affected in Δ UL97 infected cells inhibited with staurosporine similar to wt-HCMV infected cells. Finally, Co-immunoprecipitation assay with pp65 revealed that serine/threonine kinase expression is clearly affected by staurosporine as well as by deletion of the UL97 kinase activity. Taken together, these data provide evidence for an essential role of cellular serine/threonine kinase activity in HCMV assembly. Furthermore, our results propose a possible therapeutic role of kinase inhibitors in designing anti HCMV drugs.

الخلاصة

قبل الخروج من الخلية يحتاج فيروس (HCMV) إلى بناء منطقة لتجميع الفيروس داخل الخلية ، هذه المنطقة في حالة تكوين النوع البري من الفيروس في خلايا الغلفة الليفية عبارة عن شكل يلاصق النواة و يشبه اللبنة ، هذا الشكل يعتمد على ناتج مفسر موجود داخل الفيروس (pUL97) يؤثر على وحدات السيرين/ثريونين ، كما لوحظ أن هذا الشكل يتغير عند استعمال كل من (NGIC-I) أو (المثبط للمفسر سي) .

في هذا البحث تم استعمال مجموعة من المثبطات لمفسرات الثريونين/ثريونين لملاحظة وجود تغير في منطقة تجمع الفيروس في الخلية ، بعد استعمال الفحص الخاص بكمية تثبيط هذه المفسرات على الفيروس ، مثبط المفسرات المسمى ستاوروسبورين – والذي يعمل على مجموعة من المفسرات- هو الوحيد الذي لوحظ فيه وجود تغير في منطقة التجمع وشكل بروتينات الفيروس بالنسبة لأجسام جولجي. حيث أدى استعمال الستاوروسبورين إلى وجود فقائيع بالقرب من منطقة التجمع مع محافظتها على شكل اللبنة ، هذه الفقائيع تظهر بعد ما يقارب الستين ساعة من عدوى الفيروس للخلية ، كما أدى استعماله إلى تغير في شكل النواة إلى شكل يشبه الحذاء . هذه التأثيرات لوحظ فيها إمكانية عكسها عند إزالة تأثير المثبط بشكل يدعم كون المثبط أثر فقط على الخلايا التي أصابتها عدوى الفيروس ، وفي استعمال كميات أقل من الفيروس لإحداث العدوى دليل آخر على كونه يؤثر فقط على الخلايا المصابة بالفيروس. لحساب كمية الفيروس الناتجة عن العدوى تم استعمال نوعين من الفحوصات ، الأول يقوم بفحص الكمية الناتجة من المادة الوراثية في حين يقوم الآخر بحساب كمية الفيروس الناتجة من خلال عد الخلايا التي أصابتها عدوى الفيروس ، حيث لوحظ في هذا الأخير تثبيط أعلى من الفحص الأول لكونه يقوم بحساب الفيروس الفعال فقط في حين يقوم الأول بفحص المادة الوراثية سواء كان الفيروس فعال أو لا .

على الرغم من تأثير هذا المثبط على النوع البري من الفيروس إلا إن هذا الأثر لم يظهر على الفيروس المعدل (ΔUL97) لا من خلال شكل منطقة التجمع ولا حتى كمية الفيروس الناتجة في الفحصين الخاصين بحساب كمية الفيروس الناتجة عن العدوى ، إلا أن هناك أثر لإستعمال المثبط في تكوين البروتين الفيروسي (pp65) بشكل يشابه الأثر في النوع البري.

بالمحصلة فإن المفسفات الخاصة بالخلية تلعب دور في تجميع الفيروس بداخل الخلية ، و إستعمال مثبطات لهذه المفسفات من الممكن أن يكون له أثر علاجي على هذا الفيروس.

1. Introduction

1.1 Historical background

Herpes Virus Infections have been prevalent as early as ancient Greek times. The Greek word "herpes" means "to creep or crawl" in reference to the spreading nature of the herpetic skin lesions. Herpes simplex virus infections of humans have been recognized in ancient times (Nahmias and Dowdle, 1968; Wildy, 1973). Interestingly, Astruc, a physician to the King of France, identified herpes as a cause of genital infection in the 18th century (Hutfield et al., 1966). Without realizing the fact that he had just isolated HCMV infected cells; Ribbert et al. described enlarged cells in the kidneys of a stillborn (Ribbert et al., 1904). Subsequently, in 1893, 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).

Herpes viruses are a leading cause of human viral diseases; it comes in the second rank after influenza and cold viruses (Murray et al., 2002). Following primary infection all herpesviruses undergo a latent infection. During latency, the virus remains dormant and is able to evade the host immune system. Lytic replication of latent herpes viruses may start at any time point in a person's life due to known and unknown circumstances, causing a list of different diseases and thus making treatment approaches difficult (Murray et al., 2002).

1.2 Classification of Herpesviruses

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

Herpes viruses are mainly known with common names, which are widely accepted, however all these viruses have taxon names (Davison, 2010). Following the host-derived term; i.e. human, the word herpesvirus was added, followed by an Arabic number, which bore no implied meaning about the taxonomic or biological properties of the virus (Davison, 2010). This led to the use of an almost consistent dual nomenclature in the literature for the most common herpesviruses (Davison, 2010).

1.2.1 Alphaherpesvirinae

There are few genera, which are ordered under this subfamily, however, Genus Simplexvirus and genus Varicellovirus are the most common ones causing common disease to humans.

1.2.1.1 Genus Simplexvirus

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. Both viruses cause infections all over the world, independent from developed or undeveloped status of the country (Black, 1975). HSV disease ranges from the usual case of mild illness, not visible in the majority of patients, to a few cases of sporadic, severe, and life-threatening disease in infants, children, and adults (Roizman et al., 2007). HSV-1 and HSV-2 are usually transmitted by different routes and may be manifest on different areas of the body. Humans seem to be the main reservoir for transmission of these viruses to other humans, since no animal vectors for human HSV infections hadn't been identified yet. Herpes virus is transmitted from infected to susceptible individuals during close personal contact. Primary HSV-1 infections usually occur in early ages, less than 5 years, and are most often asymptomatic. The mouth and lips are the most common sites of HSV-1 infections; however, any organ can become infected with this virus. Gingivostomatitis is usually the manifestation of clinical disease. Furthermore, primary infection in young adults had been associated with pharyngitis and mononucleosis-like syndrome (Glezen et al., 1975; McMillan et al., 1993).

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). Nevertheless, cases of genital herpes caused by HSV-1 are increasing (Smith et al., 1972; Kalinyak et al., 1977; Wolontis and Jeansson, 1977; Corey et al., 1983). However, genital HSV-1 infections are usually both less severe clinically and less prone to recur (Reeves et al., 1981; Corey et al., 1983). Furthermore, herpes simplex encephalitis is one of the most devastating of all HSV infections being the most common cause of sporadic, fatal encephalitis (Olson et al., 1967).

1.2.1.2 Genus Varicellovirus

Varicella-zoster virus (VZV), human herpes virus 3 (HHV3) is the most common virus of genus Varicellovirus. VZV causes in its primary infection varicella. Varicella is commonly called chickenpox, characterized by fever and a generalized, pruritic vesicular rash which commonly appears in the childhood age (Sanfillipo et al., 2003). It has a high tropism for T lymphocytes, which aids in viral dissemination to the skin. Like other herpes viruses, VZV causes latency in cells of the dorsal root ganglia. Upon reactivation of latent infection, VZV causes herpes zoster, commonly named shingles, which is an illness that affects mostly adults and immunocompromised patients of all ages. Unlike varicella, herpes zoster infections cause an acute pain that can be severe and prolonged, if herpes zoster Pain last for more than 30 days they call it post herpetic neuralgia (Stankus et al., 2000).

1.2.2 Betaherpesvirinae

There are few genera, which are ordered under this subfamily, however, Genus Cytomegalovirus is the most common one represented by the human cytomegalovirus (HCMV), taxon human herpes virus 5 (HHV5). HCMV is the virus of interest in this work and will be discussed in details below. The name of the virus comes from the fact that cells infected with this virus become enlarged (cytomegalia).

1.2.3 Gammaherpesvirinae

The most common genus causing human diseases in this subfamily is the genus Lymphocryptovirus represented by the commonly known Epstein-Barr virus (EBV); human

herpes virus 4 (HHV4) and genus Rhadinovirus represented by the commonly known Kaposi's Sarcoma-associated herpesvirus (KSHV); human herpes virus 8 (HHV8).

1.2.3.1 Genus Lymphocryptovirus

Epstein-Barr virus (EBV) is the main representative and known virus of the genus Lymphocryptovirus. 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 the oral route. Most children from 1-3 years are being infected from other family members (Gratama et al., 1990). This primary infections remains asymptomatic, however when primary infection takes place after the age of ten, EBV causes a mild to severe infectious mononucleosis (IM) in 25% of this delayed primary infection (Crawford et al.,2006). This comes up as an infection to the B-lymphocytes (Nilsson et al., 1971; Gerber et al., 1972). In B-lymphocytes, EBV remains latent and may cause different types of lymphomas (Morrison, 2004). 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).

1.2.3.2 Genus Rhadinovirus

Kaposi's Sarcoma-associated herpesvirus (KSHV) is the most known virus of the genus Rhadinovirus. Kaposi's sarcoma (KS) was first described in the late 19th century in eastern Europe and defined as complex, angioproliferative and inflammatory lesion (Kaposi, 1872), during the HIV epidemic it was first hypothesized that the HIV virus was the causative agent of

KS , but since some of the cases with HIV positive persons didn't develop KS, it was more prominent in patient who got the virus via the sexual route than any other HIV route of transmission (i.e. transfusion or parentally) (Beral et al., 1990). Thus they started searching for another sexually transmitted agent for KS until in 1994 by using representational difference analysis, they found a DNA that absent in normal cell but found only in the KS lesion which was an identification of a partial KSHV genome (Chang et al.,1994). Within the next 2 years the whole genome was cloned (Zhong et al., 1996; Neipel et al.,1997; Nicholas et al.,1997; Neipel et al.,1998), and later on the genome was sequenced (Russo et al.,1996).

KSHV infects the endothelium originated cells which is called spindle cells (Boshoff et al., 1995; Staskus et al., 1997; Sturzl et al., 1997). These cells then produce proinflammatory and angiogenic products (Ensoli et al., 1989).

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). In thin sections of infected cells or by negative staining, the HCMV envelope appears more pleiomorphic than the envelope of other herpes viruses (Wright, et al., 1964), allowing intact HCMV virions to be distinguished

from other herpes viruses based on morphology. HCMV has a double-stranded DNA genome of more than 240 kbp (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), which composed of same viral proteins but lack viral DNA.
- C- Dense bodies, which are uniquely characteristic of HCMV infection and are non-replicating, fusion-component enveloped particles, composed primarily of the regment protein pp65.

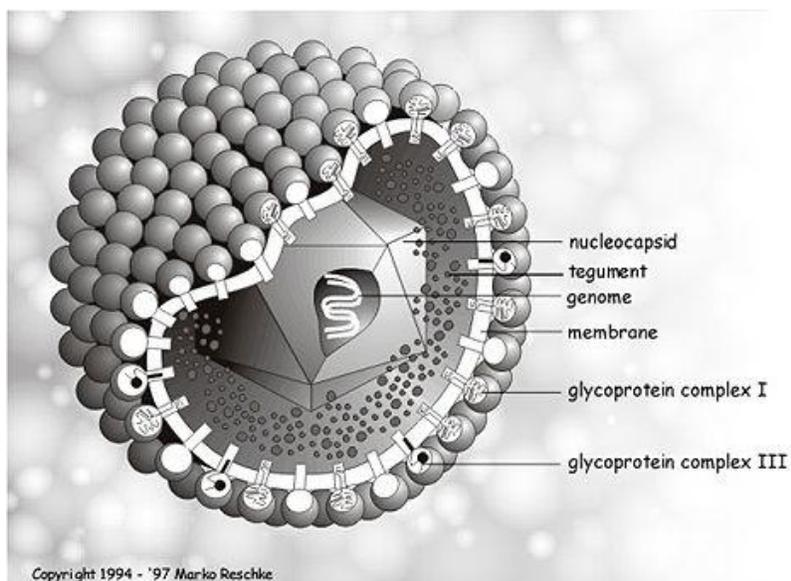


Figure 1.1: An illustration that shows HCMV structure. This graphic was produced by the human cytomegalovirus study group and Dr. Marko Reschke in Marburg, Germany, <http://www.biografix.de/>).

1.3.2 Glycoproteins

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, and many reviews have been written about the synthesis, mapping, function, and immunogenicity of the more prominent envelope glycoprotein's (Mocarski, 1993; Britt and Mach, 1996). There are approximately eight major glycoprotein types in the HCMV envelope.

1.3.2.1 Major glycoprotein (gB; pUL55)

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

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

1.3.2.2 Glycoprotein H (gH, pUL75)

HCMV gH, or gpUL75, is a 742- to 743-amino-acid envelope glycoprotein (Pachl et al., 1989) forming part of a second, relatively abundant glycoprotein complex to which neutralizing antibodies may be directed (Rasmussen et al., 1984). The gH complexes with at least one other herpesvirus-common glycoprotein, denoted gL, facilitates transport to the cell surface (Kaye et al., 1992; Spaete et al., 1993).

1.3.2.3 Glycoprotein L (gL, UL115)

HCMV gL is encoded by UL115 and is a 278-amino-acid (32-kd) 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).

1.3.2.4 UL16 glycoprotein

The HCMV UL16 gene encodes a glycoprotein which interferes with the immune response to the virus-infected cell. In vitro, UL16 interacts with MICB (MHC Class I-Related Chains) and ULBPs (UL16-Binding Proteins) which are expressed on NK cells and CD8+T cells. 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. Herpes viruses are enveloped

viruses. They bud from the inner nuclear membrane which has been modified by the insertion of herpes glycoproteins (in the mature virus, these glycoproteins determine the cell to be infected because of the availability of the appropriate receptors) (Mocarski et al., 2007).

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).

At least 25 proteins are located in the tegument layer between the virion capsid and envelope. They are products of 11 open read frames (ORFs) (UL25, UL26, UL32, UL47, UL48, UL48.5, UL82, UL83, UL85, UL88, UL99), they have been detected when virion/dense body polypeptides were electrophoretically separated, and all appear to be both phosphorylated (Roby and Gibson, 1986) and highly immunogenic (Landini, 1992). 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 numbers are herpesvirus-common. Two tegument proteins, pp150 (ppUL32 or basic phosphoprotein), a 1,048-amino-acid (150- to 155-kd) protein, (ppUL83 or lower matrix protein), a 561-amino-acid (65- to 68-kd) protein, are the most abundant proteins made during replication.

1.3.3.1 pp65

PP65 is the major tegument protein, in antigenemia assays. It is the target antigen that is used for rapid diagnosis of HCMV clinical infection. The large amount of pp65 produced by laboratory strains of virus is associated with abundant dense body production. Although all tegument proteins are phosphorylated, pp65 is a major phosphate acceptor in infected cells as well as a primary target for phosphorylation in vitro by the virion-associated protein kinase (Somogyi et al., 1990). The HCMV tegument aggregates-as shown by the analysis by mass spectrometry-are not only formed principally of the tegument proteins pp65 and ppUL25 but also contained additional virion structural proteins which include the major capsid protein (Prichard et al., 2005).

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

1.3.3.2 UL97 kinase

UL97 is a protein kinase encoded by (HCMV); it is an important target for antiviral drugs (Kamil and Coen , 2007). Upon using (HCMV) UL97 kinase deletion mutant (Δ UL97) it was indicated that there is a multi-step role for this kinase in both early and late phases of the viral life cycle, namely, in 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 Δ UL97 virus (Azzeh

et al., 2006). Also (HCMV) UL97 protein has activities similar to cellular cyclin-cyclin-dependent kinase (CDK) complexes. UL97 also phosphorylate and inactivate the retinoblastoma tumor suppressor, stimulated cell cycle progression in mammalian cells, and rescued proliferation of *Saccharomyces cerevisiae* lacking CDK activity. UL97 is not inhibited by the CDK inhibitor p21 and lacks amino acid residues conserved in the CDKs that permit the attenuation of kinase activity. Thus, 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 thus activity represents an important step in the production of mature virus particles (Prichard et al., 2005).

Recombinant HCMV that do not express UL97 kinase activity exhibit a distinctive plaque morphology characterized by the formation of highly refractile bodies late in infection. These structures were also observed in infected cells treated with the UL97 kinase inhibitor maribavir (Prichard et al., 2005).

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

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 is thought to result from

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 that encoded by the UL99 open reading frame, that is myristoylated and phosphorylated (Sanchez et al., 2000a). pp28 is essential for assembly of infectious virus, and nonenveloped virions accumulate in the cytoplasm of cells infected with recombinant viruses with a UL99 deletion. pp28 is localized to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) in transfected cells, while in infected cells, it is localized together with other virion proteins in a juxtannuclear compartment termed the assembly compartment (AC) (Sanchez et al., 2000b; Azzeh et al., 2006; Seo and Britt, 2006).

1.3.3.4 pp150 (ppUL32)

The other 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).

pp150 (ppUL32) is a prominent betaherpesvirus-conserved virion tegument protein, that accumulates –late times during infection- within a cytoplasmic inclusion adjacent to the nucleus (AuCoin et al., 2006). The tegument also contains a number of functionally uncharacterized proteins. A protein implicated in genome encapsidation, pp130 (ppUL56) is virion associated (Bogner et al., 1993; Bradshaw et al., 1994). Based on drug resistance studies (Krosky et al.

1998), to package viral DNA during virion maturation pp130 may complexes with the CMV terminase (ppUL89). One additional protein, pp67, which includes some UL65 sequence (Davis and Huang, 1985), has not been consistently observed in characterized virus strains. The association of UL25, UL26, UL47, and UL88 products with the tegument has been determined by sequencing proteins directly isolated from virus particles.

1.3.4 Capsid

The capsid is composed of seven proteins: Major capsid protein (MCP) 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). Also the assemblin/assembly play major roles as scaffolding proteins, which have critical roles in capsid assembly. The primary scaffolding components of cytomegalovirus, is the assembly protein precursor (pAP, pUL80.5) and the maturational protease precursor (pPR, pUL80a), which contains two nuclear localization sequences (NLS1 and NLS2), at least one of which is required in co expression experiments to translocate the major capsid protein (MCP, pUL85) into the nucleus (Nguyen et al., 2008).

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

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). Although HSPGs are absolutely required for HCMV entry; they are not sufficient since this interaction is not stable and subjected to dissociation (Compton et al., 1993). The stable interaction was suggested earlier to be mediated via cellular epidermal growth factor receptor (EGFR) and HCMV viral glycoprotein gB (Wang et al., 2003; Wang et al. 2005). However, recent studies (Isaacson et al., 2007; Feire et al., 2010) suggest that the HCMV binding event involves viral glycoproteins including gB interacting with $\beta 1$ and $\beta 3$ heterodimers without the involvement of EGFR. After binding to the cell surface, the virus penetrates the host cell membrane by fusing it with the virion envelope by a process not fully understood, yet known to involve 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 means which is not well understood.

Once the viral DNA is inside the nucleus, gene expression begins with immediate early (IE), or α genes, which are expressed with the assistance of tegument proteins, but do not require the expression of any other viral genes. The viral life cycle takes approximately seventy two hours (Salvant et al., 1998), therefore genes are referred to time point of their expression during this long life cycle. Genes expressed at early stages like IE1, IE2 are referred to as immediate early

genes. Genes expressed at early stages (within the first 48 h) like the tegument protein pp65 are referred to as early, delayed early genes. Genes expressed at late stages of infection as the tegument protein pp28 are referred to as late, true late genes. 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 synthesis of long concatemeric DNA molecules, which provide the templates for late (L) or γ gene expression. L gene products are primarily needed for virus assembly and egress as well as the virion structural components (Mocarski et al., 2007). Late gene expression occurs once the virus has begun 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 as yet undefined pathway. Completely assembled virus particles bud off from the cell into the surrounding area and can then go on to infect other permissive cells.

Following primary infection, HCMV can enter into a latent (or dormant) state during which time infectious virus is not produced. The latent phase of infection lasts for the life of the host. The immune system is unable to clear this latent infection. Despite its importance to human disease, the mechanisms underlying latency are very poorly understood. Reactivation is the process by which the virus transitions from a latent state back to a productive infection state (Mocarski et al., 2007). This results in 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. There is no vaccine, drug or

treatment that prevents or interrupts viral latency, nor is it known how the virus is able to establish latency to successfully persist within the human host.

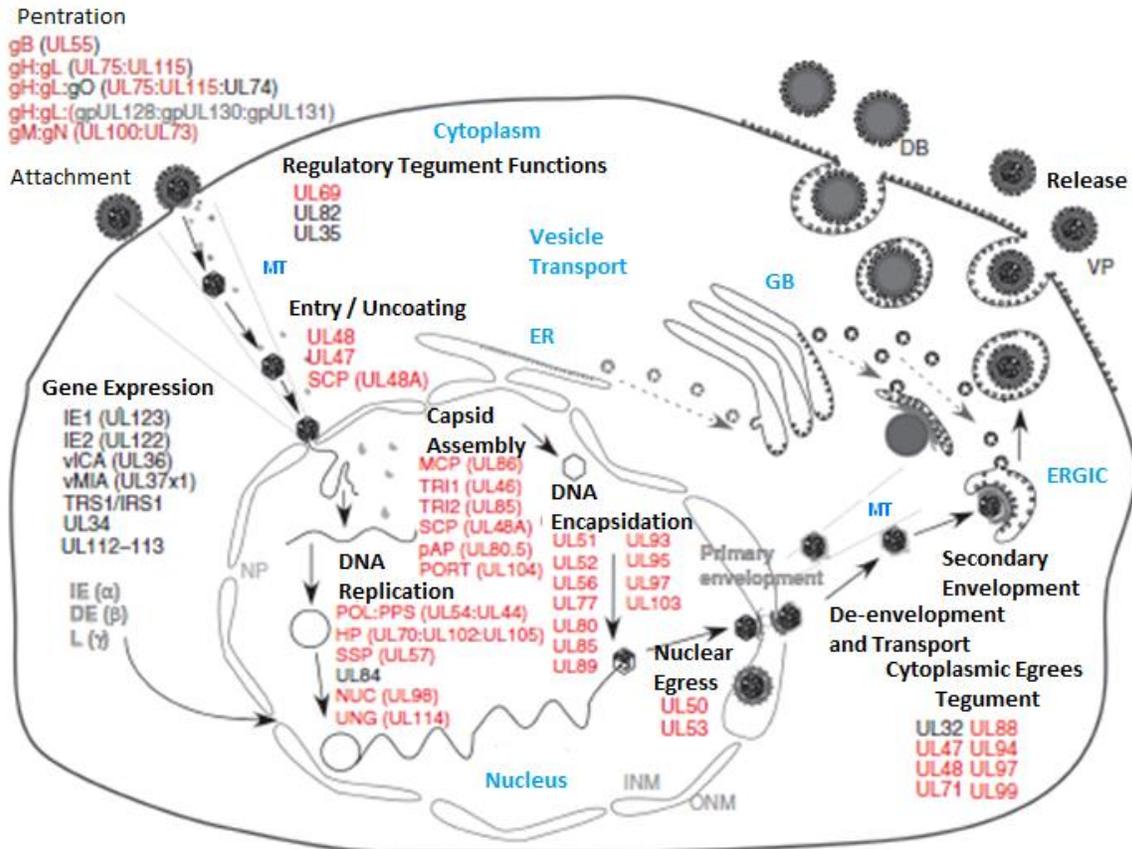


Figure 1.2: Summary of the HCMV life cycle. The viral entry begins as a fusion at the plasma membrane (attachment and penetration). Following uncoating of the capsid in the cytoplasm, the virus penetrates the nucleus where the DNA replication takes place. Capsid assembly and integration of the DNA in the capsid (Encapsidation) occurs also in the nucleus. This capsid soon buds from the nucleus via nuclear egress as the virus get the primary envelopment, once in the cytoplasm another uncoating takes place. The virus then undergoes secondary envelopment and the assembly takes place to integrate the tegument protein. Progeny virus is released from

the cell to infect other cells or remains latent (Mokarski et al., 2007). Major steps in HCMV life cycle progression indicated in black arrows.

1.5 HCMV egress and assembly

Once HCMV DNA is synthesized, 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 (Mokarski et al., 2007).

Following 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, 2006). This is a two-stage envelopment/deenvelopment/re-envelopment process (Sanchez and Spector, 2002; Mocarski et al., 2007). This model suggests that nuclear egress starts with primary envelopment at the inner nuclear membrane, followed by a deenvelopment 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 ERGIC membranes building a cytoplasmic assembly complex (AC).

The cytoplasmic assembly complex (AC) (Das et al., 2007) in HCMV-infected cells is a juxtannuclear 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.

Currently little is known about how nucleocapsids engage microtubules to traverse the cytoplasm, how final envelopment at ERGIC membranes occurs, or how tegument proteins might be added before final envelopment. However, some studies suggest that the conserved, myristoylated, and palmitoylated protein (UL99-encoded pp28) plays an important role in the secondary, or final, envelopment, even though although mutants of the gene did not exhibit a complete block to maturation or an altered cytoplasmic inclusion (Silva et al., 2003; Britt et al., 2004 ; Jones and Lee, 2004).

1.6 Interaction of cellular kinases with HCMV

Human Cytomegalovirus "ensures" that the cellular machinery efficiently supports viral reproduction by reprogramming the cellular factors, such as regulatory protein kinases towards virus-specific regulatory pathways (Fortunato et al., 2000; Bain and Sinclair, 2007). The activities of CDKs 1, 2, 7 and 9 are crucial for HCMV replication (Bresnahan et al., 1997; Chen et al., 2001; Sanchez et al., 2003; Sanchez et al., 2004; Sanchez and Spector 2006; Kapasi and

Spector 2008; Rechter et al., 2009; Hamirally et al., 2009). In the course of infection, they phosphorylate the tegument protein pUL69 (Rechter et al., 2009) and mediate phosphorylation of the tegument protein pp65 (Sanchez et al., 2007). On the other hand, the tyrosine kinase affects virus/cell fusion (Keay and Baldwin, 1996). HCMV utilize the PI3 kinase pathway to inhibit apoptosis (Yu and Alwine, 2002) and to induce viral replication (Johnson et al., 2001). Protein kinase C was shown earlier to play a role in ongoing HCMV viral infection (Slobbe-van Drunen et al., 1997), in regulation of the HCMV major immediate early promoter (Kristoffersen et al., 1994; Liu et al., 2010), in virus/cell fusion (Keay and Baldwin, 1996) and in nuclear egress (Milbradt et al., 2010; Marschall et al., 2011). Keay and Baldwin (Keay and Baldwin, 1996) also demonstrated that tyrosine kinases play a major role in HCMV/cell fusion. A possible role of protein kinase A in HCMV infection was also suggested by Kristoffersen et al. (Kristoffersen et al., 1994). A very recent report revealed that the inhibition of calmodulin-dependent kinase kinase (CaMKK), but not calmodulin-dependent kinase II (CaMKII) attenuated production of HCMV viral progeny and blocked viral DNA replication (McArdle et al., 2011).

Furthermore, cellular protein kinases encoded by the host cell and virus contribute to primary envelopment and deenvelopment and nuclear egress of HCMV (Krosky et al., 2003; Mocarski et al., 2007). All of these data suggest a redundancy and possible interplay exist between viral and host kinases (presumably in conjunction with host phosphatases) as determinants of efficient egress.

1.6.1 Serine threonine kinases

Protein kinases are large group of enzymes which function is to transfer phosphate group from a nucleoside triphosphate (ATP or GTP) to an amino acid in a protein substrate. Protein kinases

are subdivided depending on the substrate amino acids; kinases phosphorylate serine, threonine, or a tyrosine residue Protein. The catalytic core of protein kinases consists of 12 conserved sub domains, the phosphorylation results in a functional change in the targeted protein as it interferes with its cellular location, or enzymatic activity, and/or association with other proteins (Hanks and Hunter, 1995). Kinases regulate the majority of cellular pathways. About 2% of the human genes; expressing about 500 protein kinase genes, have the ability to modify about 30% of the human protein (Manning et al., 2002).

It may be implicated that cellular serine threonine kinases may play a major role in HCMV infection, however literature in this regards is rather rare. The only research made on this issue showed that the abundance of Serine/threonine protein kinase 1 (SRPK1) was increased during HCMV (Gaddy et al., 2010) and that HCMV infection was associated with an increase in threonine phosphatase activity in cells (Hakki and Geballe, 2008).

1.6.1.1 Staurosporine

Staurosporine is an alkaloid - indole carbazole - that was firstly isolated in 1977 from broth culture of the fungus called *Streptomyces staurosporesa* (Omura et al., 1977). Its effect depends on the concentration by which it is used. At 0.7 nM it acts as protein kinase C inhibitor, at higher concentration (1-20 nM), it can inhibit other kinases such as PKA (Protein kinase A), PKG (protein kinase G), CAMKII (Ca²⁺/calmodulin-dependent protein kinases II) and MLCK (Myosin light chain kinase) (Ruegg and Burgess, 2002). At 50-100 nM, it can function as neurotrophin agonist, however with a concentration of 0.2- 1 μ M, it leads to cell apoptosis (Couldwell et al., 1994; Yue et al., 1998).

Studies (Navarro et al., 1998, Kim et al., 1999) investigating the effect of staurosporine in HCMV life cycle are rare. Two studies have revealed that staurosporine played a minor role in HCMV-induced cellular gene transcription .

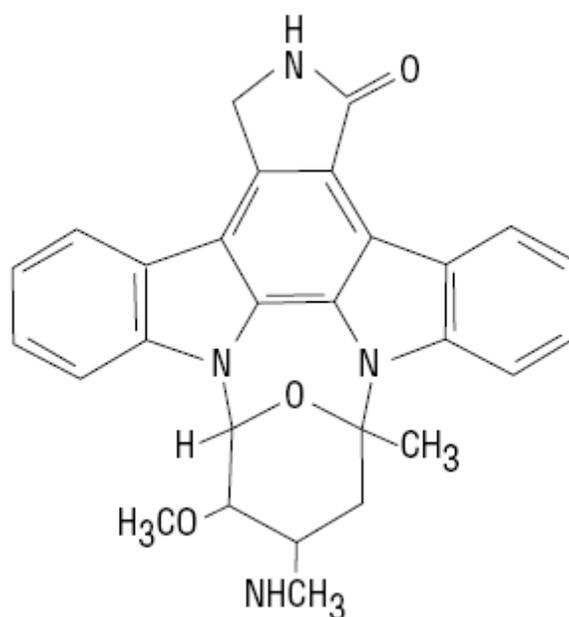


Figure 1.3: Molecular structure of Staurosporine .

1.7 Epidemiology of Human CMV Infection

Seroepidemiologic studies (Ahn, et al., 1997; Heise, et al., 1998) show that HCMV is universally distributed among human populations from developed, industrial societies to isolated aboriginal groups. Although prevalence of HCMV infection increases with age in every group that has been studied, the overall prevalence of infection and the age at initial acquisition of virus vary greatly according to living circumstances. In general, prevalence is greater and HCMV is 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 prevalence rates that are slightly higher 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 as high as 95%-99% in Turkey (Hizel et al., 1995), in Benin (Rodier et al., 1995), in South Korea (Sohn et al., 1992) and in 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). Seasonal variation in incidence of HCMV infection has not been recognized, and epidemics have not been described, aside from reports of very high infection rates in institutional populations such as day care centers (Mocarski et al., 2007).

Although HCMV is predictably transmitted in settings where susceptible persons 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. Following initial acquisition of HCMV, infectious virus is present in urine, saliva, tears, semen, and cervical secretions for months to years. Not surprisingly, high rates of CMV infection occur in settings where close contact with body fluids is expected, such as between sex partners (Chandler et al., 1985; Stanberry et al., 2004), among children in day care centers, and between preschool-aged (Pass et al., 1984; Murph et al., 1986; Adler, 1988;)

1.8 Diseases caused by HCMV

Congenital cytomegalovirus (CMV) infection is the most common and the most important of all congenital infection since current estimates showed that about 30,000-40,000 infant/year are born with congenital cytomegalovirus infection annually in the United States (Murphsu et al.,

1998). 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, the average rate of transmission from the mother to the fetus is 40%; about 65% of these infants will end up having cytomegalovirus disease at birth (Schleiss, 2010). However if it was a recurrent maternal infection, the risk of transmission to the fetus is lower, ranging from 0.5-1.5% (Schleiss, 2010). Most of these infants appear normal at birth (i.e. silent infection).

1.8.1 Cytomegalic inclusion disease (CID)

It is the most severe case of congenital CMV infection. Approximately 10% of infants with congenital infection have a clinical evidence of this disease at birth (Intern, 1965). CID almost always occurs if the pregnant women get a primary CMV infection; sometimes, however, rare cases are described in women resulting from reactivation of infection during pregnancy.

1.8.2 Asymptomatic congenital human cytomegalovirus

Most infants that have a congenital cytomegalovirus infection are born to women who have already previous immunity to cytomegalovirus. The appearance of these infants from a clinical view seems that they are healthy at birth; however, compared to uninfected infants they may have subtle growth retardation (Fowler et al., 1996) and these infants are at risk for neurodevelopmental sequelae.

The major consequence of congenital cytomegalovirus infection is sensor neural 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., 1996). Routine newborn audio logic screening may not detect cases of cytomegalovirus–associated hearing loss.

1.8.3 Perinatal infection

Perinatal acquisition of cytomegalovirus usually occurs via infected secretions of HCMV in the birth canal or via breastfeeding; however in cases of low birth weight it is preferred to check the breast milk for the presence of the virus (Schleiss et al., 2006). Most infections are asymptomatic. Indeed, in some reviews, cytomegalovirus acquired through breast milk has been referred to as a form of natural immunization. (Schleiss et al., 2006; Schleiss, 2010)

1.8.4 Human cytomegalovirus mononucleosis

Typical cytomegalovirus mononucleosis is a disease found in young adults (Henle et al., 2006), which leads to fever and severe malaise, an atypical lymphocytosis and mild elevation of liver enzymes are present. 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., 2011)

However to differentiate clinically between cytomegalovirus mononucleosis and Epstein-Barr virus (EBV)-induced mononucleosis is somehow difficult (Jenson, 2000). The cytomegalovirus mononucleosis is typically associated with less splenomegaly and less pharyngitis. As with EBV mononucleosis, the use of β -lactam antibiotics in association with cytomegalovirus mononucleosis may precipitate a generalized morbilliform rash.

1.8.5 Transfusion-acquired human cytomegalovirus infection

Post transfusion cytomegalovirus infection has a presentation similar to that of cytomegalovirus mononucleosis with incubation period ranging from 20-60 days (Granados et al., 1998). To decrease the likelihood of transmission via transfusion, it is recommended to use seronegative blood donors, frozen deglycerolized blood, or leukocyte-depleted blood for high-risk patients (i.e. Neonates, immunocompromised patients, and organ transplantation patients) (Yeager et al., 1981).

1.8.6 Human cytomegalovirus infections in immunocompromised patients

Immunocompromised patients has a high risk of getting infected by cytomegalovirus, the severity of this infection is correlated to the severity in the immune deficiency. Infection can be an acquired infection via transfusion or organ transplant from a seropositive donor or may occur from reactivation of latent viral. Also mixed Infections may arise, with donor (Revello and Gerna, 2002) and recipient isolates both present. After Viral dissemination, a multiple organ system may involve, the most important clinical manifestations consist of pneumonitis, GI disease, and retinitis.

1.8.7 Other diseases caused by HCMV

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, 2010). The mortality rate is based on the degree of

immunosuppressant and was reported to be high in bone marrow transplant recipients (Schleiss, 2010).

Cytomegalovirus is capable of causing a multiple GI tract diseases which include esophagitis, gastritis, gastroenteritis, pyloric obstruction, hepatitis, pancreatitis, colitis, and cholecystitis. With a symptoms that may 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), until the highly active antiretroviral therapy (HAART) was proposed as treatment for retinitis in HIV infection, with an overall lifetime prevalence of more than 90% (Jennifer et al., 2003).

1.9 HCMV antiviral drugs

Nucleoside analogues aciclovir, adefovir, cidofovir, ganciclovir, penciclovir , valaciclovir, and the pyrophosphate analogue foscarnet , there are four antiviral agents currently approved by the FDA for treatment of CMV (Mocarski et al., 2007). These drugs had shown to reduce or eliminate viremia or CMV shedding and to prevent or control CMV disease in specific situations in immunocompromised patients. Each agent, however, also has the potential for significant toxicity and, therefore, their use is limited to patients who are at risk for serious (disabling or life-threatening) disease (Eddleston et al., 1997; McGregor and Alistair, 2010). Antiviral treatment of severe congenital CMV infection with ganciclovir is also advocated by some, based on evidence that treatment will prevent 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. Foscarnet or cidofovir are only given to patients with CMV resistant to ganciclovir, because foscarnet has bad nephrotoxicity.

Cytomegalovirus Immune Globulin 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., 2005).

Maribavir is a benzimidazole L-riboside antiviral compound currently in clinical development (Lu and Sun, 2004). Maribavir 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). However, in February 2009, ViroPharma announced that the Phase III study failed to achieve its goal, showing no significant difference between maribavir and a placebo at reducing the rate at which CMV DNA levels were detected in patients (www.viropharma.com/)

To date all the Anti HCMV drugs targeted the viral DNA polymerase, which has a high toxicity, once resistance is emerged, treatment chances are very narrow. A promising drug, AIC246,

introduced recently by the 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., 2010; Goldner et al., 2011; Kaul et al., 2011).

1.10 Research significance

So far, the role of CDKs in HCMV life cycle was investigated. However, the role of other cellular kinases, specifically in viral assembly, was not studied yet. This current research will specifically investigate the role of cellular kinases in HCMV assembly.

Replication, assembly and egress of HCMV are regulated by both cellular and viral kinases. Nuclear egress of capsids during HCMV infection is relatively well understood (Dal Monte et al., 2002; Marschall et al., 2005, Sanchez et al., 2007, Camozzi et al., 2008). However, it is yet unknown whether the tegument and glycoproteins involved in assembly complex (AC) undergo phosphorylation by UL97 or other cellular kinases. Since replication of UL97 deletion mutants are not completely inhibited, it is speculated that cellular protein kinases complement some or all UL97 functions, enabling slow replication of the virus (Prichard et al., 2005). Elucidation of the roles of pUL97 and that of cellular protein kinases involved in cytoplasmic assembly is critical for designing potentially new class of anti herpesviral drugs.

An example of a successful inhibitor designed to block a specific targeted kinase comes from the field of leukemia. Gleevec is an agent that was designed to specifically inhibit the BCR-ABL tyrosine kinase activity. This has revolutionized the treatment of chronic myelogenous leukemia

(CML), and perhaps hallmarked the initiation of molecularly targeted therapy (Druker et al., 2001). Taking into consideration that resistant viral mutants to currently available drugs may lead to severe disease and death in immunocompetent patients, development of inhibitors of protein kinases involved in the life cycle of a virus is of major therapeutic interest.

1.11 Objectives

1- To identify cellular protein Kinases, which complement the viral protein kinases or interact with them. A possible interaction between cellular protein kinases and the HCMV UL97 kinase, or cellular kinases with HCMV assembly events will be a key contribution in understanding the poorly studied assembly steps in HCMV life cycle.

2- To reveal the role of cellular protein kinases in viral cytoplasmic assembly, on viral load and viral titer.

3- To illuminate a possible interaction between specific viral proteins involved in assembly with cellular kinases.

4- To delineate whether HCMV infected cells are affected by cellular kinase inhibitors or not and whether this effect is basically related to viral infection.

2. Materials and Methods

2.1 Cell culture

Cells in culture are derived from either primary tissues or cell suspensions. Primary cell cultures typically have a short life span, whereas continuous cell lines are, by definition, abnormal and are often transformed cell lines or isolated from specific cancer tissues.

All cell culture work was performed in laminar flow purifier safety cabinet (purifier class II biosafety cabinet, Labconco, USA).

2.1.1 Cell lines and media

Human foreskin fibroblasts (HFF) (kindly provided by Dr. Nina Mayorek, Hebrew University) is a primary cell line isolated from new born male's foreskin. HFF cells were cultured in culture flasks (Easy Flask, 25 cm², 75 cm², 175 cm², Nunc, Denmark) or well plates in complete DMEM medium. Complete DMEM medium contains DMEM (01-055-1A, Beit Haemek), 1:1000 dilution of Pen/Strep (03-031-5C, Penicillin: 100,000 units/ml, Streptomycin: 100 mg/ml, 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 (see below). Cells were cultivated in 96% humid CO₂ incubator (5% CO₂) at 37°C (Hera cell incubator).

2.1.2 Propagation and Passage of HFF Cell

Cells were propagated by splitting into 2 or more culture flasks, or into different well plates. For this, 0.25% Trypsin-EDTA (03-052-1A, Beit Haemek) was added to the confluent cells in flask for 2 minutes. Cells were loosened by gently up and down pipetting using sterile disposable plastic pipette. Complete medium was added to the non adhering trypsinized cells, gently mixed and distributed to the new flasks. The new cells are one passage higher than the one they were split from. Since HFF is a primary cell line, it was propagated up to passage 18 in our lab. Experiments were performed on passages 10-18.

2.1.3 Freezing and Thawing of HFF Cell

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

For a medium flask, 75 cm²

- 1- The medium was removed.
- 2- 3 ml 0.25% of Trypsin were added.
- 3- Detached cells in Trypsin were transferred into 15 ml tube .
- 4- Tubes were centrifuged at 1500 rpm for 10 min.
- 5- The supernatant was discarded carefully.
- 6- Pellet was gently re-suspended in 3ml freezing medium .
- 7- Re-suspended cells were transferred into cryotube (Nunc, Denmark).
- 8- Cells in cryotubes were first frozen at -70°C and transferred a day later to liquid nitrogen (slow freezing).

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

2.2 Viruses

In this work, HCMV viral strains, either as wild type (wt) or as deletion mutant, were used. HCMV strain AD169 (wt-HCMV; American Type Culture Collection) and pp65 deletion mutant (Δ pp65) were a kind gift from Prof. Bodo Plachter, University of Mainz-Germany. The UL97

deletion mutant, Δ UL97, 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

Viruses were propagated in HFF cells. Initially HFF cells were infected with AD169, Δ pp65 or Δ UL97 at low multiplicity of infection (moi). Moi represents the number of viruses divided by the number of cells. In this work viruses were propagated in 175 cm² flasks, which contain 3x10⁷ 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 CO₂ incubator (5% CO₂) at 37 °C. After 2 h, viral suspension was removed from cells and replaced by 30 ml complete medium. 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; then the 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 °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

The plaque assay technique was employed to determine the viral titer (viral count/ml or total viral count). 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 CO₂ incubator (5% CO₂) at 37°C. Meanwhile, 2.5% agarose was dissolved in dH₂O (distilled water) and cooled down to 50°C. Complete medium was also pre-warmed up to 50°C. A 0.25% agarose was prepared in the pre-warmed complete medium just exactly at 2h after 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. Finally, plates are left at room temperature (RT) for 30 min to allow agarose to polymerize and then transferred to 96% humid 5% CO₂ cell culture incubator, at 37 °C. One week later, another 1ml of agarose-containing medium is added following the procedure above. The agarose in the medium should hinder virus progeny from spreading to neighboring cells and allows counting the plaques in the correct dilutions resulting from the infection independently in different cell groups.

At 2 weeks pi, viral plaques are counted in those wells with those viral dilutions, which allow to count separate plaques viral dilutions, that allow counting 1-20 plaques per well are usually chosen to determine viral titer.

The viral titer, also referred to as plaque forming unit (pfu) was calculated using the equation below:

$$\# \text{ of plaques} / d \times V = \text{pfu/ml}$$

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 either HCMV viral type at moi of 0.5. Usually, the number of cells contained in any type of cell culture plate or flask is known, if this was not the case, cells were counted after trypsinization using the Neubauer chamber. Since cell number in each cell culture unit is known and so the pfu of the virus using the methodology mentioned in 2.2.2, the calculation of moi was possible.

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)	Post infection medium (ml)
175 cm ² (big flask)	20	4	20
75 cm ² (medium flask)	10	2	10
25 cm ² (small flask)	4	1	4
6 well plate (per well)	3	1	3
12 well plate (per well)	1	0.4	1
24 well plate (per well)	1	0.2	1
Chamber slide (per well)	0.5	0.15	0.5

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 infecting cells. For infection, medium was removed from cells and virus containing medium at the specific moi was added instead. The amount of virus containing medium added to the cell depends on the type of cell culture unit used as mentioned in table 2.1. 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 2.8). The amount of this medium also depends on the type

of cell culture unit used as mentioned in table 2.1. 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 Immunofluorescence (IF)

For immunofluorescence experiments, cells were either grown on 8-well glass slides (Labtek chamber slide, Nalge Nunc International, Naperville, IL) or on 12 well plate containing glass slides. For this, 18 mm Ø glass slides were sterilized and inserted into each well under sterile conditions in the laminar flow before splitting the HFF cells on them. The cells were infected at moi of 0.5 pfu, once cells were 90% confluent. Two hours after infection, virus containing medium 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 Na₂HPO₄, 0.24g KH₂PO₄ in 1000 ml dH₂O, pH 7.4) and fixed with 3.7% paraformaldehyde (diluted in PBS) for 30 min at RT. After three washes with PBS, cells were permeabilized with 0.1% Triton X-100 (T8787, Sigma-Aldrich, diluted in PBS) for 1-2 min at RT. Cells were then re-washed for five times with PBS and blocked with 1% bovine serum albumin (BSA, 0175, AMRESCO Inc., USA, prepared in PBS) for 60 min at RT. First antibody was diluted in 0.5% BSA and incubated with cells for 2 hrs at RT or over night at 4°C. In case of co-localization experiments of IF, the first two antibodies of the different antigens were pre-mixed before adding them to the cells. After incubation with the first antibody, cells were washed 5 times with PBS, while the last wash

was left on cells for 30 min. 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 1 μ g/ml 4', 6-diamidino-2-phenylindole (DAPI, 268298, Calbiochem, Germany) for 10 min at RT. Cells were washed 5 times with PBS. Finally, cells were covered with mounting anti-fading solution (0.5% g n-propyl gallate; P3130, Sigma-Aldrich, Germany; 100mM Tris pH 9 , 70% glycerol), (Giloh and Sedat, 1982).

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.

2.4 Monoclonal, polyclonal antibodies and fluorescing markers

Different antibodies and fluorescing markers were used in this work. The variable monoclonal and polyclonal antibodies used in this work were to perform either Immunofluorescence (IF) or Western blot (WB) experiments.

Primary mouse monoclonal antibodies (mAbs) against HCMV: pp28 (CA004-100), pp65 (CA003-100), and Immediate early (IE1) antibody (CH443) 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. GOLPH4-Golgi marker (ab28049), TGN46 (ab50595), Phosphoserine/threonine (ab17464), β -Actin-Loading control (ab8227) were purchased from Abcam (Abcam, United Kingdom). 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).

2.5 Fluorescence Image capturing

In order to visualize immunofluorescence staining and capture it, an Olympus BX60 and Olympus digital camera DP71 (Olympus, Japan) were used. 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).

2.6 Cell lysis for protein extraction

In infection experiments, cells were infected as mentioned in 2.2.3. For cell lysis medium was removed at 96 hpi or from mock infected cells. Cells were washed with 5 ml ice cold PBS, 2 ml or 1 ml for medium flask, small flask or 6 well plate respectively. 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 (Biologix, KS, USA). Cells were down centrifuged for 10 minutes at 3000 rpm and 4°C and re-suspended in ice cold cell lysis buffer. Cell lysis buffer was partially freshly prepared prior experiment and kept on ice as explained below.

Buffer stock for cell lysis (50mM Tris pH 7.4, 150mM NaCl, 10% glycerol) was prepared, filtered and stored at 4°C. 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.

Cells were incubated for 30 minutes on ice and gently mixed in 10 minutes intervals. Finally, cell debris was down centrifuged for 10 minutes at 13000 rpm and 4°C. Supernatant was kept in 30 µl aliquots frozen at -70 °C.

2.7 Western Blot analysis (WB)

WB is a method to detect proteins in a sample using mono- or polyclonal antibodies, which binds specifically to these proteins. Western blot analysis consists of separation of proteins using polyacrylamide gels, followed by blotting and immuno-detection and finally visualization using ECL reaction as detailed below.

2.7.1 Preparation of the polyacrylamide Gels

In this work BioRad vertical gel electrophoresis system was used (165-3302 Mini-PROTEAN 3 Electrophoresis Module, BioRad, CA, USA).

10% resolving gel (amounts below are for 2 gels):

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

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

Once polymerized, 5% stacking solution was prepared:

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

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

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

2.7.2 Sample Preparation

1- 10 μ l of 4x Laemmli 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 μ l of protein aliquot (see 2.6).

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

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

4- Proteins were run at 80 V (constant Ampere) for 30 min then at 130 V until the smallest marker's protein (20kDa) reached the bottom of the gel or longer if high kilo Dalton protein was to be detected.

2.7.3 Blotting

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 methanol followed by washing in dH₂O and finally well covered in transfer buffer (3g Tris base, 14.4g Glycin, 800ml dH₂O and 200 ml Methanol). Whatmann paper was also well covered with transfer buffer.

3- Stacking gel was separated from Resolving gel by cutting.

4- Blot sandwich was prepared as following:

2whatmann \Longrightarrow Membrane \Longrightarrow Gel \Longrightarrow 2whatmann

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

6- Membranes were blocked in 1% drinking milk or in 1% skim milk (prepared in PBS containing 1% Tween, Skim milk powder, Fluka 70166, Sigma-Aldrich, Germany). Blocking was either for 30 min at RT (shaking) or over night at 4 °C .

7- Milk was replaced by first antibody diluted in PBS containing 1% Tween and incubated for 2h shaking at RT.

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

9-Secondary antibody was diluted in PBS containing 1% Tween for 30 min at RT shaking.

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

2.7.4 ECL Reaction

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

2- Tube A: 3.3 μ l of 30% H_2O_2 , tube B : 60 μ l Luminol (250 mM in DMSO, A8511, Sigma-Aldrich, Germany) + 26.6 μ l p-coumaric acid (90 mM in DMSO, C9008, Sigma-Aldrich, Germany).

3- Solutions in tubes A+B were added simultaneously to the membranes and hand shaken with membranes for 1 min.

4- Solution was poured off.

5- Membrane was placed in a Nylon file.

6-In dark room, membranes were covered with X-ray negative film and casted for 30 seconds to 5 min.

7- Negative was developed using a developing machine. If signals were strong, a further shorter incubation of the membrane with negative was performed, if the signal was week, a longer incubation was performed before re-developing.

8-Membranes were kept in $-20^{\circ}C$, stripped and blotted with another antibody if needed.

2.7.5 Membrane Stripping

If another protein with another antibody was to be detected, membranes were stripped. Stripping membranes is a method used to denaturize 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

dH₂O), shaken, washed 5 times (15 min each) with PBS before a new block with 1% milk or in 1% skim milk (see 2.7.3) followed and continued as mentioned in 2.7.3, step 6.

2.7.6 Gel and membrane Staining

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

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

Destaining buffer: methanol: acetic acid 1:1

For membrane staining with ponceau S (P7170, Sigma-Aldrich, Germany), a sufficient amount of 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) covered the membrane at RT for 5 min. The membrane was then transferred to an aqueous solution containing 5% acetic acid for 5 min and washed twice for 10 minutes each. Protein bands appear reddish on the membrane.

2.7.7 Co-Immunoprecipitation (Co-IP)

- 1- 2-4 µl of precipitation Antibody were added to 400 µl lysate (from medium flask).
- 2- 50 µl (50 mg/ml) ready pre swelled Protein A/G plus –agarose (sc-2003,Santa Cruz Biotechnology, USA) was added to tube in step 1.
- 3- Mixture was incubated over night (ON) in rotation shaker at 4°C.

4- Precipitate was centrifuged at 10,000 rpm, 4°C for 2 min.

5- Wash steps:

In 1 ml Co-IP-Buffer (3 x), centrifuge each at 10,000 rpm, 4°C, 2 min .

In 1 ml H₂O bidest steril (1 x), centrifuge at 10,000 rpm, 4°C, 2 min.

6- Pellet was resuspended in 25 µl laemmli buffer (see 2.7.2, step 1).

7- Protein denaturation was done at 95°C, 10 min.

8- Half of the amount was used for WB, and the rest was stored at -20 °C.

9- Western blot was performed as detailed in 2.7.1-2.7.4.

10- Another or the same antibody was used for the blot.

Co-IP Buffer: 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol were prepared, filtered and stored at 4 °C. Just before use, 0.1% 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).

2.8 Drug inhibition assay; serine/threonine inhibitor set

To investigate the role or/and influence of serine/threonine kinase on HCMV infected cells, a serine/threonine inhibitor set (cat#539572, Calbiochem, Merck, Germany) was utilized. This set contains 250 µg of protein kinase C inhibitor, Bisindolylmaleimide I (Cat. No. 203290); 1 mg of protein kinase A inhibitor, H-89, Dihydrochloride (cat#371963); 1 mg of PKG inhibitor, Protein

Kinase G Inhibitor (cat#370654); 1 mg of MLCK inhibitor, ML-7 (cat# 475880); 1 mg of CaM kinase II inhibitor, KN-93 (cat#422708); and 100 µg of the broad range Serine/Threonine Kinase inhibitor, Staurosporine (cat#569397). With exception of Kinase G Inhibitor, which was prepared in Ultra pure water, stock solutions of each drug were prepared in DMSO. Therefore, DMSO or pure water was used in drug control experiments. Drug titration assays were initially performed, in order to identify the concentration, which does not harm the cells, but induce a detectable effect on the assembly complex as recorded via IF staining mainly. In such titration assays, drug in different concentrations diluted in complete medium was added at 0 hpi to the infected cells, changes in the assembly complex were recorded at 96 hpi via IF experiments using Golgi markers (see 2.3 and 2.4). The stock and final working concentrations of each drug solution are detailed in Table 2.2.

Table 2.2: Stock and working solution of the serine/threonine inhibitor set

Serine/threonine inhibitor	Stock solution	Working concentration
Bisindolylmaleimide I	2.424 mM	4.848 μ M
H-89 , Dihydrochloride	1.926 mM	1.926 μ M
KN-93	1.996 mM	1,996 μ M
ML-7	2.209 mM	2.209 μ M
Protein kinase G inhibitor (PKGI)	1.06 mM	10.6 μ M
Staurosporine	2.143 mM	42.8 nM

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 then either infected or mock infected at moi of 0.5, 2 h later virus containing medium was aspirated. After washing cells twice with complete medium, complete medium containing either the drug or

DMSO control was added. With exception of kinetic experiments, the effect of kinase inhibitors was detected at 96 hpi.

At 96 hpi supernatant was frozen, while cells were subjected to IF (see 2.3) or WB (see 2.7). Frozen supernatants of drug inhibition versus no inhibition were thawed and subjected to plaque assay and viral load analysis.

2.8.1 Golgi disruption assay

Different Golgi markers were shown to co-localize with HCMV assembly complex in vitro (Sanchez et al, 2000a, 2000b, Azzeh et al, 2006, Das et al, 2010). Therefore, disruption of Golgi complex using Brefeldin A (BFA, cat#B7651, Sigma-Aldrich, Germany) was a useful technique to highlight the role of Golgi in HCMV viral assembly. BFA is a fungal metabolite which disrupts the structure and function of the Golgi apparatus (Bershadsky and Futerman, 1994). BFA is also known to be an activator of the sphingomyelin cycle, and BFA-mediated apoptosis has been observed in human tumor cells. A stock concentration of 10mg/ml of BFA was made in absolute Ethanol. A working concentration of 4 µg/ml was prepared freshly in complete medium.

Cells were infected as detailed in 2.2.3 and subjected to destruction assay at 24 hpi, 48 hpi, 72hpi and 96 hpi. At the desired hpi time point, supernatant was removed from cells and was replaced with the appropriate amount of complete medium containing a final concentration of 4 µg/ml

BFA. After 2 h incubation in 5% CO₂ and on 37 °C, cells were subjected to IF analysis as detailed in 2.3.

2.8.2 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 statistically 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.

2.9 Kinetic experiments

Kinetic experiments are necessary to detect the effect of a variable at different time point of the experiment. In this work kinetic experiments were performed to detect the influence of serine/threonine kinase inhibitors during HCMV infection. For this, infection was performed as detailed in 2.2.3 and 2.8 or mock infected on 12 well plates for IF analysis and 6 well plates for WB analysis. Slides of 12 well plates were removed at different time points; 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 °C and later subjected to viral load analysis. For Western blot kinetic analysis, infected versus mock infected cells were lysed (see 2.6) 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 2.7. In some cases the drug was added before infection and then cells were infected as in 2.2.3 and subjected to kinetic experiment.

2.10 DNA extraction

In this work, DNA was either extracted from the eukaryotic cells, viral supernatant or from transformed bacteria cells. These two methods vary and are illustrated below.

Viral supernatants or lysed cells were thawed and subjected to total DNA extraction using QIAamp DNA Mini Kit (Qiagen, Germany). In most cases 200 μ l of viral supernatant were aliquoted and subjected directly to DNA extraction, while lysed cells were adjusted to 200 μ l using PBS. The extraction steps were performed according to the manufacturer's instructions as follows:

1. 20 μ l Qiagen Proteinase K were pipetted into the bottom of a 1.5 ml microcentrifuge tube.
2. 200 μ l samples were added to the microcentrifuge tube. If the sample volume was less than 200 μ l, PBS was added to adjust the amount.
3. 200 μ l Buffer AL were added to the sample, mixed by pulse-vortexing for 15 s.
4. The mixture was incubated at 56 °C for 10 min, followed by a short spin down

5. 200 μ l 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 2 ml 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 μ l 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 μ l Buffer AW2 were added, and filtrate was discarded and centrifugation step was repeated.
9. Finally, the QIAamp Mini spin column was placed in a clean micro-centrifuge tube, 200 μ l 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 °C.

2.11 Viral load; Real time PCR method

Viral supernatant were thawed and total DNA was extracted from 200 μ l (see 2.10). DNA was either frozen at -20 °C or directly subjected to viral load analysis. Viral load analysis is a method, by which the number of DNA copies can be detected using the real time PCR technique.

Real-time PCR for viral load detection was performed using an ABI Real Time PCR 7500 (Applied Biosystems, USA). All positive controls, negative controls and samples were tested in duplicate manner. A total of 20 μ l reaction mixture consisted of 5 μ l control (positive control; standard, negative control, ultra pure water) or sample's DNA, 10 μ l PCR master mix (Power SYBER Green, cat# 4367659, Applied Biosystems, United Kingdom), 1 μ l of each primer (forward and reverse, each at 10 pmol/ μ l, Metabion, Germany), and 3 μ l ultra pure water.

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

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.

Calculations for the viral load: Real Time PCR result $\times 5$ (200 μ l sample out of 1000 μ l) $\times 40$
(5 μ l DNA out of 200 μ l) = Result $\times 200$ = copies/ml

3. Results

To check the role of the cellular kinases in HCMV life cycle and specifically its assembly, different cellular kinase inhibitors (table 2.2) were tested utilizing drug inhibition assay experiments (see 2. 8). The effect of these kinase inhibitors was detected initially via IF. For this purpose pp28 viral tegument protein, which resides in the AC was used in all elementary experiments. Once an effect was detected, further experimental investigations were progressed for evaluation.

All results shown below were confirmed in at least three independent experiments.

3.1 Subcellular distribution of pp28 in AD169 infected cells

pp28, a marker of the AC (Azzeh et al., 2006) appears as a compact juxtannuclear "bulb"-like structure in AD169 infected HFF. Inhibition of the UL97 viral kinase in AD169 infected cells or infecting cells with the UL97 deletion mutant (Δ UL97) result in modification of the subcellular distribution of pp28. pp28 assumes a "crown"-like structure punctuated with vacuoles (Azzeh et al., 2006). Since pp28 was used as a reference in all kinase inhibitor experiments, it was important to emphasize its natural subcellular distribution in AD169 infected HFF cells. For this infected cells were stained at 96 hpi with anti pp28 (prepared in mouse) and followed by anti mouse Cy2 conjugated antibody. The staining was visualized using Olympus BX60 IF microscope and DP71 digital camera. As clearly shown in Figure 3.1, pp28 distributed as published earlier; as a "bulb"- like structure at the AC.

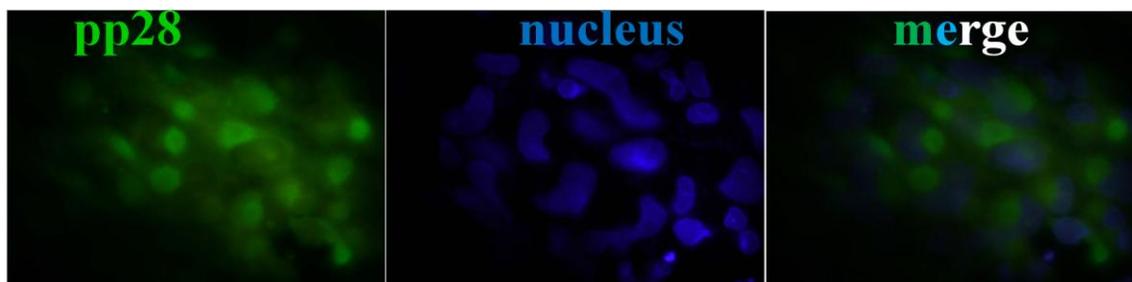


Figure 3.1: Subcellular distribution of pp28 in AD169 infected cells. HFF cells were infected with AD169 at moi of 0.5; virus containing media was removed after 2 h and replaced with media (see 2.2.3). At 96 hpi cells were subjected to IF analysis as detailed in 2.3. 1st antibody against pp28 was diluted in 0.5% BSA and added to the blocked cells for 2 h at RT. After intensive washes, the 2nd antibody was added for 30'; Cy2 anti-Mouse. Nucleus was counter-stained with DAPI (1 μ g/ml). Staining was visualized with Olympus BX60 and Olympus DP71 digital camera.

3.2 Influence of Bisindolylmaleimide I on subcellular distribution of pp28

Cellular kinase inhibitor experiments was started using the kinase inhibitor Bisindolylmaleimide I (BIM); protein kinase C inhibitor. Since protein kinase C was shown earlier to play a role in ongoing HCMV viral infection (see 1.6), it was expected that it may interfere with the building of the assembly complex or may interact indirectly with kinases modulating the AC. As clearly seen in figure 3.2, BIM failed to induce any remarkable effect on the subcellular distribution of pp28 or the nucleus of AD169 infected HFF. The subcellular distribution of pp28 was similar to that seen in non-inhibited infected cells (figure 3.2).

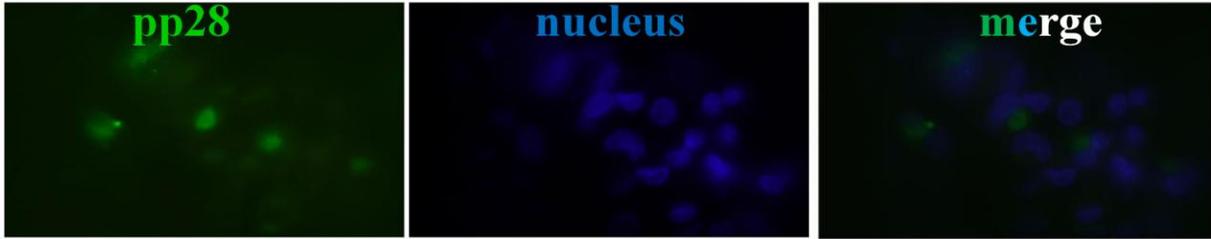


Figure 3.2: Influence of Bisindolylmaleimide I on subcellular distribution of pp28. Cells were infected and subjected to drug inhibition (see 2.8) using BIM at 4.848 μ M final concentration. At 96 hpi, cells were subjected to IF analysis and stained as in figure 3.1.

3.3 Influence of H-89 on subcellular distribution of pp28

The kinase A inhibitor was also suspected to play a role in HCMV infection (see 1.6) and was therefore tested for its possible role in HCMV assembly. H-89, PKA inhibitor was added to the infected cells after viral absorption as detailed in 2.8. Similar to BIM, H-89 did not affect the subcellular distribution of the tegument protein pp28, nor did it affect the shape of the nucleus (figure 3.3).

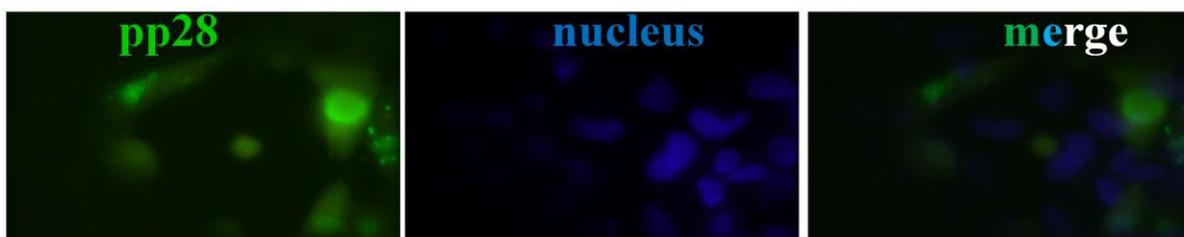


Figure 3.3: Influence of H-89 on subcellular distribution of pp28. Cells were infected and subjected to drug inhibition as detailed in 2.8 using H-89 at 1.926 μ M final concentration. At 96 hpi, cells were subjected to IF analysis and stained with pp28 antibody, followed by anti mouse Cy2 antibody (green) and counterstained with DAPI (blue) as detailed in figure 3.1.

3.4 Influence of KN-93, ML-7 and protein kinase G on subcellular distribution of pp28

Our literature research did not reveal any implication for a role of myosin light chain kinase (MLCK), PKG or Ca^{2+} /calmodulin-dependent kinase II in HCMV infection or life cycle. Nevertheless, we attempted to check whether one of these kinases may play a role in HCMV assembly. KN-93 inhibits Ca^{2+} /calmodulin-dependent kinase II, while ML-7 inhibits MLCK. The kinase inhibitors were used in drug inhibition assays as detailed in 2.8. The subcellular distribution of pp28 was detected at 96 hpi. As clearly visible in figure 3.4 A-C, the “bulb”- like structure of pp28 is predominant after treatment with either kinase inhibitor. Taken together, these results suggest that neither of the kinases; MLCK, PKG, Ca^{2+} /calmodulin-dependent kinase II play a role in HCMV viral assembly.

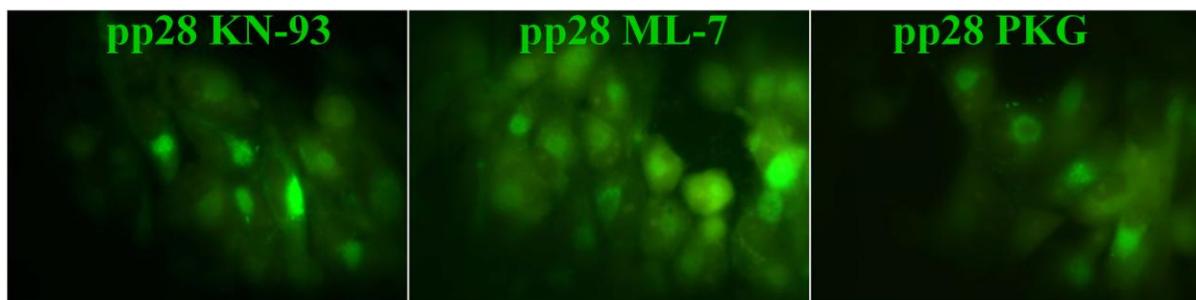


Figure 3.4: Subcellular distribution of pp28 in either KN-93, ML-7 or protein kinase G treated AD169 infected HFF. HFF cells were infected with AD169 at moi of 0.5, which was removed after 2 h and replaced with media containing either drug (see 2.8). KN-93 was used at 1,996 μM , ML-7 at 2.209 μM and PKG at 10.6 μM respectively. At 96 hpi, cells were subjected to IF analysis using 1st antibodies against pp28 and anti mouse Cy2 secondary antibody (green).

3.5 Influence of staurosporine on subcellular distribution of pp28

Staurosporine is a wide range serine threonine kinase inhibitor. Staurosporine was poorly investigated in HCMV studies. Cellular serine/threonine kinases were shown to be increased during HCMV infection (Hakki and Geballe, 2008; Gaddy et al., 2010). In our assembly experiments, staurosporine was the only kinase inhibitor that showed a major difference in the subcellular distribution of pp28, as shown in figure 3.5 A. The pp28 was remarkably punctuated with distinct large vacuoles (5-10 μm in diameter) that did not appear in the non-inhibited infected cells or in any infected cells treated with other kinase inhibitor.

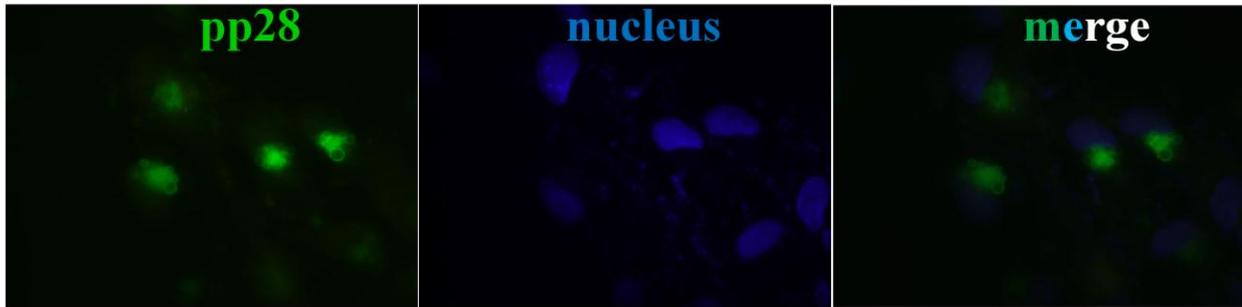


Figure 3.5 A: Subcellular distribution of pp28 in staurosporine inhibited AD169 infected HFF. HFF cells were infected with AD169 at moi of 0.5, which was removed after 2 h and replaced with media containing staurosporine (42.8 nM) (see 2.8). At 96 hpi, cells were subjected to IF analysis using 1st antibodies against pp28 and anti mouse Cy2 secondary antibody (green). Nucleus was counter-stained with DAPI (blue). Staining was visualized with Olympus BX60 and Olympus DP71 digital camera.

Our previous experiments (Azzeh et al., 2006; Zawahreh, 2009) had shown that Δ UL97 infected cells were not affected by different drugs. In order to rule out this observation in case of staurosporine, Δ UL97 infected cells were subjected to drug inhibition assay and stained with pp28 at 96 hpi. As predicted, the Subcellular distribution of pp28 was not affected by staurosporine in Δ UL97 infected cells (figure 3.5 B). In both, Δ UL97 infected and Δ UL97 infected staurosporine inhibited cells, pp28 distributed to its typical "crown"-like vacuole rich structure as shown earlier by Azzeh et al. (Azzeh et al., 2006).

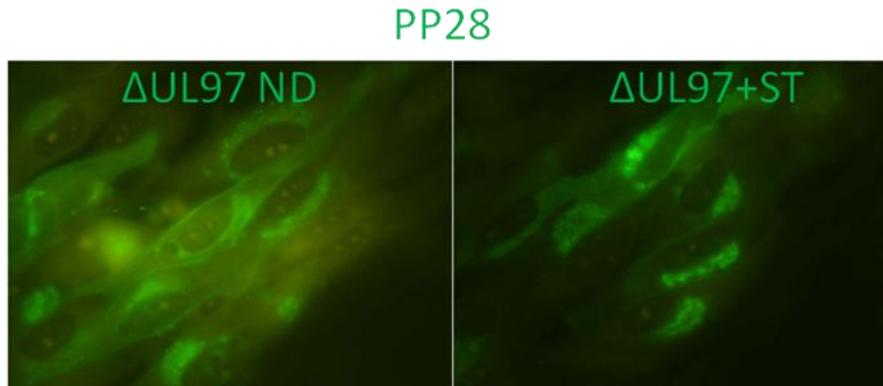


Figure 3.5 B: Subcellular distribution of pp28 in staurosporine inhibited Δ UL97 infected HFF. HFF cells were infected with Δ UL97 at moi of 0.5, which was removed after 2 h and replaced with media containing staurosporine (42.8 nM) (see 2.8). At 96 hpi, cells were subjected to IF analysis using 1st antibodies against pp28 and anti mouse Cy2 secondary antibody (green). Nucleus was counter-stained with DAPI (blue).

This experiment with staurosporine inhibition of AD169 were repeated in order to check the reproducibility of the results, but expanded to test the subcellular distribution of pp65, a major tegument protein. In both cases, pp28 and pp65 were detected in simultaneous experiments in non-inhibited cells. As demonstrated in figure 3.6 (A-B), the vacuoles rich AC structure appears continuously in staurosporine treated infected cells, when stained with either pp28 or pp65 tegument proteins.

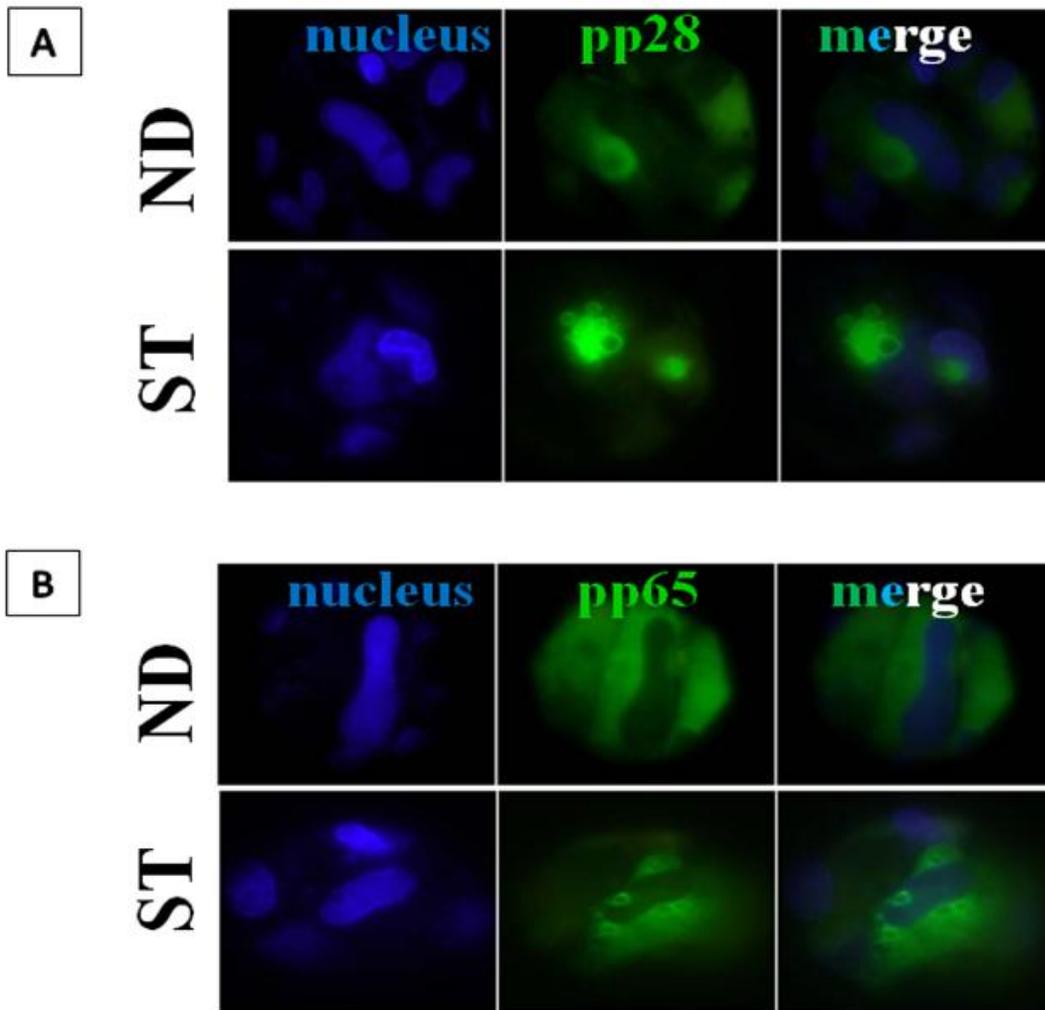


Figure 3.6 A-B: Subcellular distribution of pp28 (A) and pp65 (B) in non-inhibited AD169 infected cells versus staurosporine inhibited infected cells. HFF cells were subjected to infection assay (see 2.2.3) or drug inhibition assay using staurosporine at 42.8 nM (see 2.8). At 97 hpi, cells were subjected to IF analysis using either mouse anti pp28 (A) or mouse anti pp65 (B) as 1st antibody, followed by anti mouse Cy2 secondary antibody (green), the nucleus was counter-stained with DAPI (blue).

3.6 Staurosporine does not affect early stages of infection, nor does it affect AC if added to cells before infection

In order to test, whether staurosporine inhibition is restricted to the AC or early events of HCMV infection, IF staining experiments were performed using IE1 (marker for immediate early protein of HCMV). As clearly demonstrated in figure 3.7 A, IE1 expression in the nucleus was not affected or altered due to staurosporine inhibition.

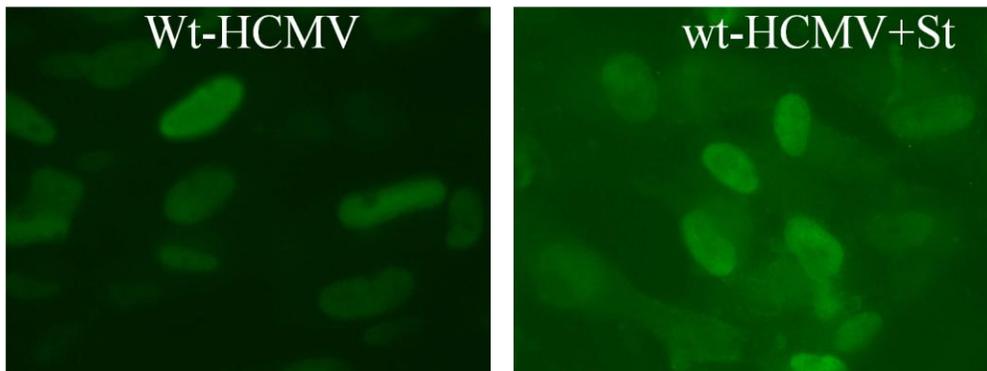


Figure 3.7 A: Staurosporine does not affect the subcellular distribution of IE1. Cells were infected with AD169 and either staurosporine inhibited or mock inhibited. At 96 hpi, cells were subjected to IF analysis using marker to the viral immediate early protein IE1 (green).

To check whether staurosporine affects the AC by affecting viral entry, cells were pretreated with staurosporine for 2 h before subjecting them to infection assay as in 2.2.3. At 96 hpi, infected pretreated cells were subjected to IF analysis using pp28 antibody as AC marker. These experiments revealed that the subcellular distribution of pp28 was not affected, once staurosporine was added before infection (figure 3.7). This also indicates that staurosporine affects the viral infected cells, rather than the cells directly. On the other hand this result also illuminate that staurosporine does not inhibit viral entry, since the subcellular distribution of pp28 remained unchanged if compared with infected non-treated cells.

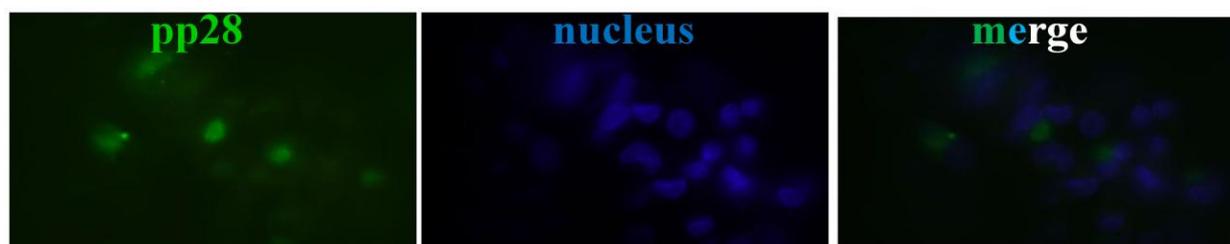


Figure 3.7 B: Subcellular distribution of pp28 in HFF cells pre-treated with staurosporine. HFF cells were incubated with staurosporine (42.8 nM) containing medium for 2 h; cells were washed 3 times and subjected to infection assay (2.2.3). At 96 hpi, infected cells were subjected to IF analysis using pp28 (green).

3.7 Influence of staurosporine on subcellular distribution of Golgi in uninfected cells

Initially FITC conjugated Wheat germ agglutinin (WGA) was tested in IF experiments on uninfected cells after or without inhibition with staurosporine, to ensure that Golgi does not lose

its integrity due to staurosporine. WGA is a carbohydrate that binds to clustered terminal N-acetylneuraminic acid residues and to N-acetylglucosamine-containing oligosaccharides on proteins (Bhavanandan and Kaltic, 1970) and decorates distal Golgi cisternae, the trans-Golgi network and the cell surface (Tartakoff and Vassalli, 1983; Virtanen et al., 1980).

HFF cells were either treated with staurosporine for 96 h or mock treated and subjected to IF analysis using WGA. As demonstrated in figure 3.8, staurosporine did not affect the typical distribution of WGA staining. WGA maintained its normal appearance, which indicates that staurosporine in the used concentration did not result in loss of cellular viability, nor did it cause cell death. Furthermore, these experiments indicate that staurosporine rather affects infected than uninfected cells, when comparing this result with figure 3.5A and 3.6.

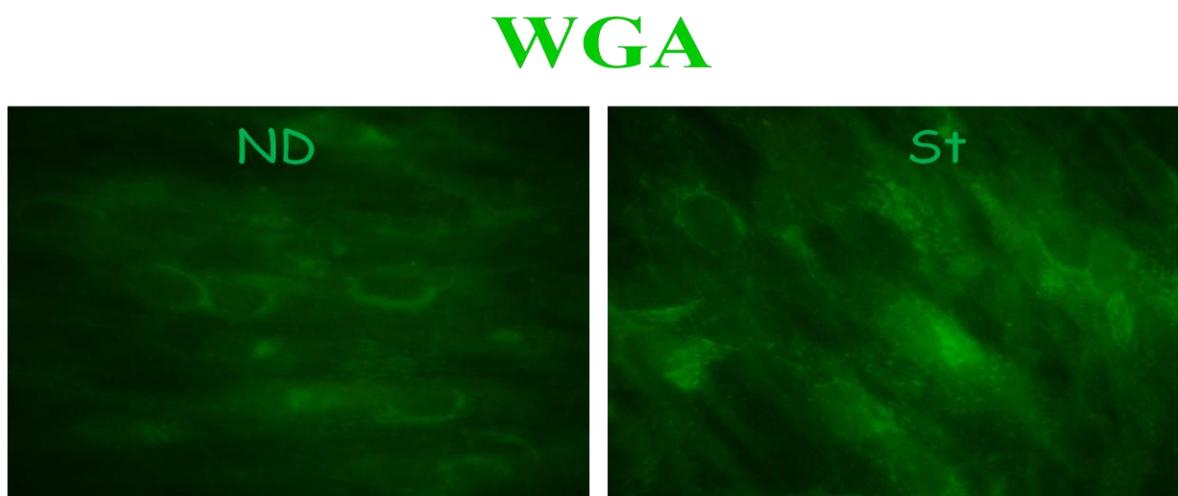


Figure 3.8: Influence of staurosporine on uninfected cells. HFF cells were non-treated (left) or treated with a final concentration of staurosporine of 42.8 nM for 96 h. Cells were subjected to WGA staining and visualized as in Figure 3.1.

3.7.1 Co-localization of Golgi marker WGA with tegument proteins in staurosporine infected cells

In order to test whether the AC in staurosporine inhibited infected cells maintains its co-localization with Golgi markers, co-localization experiments were carried out. HFF cells were subjected to drug inhibition assay and IF assay using either pp28 or pp65 antibody and WGA simultaneously. These experiments revealed that tegument protein; pp28 and pp65 co-localized perfectly with WGA in staurosporine treated cells. This indicates that the AC is not affected by the staurosporine treatment or its co-localization with Golgi marker WGA.

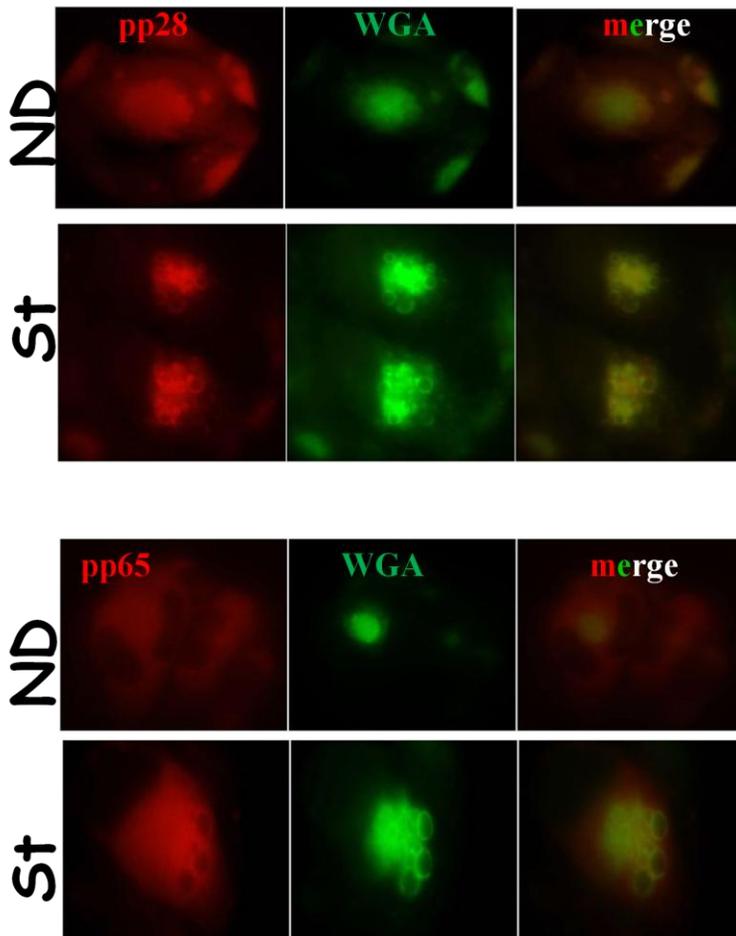


Figure 3.9 A-B: A. Co-localization of pp28 with WGA Golgi marker in MOCK inhibited HCMV infected cells versus staurosporine inhibited infected cells. B. Co-localization of pp65 with WGA Golgi marker in MOCK inhibited HCMV infected cells versus staurosporine inhibited infected cells. HFF cells were subjected to infection and drug inhibition assay (see 2.8) using staurosporine at 42.8 nM final concentration. At 96 hpi, cells were subjected to IF analysis using pp28 (A) or pp65 (B) antibody followed by anti mouse Cy 5 (red) and the FITC-conjugated WGA (green).

3.8 The reversibility of staurosporine inhibition in AD169 infected cells

To check if the AD169 infected cell can reverse the effect of staurosporine, block-release assays were performed. Hereby, staurosporine was added to infected cells as in 2.8 (block). At 96 hpi, cells were washed 4 times with PBS and finally replaced with drug free medium (release). 72 h after release, cells were subjected to IF analysis using either the major tegument protein pp65 or WGA Golgi marker. As illustrated in figure 3.10, the effect of staurosporine evoking the characteristic vacuole rich WGA or pp65 staining was released and the normal "bulb"- like appearance of both markers was restored. These results indicated that the effect of staurosporine is reversible and not permanent and restricted to infection.

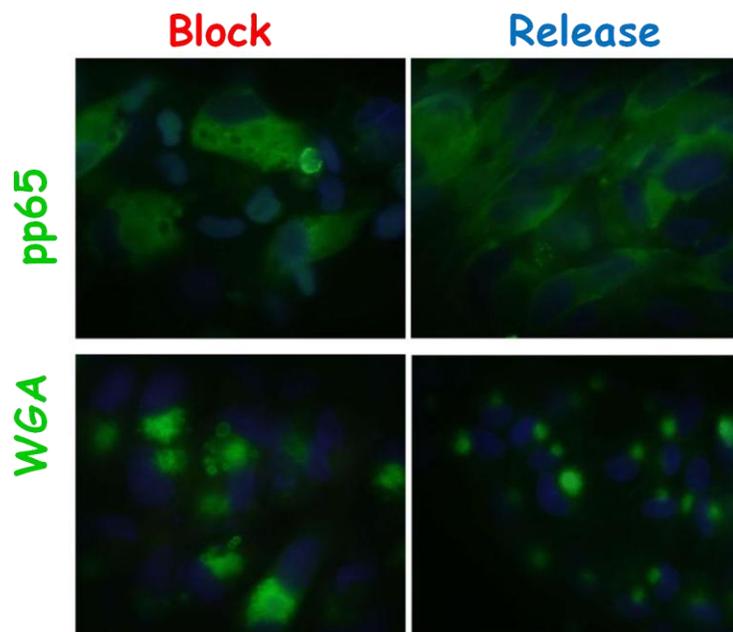


Figure 3.10: Block-Release of staurosporine inhibition. HFF cells were subjected to inhibition assay (2.8) at 42.8 nM staurosporine end concentration (block). At 96 hpi, staurosporine was washed out using PBS for 4X and drug free medium was added (release). 72 hpi after release,

cells were subjected to IF analysis using either pp65 antibody (upper row) or FITC conjugated WGA (lower row). Nuclei were counter-stained with DAPI (blue).

3.9 Subcellular distribution of *trans* Golgi and *distal* Golgi in AD169 infected HFF cells

The *trans*-Golgi network (TGN) is part of the secretory pathway of eukaryotic cells which is distinct from the Golgi stack. The TGN plays a key role in the sorting and targeting of secreted proteins to the correct destination which is a later stage of protein secretion. TGN46 cycles between the trans-Golgi network and the cell surface returning via endosomes. It is thought to be involved in regulating membrane traffic to and from trans-Golgi network. There are three isoforms of TGN: TGN46, TGN48 and TGN51. TGN46 is widely expressed and is a human specific protein; TGN48 is expressed only in promyelocytic cells and embryonic kidney, while TGN51 is expressed in fetal lung and kidney.

A rabbit polyclonal antibody to TGN46 (ab50595) was used as a marker for the Trans Golgi network. Uninfected cells, versus AD169 infected cells were subjected to IF analysis using TGN46 antibody at 96 hpi. As clearly demonstrated in figure 3.11, TGN46 distribution was strikingly affected due to HCMV infection. While it showed a typical Golgi "thread"- like stacks in uninfected cells, it assumed a compact "bulb"- like structure in infected cells as known for the AC. These results provide a strong evidence for the powerful involvement of viral assembly in the cell structure and precisely in Golgi structure.

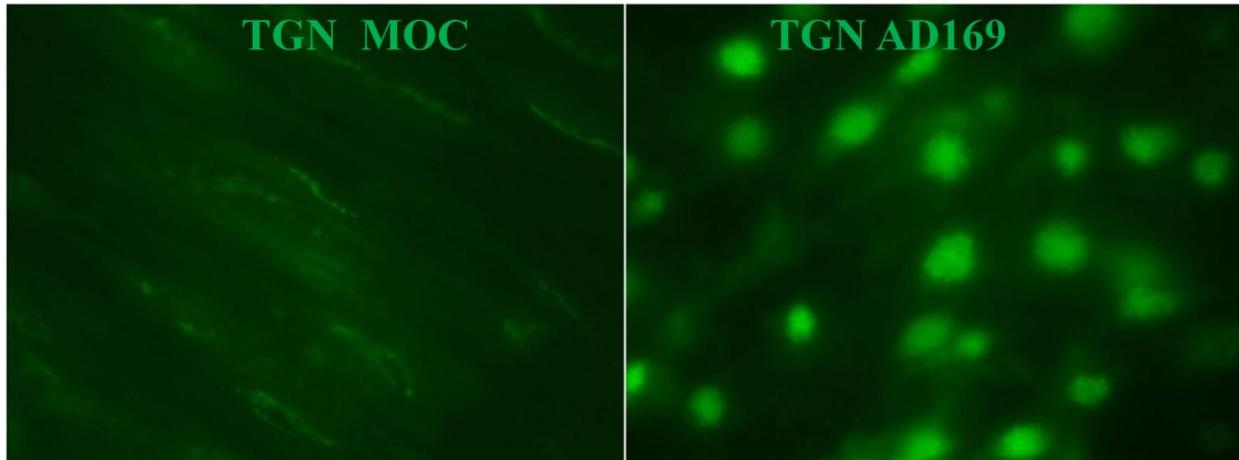


Figure 3.11: Subcellular distribution of *trans* Golgi marker TGN46 in AD169 infected cells.

HFF cells were either mock infected (left) or subjected to infection assay as in 2.2.3 (right) and to IF analysis at 96 hpi. TGN46 antibody was used to stain trans Golgi, followed by anti rabbit Cy2 antibody (green).

The subcellular distribution of TGN46 was very similar to that of AC markers. In order to prove the fact that TGN46 co-localizes to the assembly complex in staurosporine treated AD169 infected cells, co-localization experiments were performed. In these experiments, HFF cells were infected and drug inhibited as usual and staining was made for both, Golgi marker TGN46 and the tegument protein pp28. Interestingly, pp28 and TGN co-localized perfectly to the AC, and this co-localization was not affected when infected cells were treated with staurosporine (figure 3.12).

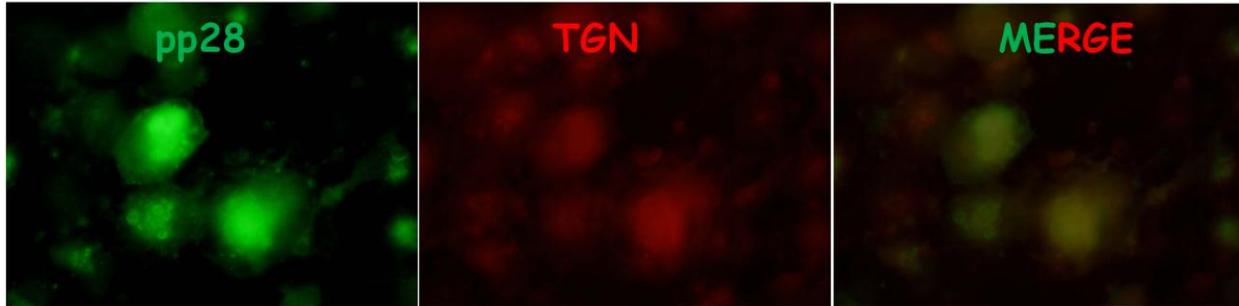


Figure 3.12: Co-localization of TGN46 with tegument protein pp28. HFF cells were subjected to infection assay or drug inhibition assay using staurosporine. At 96 hpi, cells were subjected to IF analysis using TGN46 antibody followed by Cy5 secondary antibody (red) and pp28, followed by using Cy2 secondary antibody (green).

GOLPH is a *cis* Golgi localized calcium binding protein. GOLPH4 has been a revealing marker of early Golgi vesicle trafficking (ab28049, Abcam, UK). Initially GOLPH4 was used to stain *cis* Golgi in AD169 infected cells versus mock infected cells. These experiments showed that the subcellular distribution of GOLPH4 is remarkably changed in infected cells (into a "bulb"-like structure), similar to the TGN46 and WGA (figure 3.13). In co-localization experiments with pp28, GOLPH4 distributed clearly to the AC, also in staurosporine inhibited AD169 infected cells (figure 3.14).

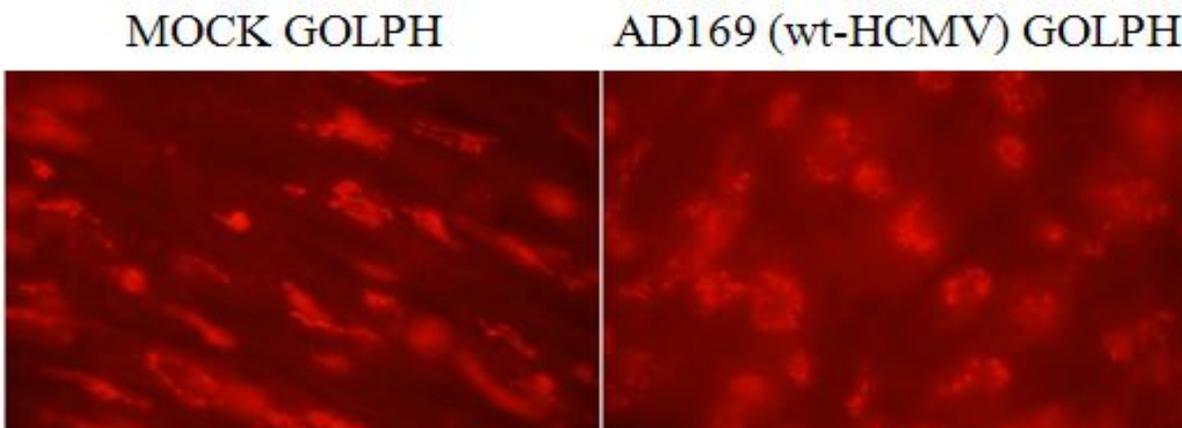


Figure 3.13: Subcellular distribution of *cis* Golgi marker GOLPH4 in AD169 infected cells. HFF cells were either mock infected (left) or subjected to infection assay as in 2.2.3 (right) and to IF analysis at 96 hpi. GOLPH4 antibody was used to stain cis Golgi and followed by anti rabbit Cy5 antibody (red).

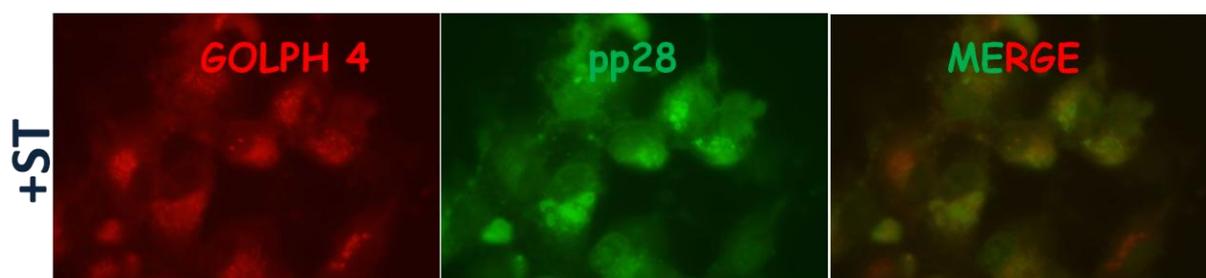


Figure 3.14: Co-localization of GOLPH4 with tegument protein pp28. HFF cells were subjected to infection assay or drug inhibition assay using staurosporine. At 96 hpi, cells were subjected to IF analysis using GOLPH4 antibody followed by Cy5 secondary antibody (red) and pp28, followed by Cy2 secondary antibody (green).

3.10 The nucleus of AD169 infected cell is also affected by staurosporine

Not only was the subcellular distribution of the tegument proteins very distinguishable in HCMV infected cells treated with staurosporine, but also the nuclear shape. The normal nucleus of uninfected HF cell appears as an oval shape, however upon infection with AD169 or Δ pp65 the nucleus turns into kidney shape (Azzeh et al., 2006; Zawahreh, 2009), and when infected with Δ UL97 or AD169+NGIC-I, it maintained its oval shape but was enlarged and punctuated by vacuoles of different sizes (Azzeh et al., 2006; Zawahreh, 2009). Interestingly, after treatment with staurosporine, nuclei of AD169 infected cells assumed a new shape; we referred to as "boot"-like shape (figure 3.15). This type of shape was observed in 40% of the AD169 infected, staurosporine treated cells (see 2.8.2 for statistical analysis). Most interestingly, the AC in the AD169 (wt-HCMV) infected, staurosporine treated cells localized to the slight invagination at the thin top of "boot"-like shaped nucleus (see also figures 3.5-6, 3.10).

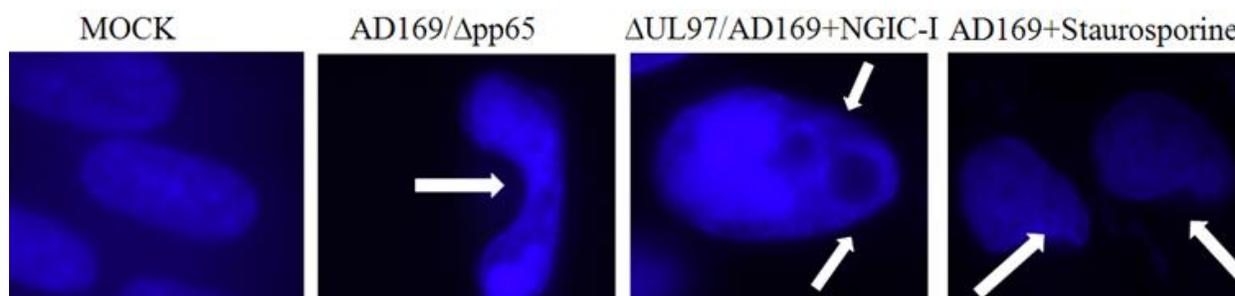


Figure 3.15: Correlation between nuclear shape and structure of assembly complex. HFF cells were either mock infected, infected with AD169 (wt-HCMV), Δ UL97, Δ pp65 deletion mutants or infected and subjected to drug inhibition assay either using NGIC-I (0.5 μ M, Azzeh et al., 2006) or staurosporine (42.8 nM). At 96hpi, cells were subjected to IF assay using DAPI nuclear staining (blue). The white arrows show the juxtannuclear area, where the AC occurs.

3.11 The role of Golgi in building the assembly complex

Brefeldin A (BFA) is a Golgi disruption agent and was used in Golgi disruption assays (2.8.1). As indicated by the name of the assay; BFA destroys the Golgi. Since the experiments above show that the AC is co-localizing with Golgi markers (see 3.7 and 3.9), disruption of Golgi should affect the subcellular distribution of the AC.

Although it was not destroyed completely as illustrated in figure 3.16, the AC was scattered within the cytoplasm accordingly with the scattered Golgi in AD169 infected and infected staurosporine treated cells. The scattering of Golgi was detected by anti- GOLPH4 (*cis* Golgi marker) and anti- TGN46 (*Trans* Golgi marker) staining, followed by anti- rabbit Cy5. The scattering of the viral AC proteins was detected using anti pp28 antibody, stained green via anti-mouse Cy2. Both, the cellular Golgi marker GOLPH4/TGN46 and the viral AC marker pp28 lost their typical compact "bulb"-like appearance to a destructed structure showing staining throughout the cytoplasm.

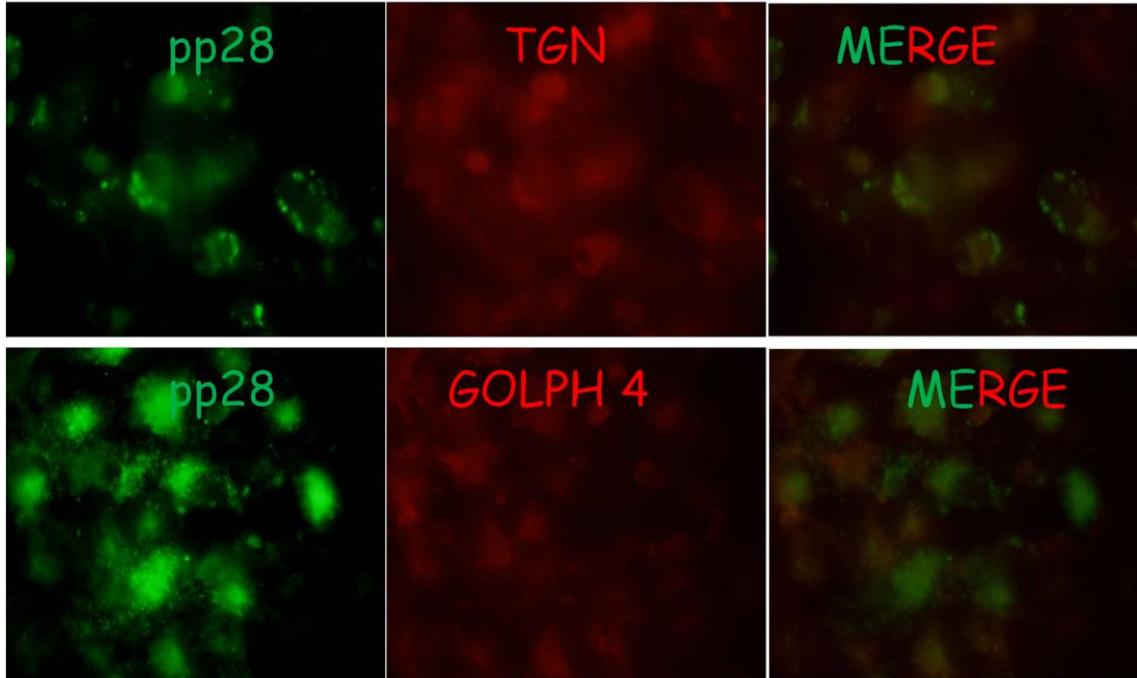


Figure 3.16: The role of Golgi in building the assembly complex. HFF cells were subjected to drug inhibition assay as in 2.8 using staurosporine. At 96 hpi, BFA (4 μ g/ml) containing complete DMEM medium was added to the infected cells and incubated for further 2 h (see 2.8.1). Finally cells were subjected to IF analysis using Golgi markers, GOLPH4 and TGN46 (red) and the viral AC marker; pp28 (green).

3.12 The influence of staurosporine on viral load

Staurosporine, as a kinase inhibitor and as demonstrated above, changed the structure of the assembly complex and therefore it was expected that this kinase inhibitor may also affected the viral load. For this, supernatants of infected AD169 cells, either treated or untreated with staurosporine were subjected to viral load analysis using the real time PCR method (2.11).

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

As clearly indicated in figure 3.17, staurosporine reduced the viral load overall, this reduction however, 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. This reveals that the staurosporine effect is clearly dependent on the viral titer used initially for infection and it is a further indication for our proposed theory that staurosporine rather inhibits infected than mock infected cells.

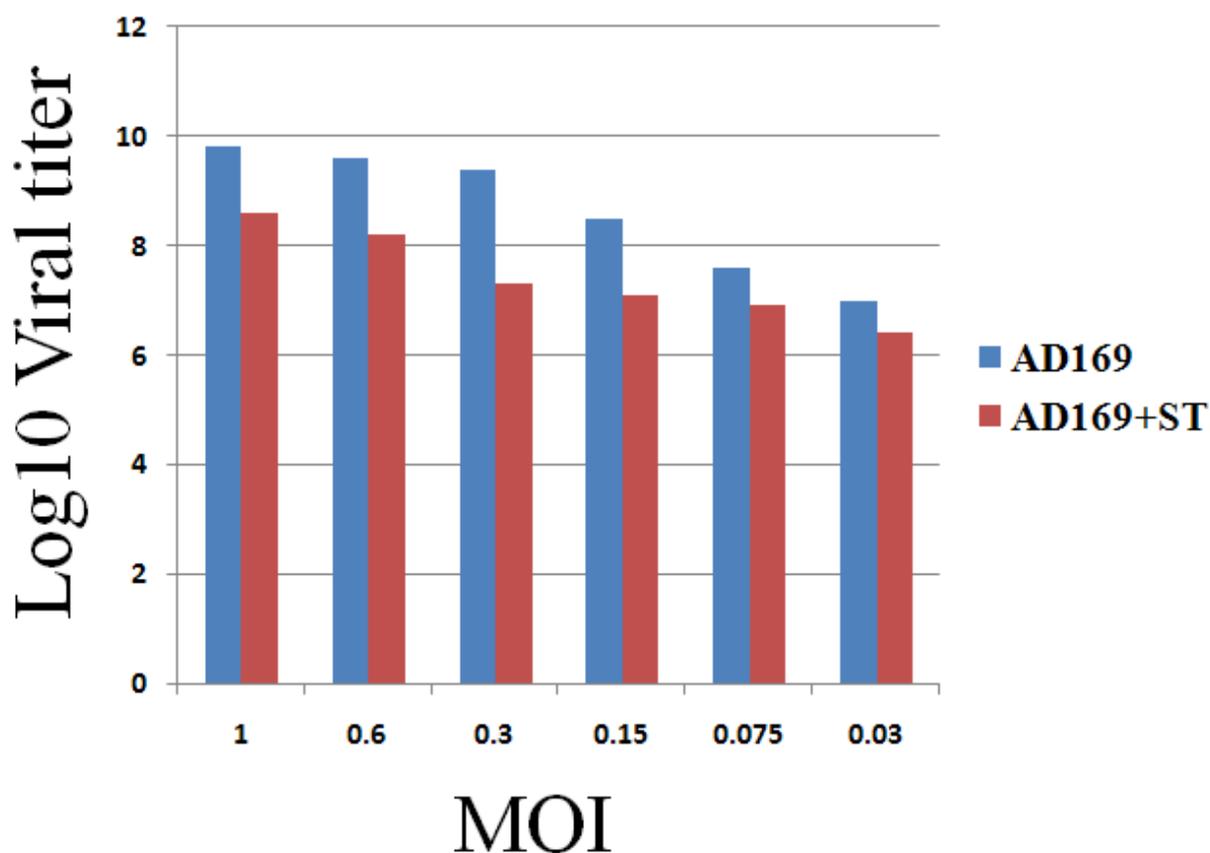


Figure 3.17: Influence of moi and drugs on HCMV viral load. HFF cells were infected with different moi of AD169 and mock inhibited or staurosporine (ST) inhibited using drug

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.

3.13 Influence of staurosporine on viral titer

Viral titration assays were performed, in order to assess the influence of staurosporine on viable progeny viruses using the traditional plaque assay method (see 2.2.2). Cells were subjected to infection assay using AD169 or Δ UL97, mock inhibited or staurosporine inhibited in drug inhibition assay. Supernatants were subjected to plaque assay analysis at 96 hpi. The viral titer was strikingly reduced in the staurosporine treated AD169 infected cells compared to staurosporine inhibited Δ UL97 infected cells (figure 3.18). In the first case the viral titer was

reduced almost 3 log, but only less than 1.5 log in the second case (figure 3.18).

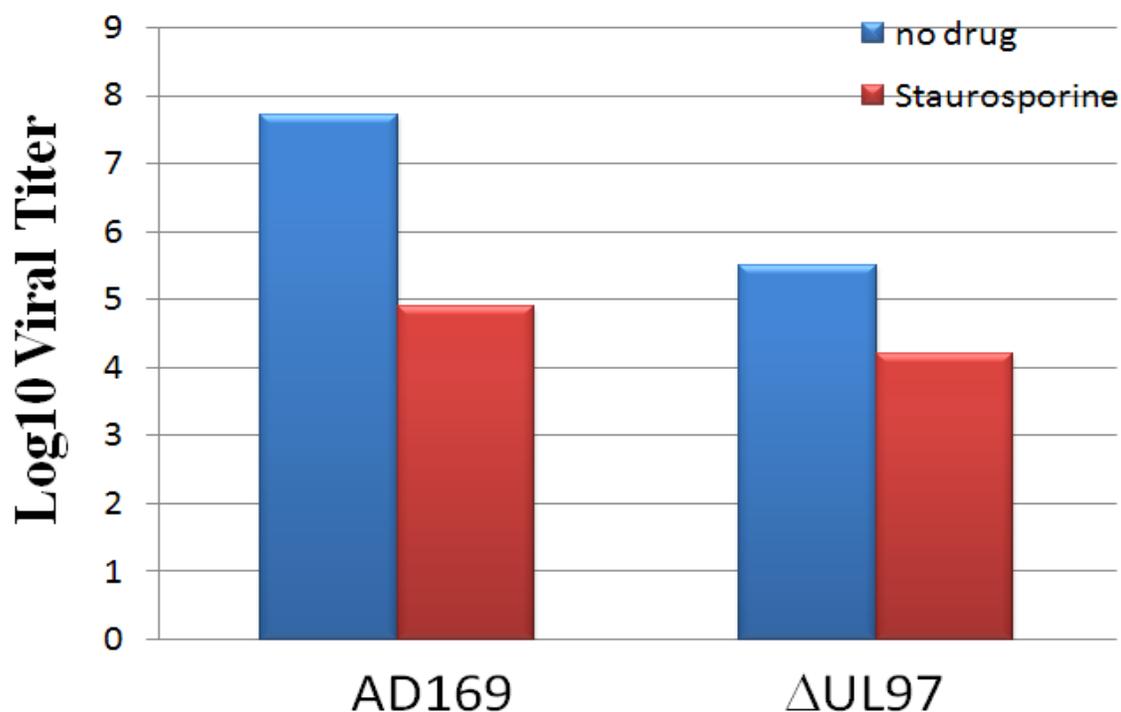


Figure 3.18: Influence of staurosporine on Viral Titration. HFF cells were infected with either AD169 or ΔUL97 and either subjected drug inhibition assay or mock inhibited. At 96 hpi viral supernatants of treated versus mock treated (untreated) infected cells were subjected to plaque assay. Plaques were counted, viral titer of AD169 infection from 3 different experiments was averagely 6.3×10^8 , 8×10^4 of staurosporine inhibited AD169 infection, 4×10^5 of ΔUL97 infection and 1.6×10^4 of staurosporine inhibited ΔUL97 infection. Log 10 of viral titers is given on the Y axis, virus type on the X axis.

3.14 Staurosporine does not affect viral entry or viral exit

To check whether staurosporine affects the viral entry, cells were pretreated with staurosporine for 2 h before subjecting them to infection assay as in 2.2.3. At 96 hpi, DNA was extracted from supernatants and subjected to viral load analysis as detailed in 2.11. As shown in figure 3.19, pre-treatment of cells with staurosporine did not affect the viral load in AD169 or Δ UL97, while adding staurosporine at 0 hpi did remarkably affect the viral load in case of AD169 (wt-HCMV) infection. Furthermore, adding staurosporine later during post infection, at 24 hpi, 48 hpi or 72 hpi did not cause reduction of viral load in either viruses.

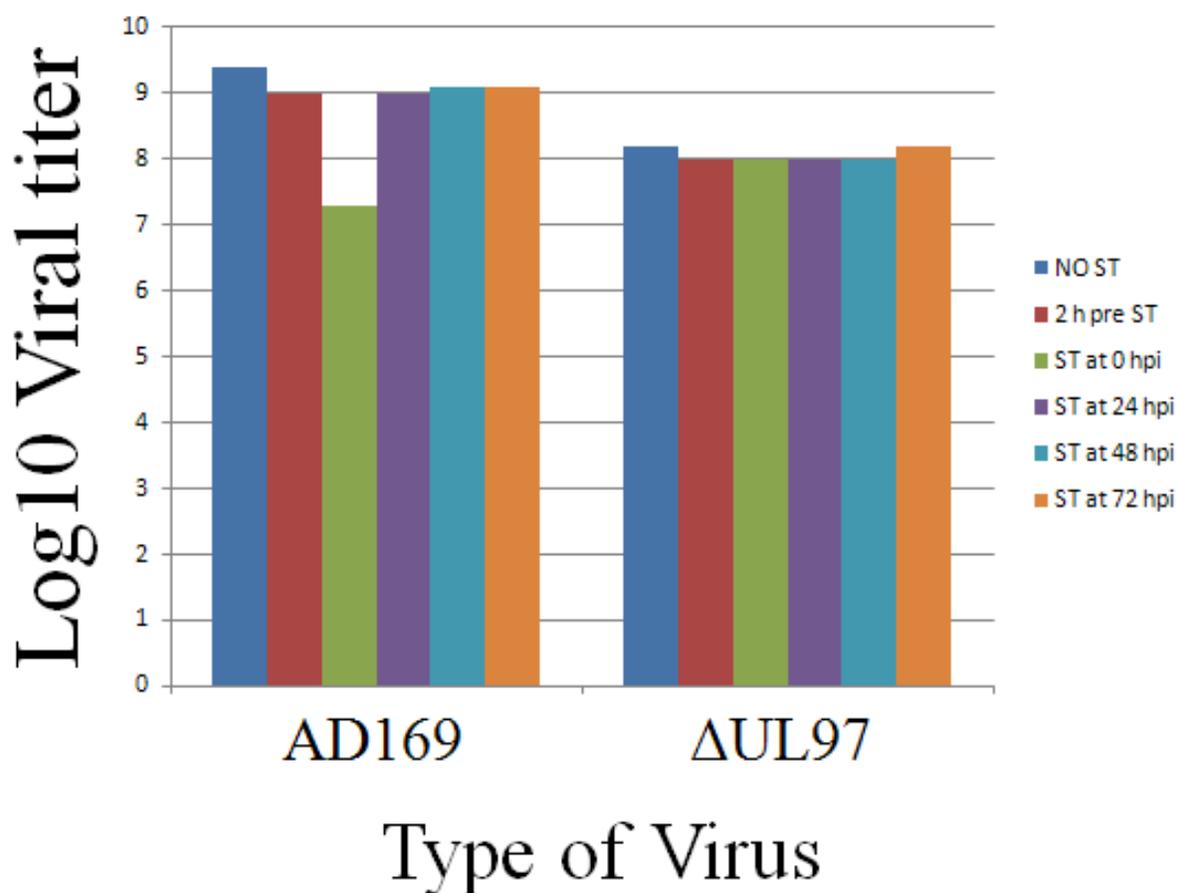


Figure 3.19: State of staurosporine inhibition in HCMV life cycle. Staurosporine was added to cells before infection with either AD169 or Δ UL97, or at different time points post infection (hpi). Supernatants were harvested at 96 hpi and subjected to viral load analysis.

To check if staurosporine affects viral exit, DNA was extracted from infected treated or mock treated cells as well as from supernatant at 96 hpi and subjected to viral load analysis. The ratio viral load in supernatant to viral load in cells was calculated and compared between AD169 infected and AD169 infected staurosporine inhibited cells. Interestingly, this ratio was constant for each experiment in both cases and had a value of 1.2-1.4. This is an indication for the fact that viral exit is not affected by staurosporine inhibition.

3.15 Kinetic development of the vacuoles in staurosporine inhibited AD169 infected cells

Kinetic experiments were performed in order to check the time point, at which the vacuoles arise. The experiments were performed as detailed in 2.9. The IF analysis using WGA staining showed clearly that vacuoles emerged at 60 hpi (figure 3.20). This means that vacuole building is a process occurring during the assembly stage, not an earlier or later stage in the HCMV life cycle.

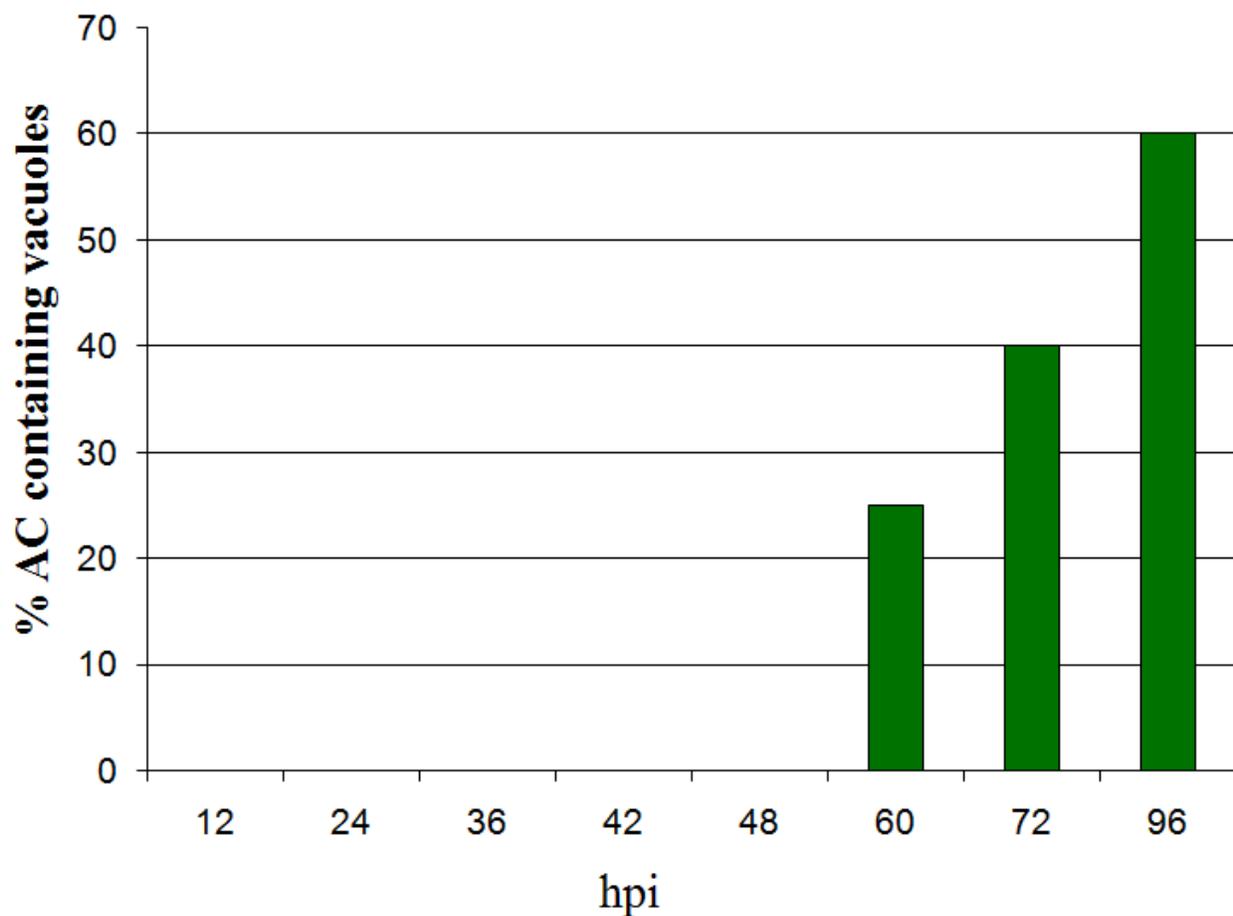


Figure 3.20: Vacuole building in staurosporine treated AD169 infected cells. HFF cells were infected with AD169 and treated with staurosporine at 0 hpi. Cells were subjected to IF analysis using WGA staining every 2 hpi as detailed in 2.9.

3.16 The influence of staurosporine on tegument protein expression

All experiments shown above emphasize that staurosporine affects viral assembly in wt-HCMV (AD169) infected cells, if the inhibition occurs at 0 hpi. The IF analysis above illustrates structural changes in the subcellular distribution of pp65 and pp28.

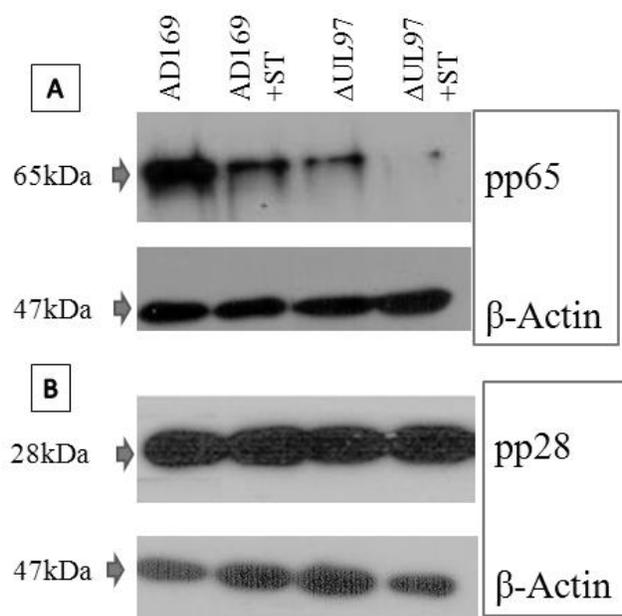


Figure 3.21 A-B: Protein expression of tegument proteins pp65 (A) and pp28 (B) with or without staurosporine inhibition. Equal amounts of total protein lysates were subjected to Western blot analysis. pp65 was detected with anti pp65 (A, upper panel), pp28 with anti pp28 (B, upper panel), both mouse first antibodies, followed by anti Mouse HRP. To ensure that equal amounts were loaded into the gel, striped membranes (2.7.5) were subjected to another immunoblot analysis (see 2.7 and 2.7.5) by using anti β-Actin (A, lower panel, B, lower panel) loading controls.

Here, we became interested in the status of protein expression of both tegument proteins. For this, cells were either infected with AD169 or ΔUL97 and inhibited or mock inhibited with staurosporine at 0 hpi. At 96 hpi, cells were subjected to western blot analysis (see 2.7) using either pp65 or pp28 antibody.

On the contrary to pp65, pp28 expression was hardly affected by staurosporine inhibition (Figure 3.21A-B). The level of expression in AD169 or ΔUL97 infected cells, versus inhibited infected cells was very similar in case of pp28 (figure 3.21A). pp65 was affected remarkably, reflecting

the result revealed by viral load analysis. pp65 expression was lower in staurosporine inhibited infected cells, but also in Δ UL97 infected cells compared to AD169 infected cells.

3.17 The influence of staurosporine on cellular proteins in HCMV infected cells

In order to check whether cellular proteins are affected by staurosporine inhibition in infected cells, infected cellular lysates were subjected to western blot analysis using TGN46 antibody. As indicated in figure 3.22, TGN46 protein expression was not affected by staurosporine inhibition. Expression level was similar in all three conditions; infection with AD169, infection with AD169 inhibited by staurosporine and finally infection with Δ UL97.

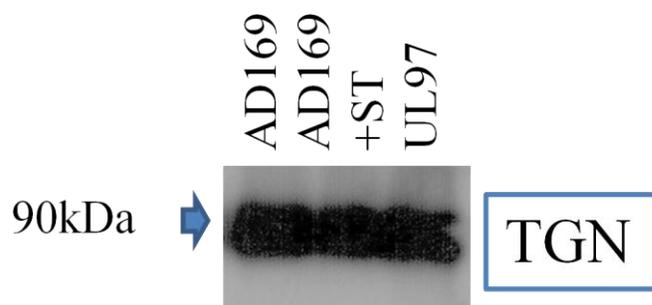


Figure 3.22: TGN46 expression in HCMV infected and HCMV infected, staurosporine inhibited cells. HFF cells were infected with either AD169 or Δ UL97 or infected with AD169 and inhibited with staurosporine. At 96 hpi, cells were subjected to western blot analysis using equal amounts of protein extracts.

3.18 The influence of staurosporine on cellular serine/threonine in HCMV infected cells

Since staurosporine is known to be a broad range inhibitor of serine/threonine kinases, it was worth investigating the status of serine/threonine kinase expression. The manufacturer (Abcam, UK) indicates that four bands of different kDa can be detected via anti serine/threonine antibody; 28 kDa, 55 kDa, 70 kDa and 80 kDa via western blot analysis. However the experiments performed here did not reveal any protein bands in direct western blot analysis. In Co-IP experiment, two protein bands were detectable (figure 3.24). Cellular lysates of infected, staurosporine treated versus mock treated were subjected to Co-IP (2.7.7) using pp65 antibody followed by western blot analysis using anti serine/threonine. The result shown in figure 3.23 revealed that the 28 kDa band was detectable in all lysates, however the 55 kDa band was clearly distinguishable in the AD169 (wt-HCMV) lysate mainly. The 55 kDa band was very weak in the other lysates.

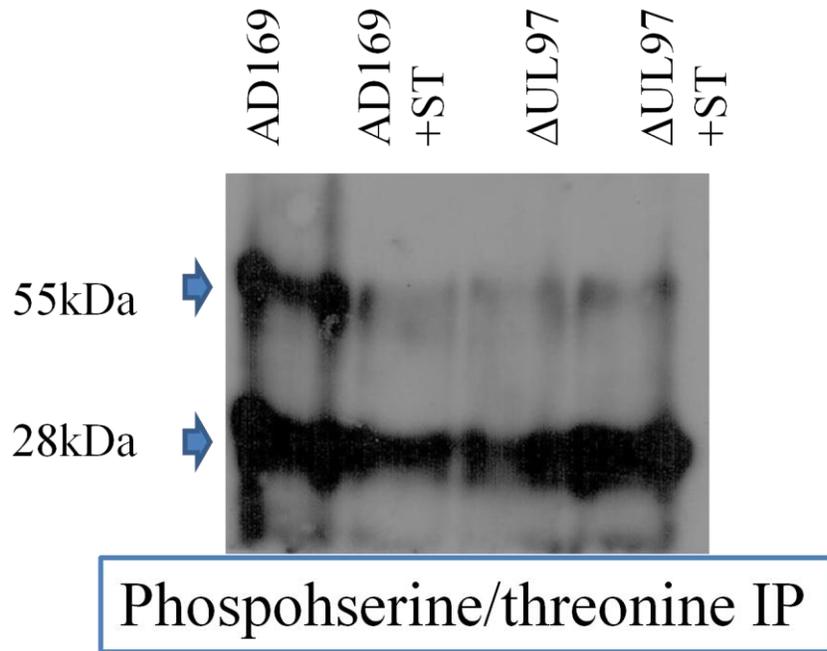


Figure 3.23: IP of serine/threonine kinase in HCMV infected and HCMV infected, staurosporine inhibited cells. HFF cells were infected with either AD169 or Δ UL97 or infected and inhibited with staurosporine. At 96 hpi, cell lysates were subjected to Co-IP with pp65 and followed by western blot analysis with anti phosphoserine/threonine using equal amounts of protein extracts (see also 2.7.6).

4. Discussion

Protein kinases play a critical role in signaling pathways in all cells. In cancer therapy, protein kinases became very promising therapeutic targets.

As the case for cancer therapy, the problematic of developing anti viral drugs remains one of the most challenging targets in viral research. For the last decades only few anti viral drugs were developed and approved, despite the toxic side effect of most of these drugs on human being. Many anti viral drugs fail even in the last clinical trials because of extreme side effects on the diseased volunteers, because of the high incidences of drug resistance viruses produced during therapy, or because of low efficacy in critical diseased patients (see www.viropharma.com on maribavir, 2009).

Although eight kinase inhibitors have been approved in the United States for cancer therapy (Ghoreschi et al., 2009), no kinase inhibitor is approved for viral diseases yet.

Better and detailed understanding of cellular viral interaction is the key to develop precise medication against viral infections and was the main aim of this work. Despite the fact that many researches were performed on cellular kinases (see 1.6, p. 20-21), most of this research showed regulatory roles specifically of CDKs in viral infection. Other researches showed that some of the CDKs directly interacted with viral proteins indicating specific roles in viral life cycle (Bresnahan et al., 1997; Chen et al., 2001; Sanchez et al., 2003; Sanchez et al., 2004; Sanchez and Spector 2006; Kapasi and Spector 2008; Rechter et al., 2009; Hamirally et al., 2009). PI3-K was also shown to play a major role in HCMV life cycle, since its inhibition inhibits viral replication and virus-induced signaling (Johnson et al, 2001).

In this work, a wide range of cellular kinases were tested for their possible role in the viral assembly, initially based on the change they made in subcellular distribution of viral tegument protein during infection.

4.1 The influence of wide range serine/threonine kinase inhibitors on subcellular distribution of HCMV tegument protein and AC marker pp28

Drug inhibition assays testing different cellular kinase inhibitors of the serine/threonine inhibitor set; Bisindolylmaleinide I, H-89, KN-93, ML-7, PKG inhibitor and staurosporine were checked for their possible role in building assembly complex. With exception of staurosporine, all the other components of the serine/threonine cellular kinase inhibitor set failed to produce a remarkable effect on subcellular distribution of pp28 or on the viral load and viral titer.

One of the kinases, against which inhibitors were used here are actually known to play a role in HCMV life cycle. Bisindolylmaleinide I inhibits protein kinase C, which was shown to play different roles in ongoing HCMV viral infection (Kristoffersen et al., 1994; Keay and Baldwin, 1996; Slobbe-van Drunen et al., 1997; Liu et al., 2010; Milbradt et al., 2010; Marschall et al., 2011; see 1.6). PKC involvement in nuclear egress was one of the most interesting roles, which may actually implicate its possible influence in viral assembly stage (Milbradt et al., 2010; Marschall et al., 2011). It was expected that PKC affects viral assembly; however, we propose that the tests made in this work may have not been sufficient to rule out a role for PKC in HCMV viral assembly. It is still possible that PKC plays a role in assembly, but one, which may not have a direct effect on AC structure, viral load or on the specific protein's tested in this work.

PKA was also shown to affect HCMV infection (Kristoffersen et al., 1994); this role however, was not clearly illuminated. The fact that PKA and the other cellular kinase inhibitors did not cause a change in the subcellular distribution of pp28 does not necessarily mean that they do not affect the viral infection at any other time point of the viral life cycle, which was not tested in this work.

Although staurosporine by itself was not used in HCMV research investigation, few publications revealed that cellular serine/threonine was increased in HCMV infected cells (Gaddy et al., 2010; Hakki and Geballe, 2008). This fact by itself is a proof for a possible role of cellular serine/threonine kinase in HCMV life cycle. Our experiments here emphasized that cellular serine/threonine kinase play a major role in HCMV assembly.

4.2 The function of staurosporine and its effect on HCMV assembly

Staurosporine is known to be a broad range serine/threonine inhibitor affecting different cellular kinases based on the concentration used (Meyer et al., 1991; Seynaeve et al., 1993; Couldwell et al., 1994; Yue et al., 1998; Ruegg and Burgess, 2002). Staurosporine was not investigated yet in the HCMV research making it very interesting to discuss the new finding presented here.

The data presented here highlight a serious role of serine/threonine in HCMV assembly based on the inhibition assays performed using staurosporine. We propose that the resulted effects were specific to serine/threonine kinase inhibition evoked by staurosporine.

4.3 Subcellular distribution of AC and other effects during staurosporine inhibition

The subcellular distribution of the AC resulted from staurosporine inhibition was very remarkable and eminent with a new/old feature, the vacuoles. Previous work made by Prichard et al. and Azzeh et al. (Prichard et al., 2005; Azzeh et al., 2006) had revealed the emerging vacuoles due to specific inhibition of the HCMV UL97 serine/threonine kinase using either maribavir (Prichard et al., 2005) or NGIC-I (Azzeh et al., 2006). The punctuation of the AC markers with vacuoles is the main common feature in all of these researches. However, the details of the AC structures are not precisely similar when inhibiting the UL97 kinase with either maribavir, NGIC-I or staurosporine. In case of Prichard et al. (Prichard et al., 2005), only pp65 AC marker was used, the resulted vacuoles were very comparable to our results shown in 3.6b and 3.9 b. In case of Azzeh et al. (Azzeh et al., 2006), the inhibition of UL97 kinase activity or its deletion resulted in a “crown”-like shaped distribution of pp28, punctuated with vacuoles. In case of staurosporine inhibition, it is not known yet whether the inhibition targets the UL97 kinase activity, but the subcellular distribution of AC remained "bulb"-like but punctuated with vacuoles on the outer edges. So in case of staurosporine, no “crown”-like shape was detected as was the case with NGIC-I.

In order to illuminate these results, a deeper look into the images through the microscope was useful. Playing with the focus of the microscope looking into the “bulb”-like structure of wt-HCMV infected cells, showed that even this "bulb"-like structure contains vacuoles, these are however very tiny to be registered compared with those seen in staurosporine inhibited infected cells. These observations are

illustrated in figure 4.1. Although we were unable to capture a sharp image of these tiny vacuoles using the regular IF microscope at the Virology laboratory-AL-Quds University, Das et al. (Das et al., 2007) had documented that the AC is highly punctuated with vacuoles using electron microscopy (Das et al., 2007). This means that vacuoles are actually there in the non-inhibited infection, but for some reason got enlarged during inhibition as clearly indicated in this work.

The work by Das et al. (Das et al., 2007) also stated that early endosomes (an organelle that is normally found near the cell periphery as a near-terminal component of the exocytic and endocytic pathways) have been relocated to the center of a concentric structure of the AC (Das et al., 2007). The AC was described as a set of concentric nested cylinders composed of a particular type of vesicle (Das et al., 2007). These vesicles contain mature virions to make their way from the center of this compartment to the cell surface (Das et al., 2007).

Therefore we propose that the enlarged vacuoles seen in staurosporine inhibited cells may be referred to a trafficking deficiency of these assembled mature virions. Once inhibition takes place the vesicles mentioned by Das et al. (Das et al., 2007) get enlarged and enlarged waiting for the cargo mature virions. Apparently there are less mature virions in the staurosporine inhibited cells, so that the cargo virions do not get into the vesicles, making them enlarged empty vacuoles.

Interestingly, block-release experiments showed that staurosporine effect is reversible. This is a further evidence stating that staurosporine is affecting the AC, since releasing it reversed the typical "bulb"-like shape.

The fact that a new shape of the nucleus was increasingly detected during staurosporine inhibition of HCMV infection (see 3.10, figure 3.15) implicates that staurosporine may play a role during nuclear egress. This is a very remarkable

finding, since other inhibitors did not emerge a new nuclear shape (Prichard et al., 2005; Azzeh et al., 2006). Since PKC was shown to be involved in nuclear egress stages (see above), we propose that the changes in nuclear shape may be due to the PKC inhibition activity of staurosporine.

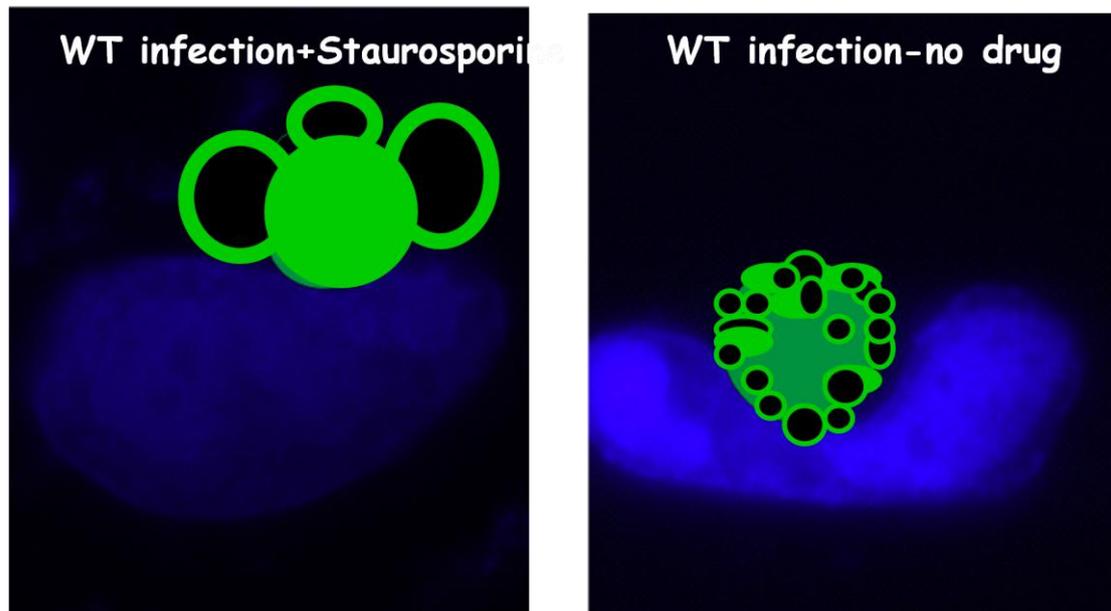


Figure 4.1. Illustration of vacuoles in assembly complex (AC). DAPI stained nucleus of either wt-HCMV infected cells or wt-HCMV infected staurosporine inhibited cells was used in the illustration, the AC was created using power point program drawing tools.

4.4 Titer of viruses in staurosporine inhibited cells

Plaque assay experiments document almost a 3 log reduction of viral titer in staurosporine inhibited infected cells compared to mock inhibited cells in case of wt-HCMV. Plaque assays reflect the number of plaques caused by viable viruses, while defected viruses may not cause any plaques. This means that there is less cargo

mature viable viruses in the inhibited cells as mentioned above. So either the staurosporine inhibition inhibits the maturation of the viruses, which we could only detect via AC experiments or it causes a defect, which delays the viral maturation keeping the vacuoles empty of mature virions and causing less production of overall viruses. We also noticed that the plaques resulted from supernatants of inhibited infection were smaller than that from mock inhibited infection. This, by itself could be a proof for the specific inhibition activity of staurosporine to HCMV viruses.

Furthermore, staurosporine did not inhibit viral entry or viral exist as detected via IF and viral load analysis (see 3.6 and 3.14). The inhibition activity of staurosporine seems to be restricted to the early late to late stage of viral life cycle. This fact is furthermore stated by the kinetic experiment (see 3.15, figure 3.20). The vacuoles building were only detected at late stage of infection; at 60 hpi. Also, adding staurosporine at later time points during infection (see 3.14, figure 3.19) did not emerge the vacuole building, indicating again that staurosporine affects mainly late stages in infection. Experiments performed at the Virology laboratory with NGIC-I and Δ UL97 showed also similar results regarding the late stage of vacuole building (unpublished data).

Another confirmation for the specific inhibition activity of staurosporine to HCMV was revealed via moi dependent inhibition assays. Viral load results of such assays showed evidence for specific virus inhibition by staurosporine, since the lower the viral moi was, the smaller the inhibition effect.

If compared with the viral load experiments, plaque assay showed stronger inhibition of the same viral supernatants. This is explained by the fact that viral load experiments measure the DNA, even that of defected viruses, while plaque assay

detects plaques caused by viable viruses. This also means that staurosporine does not affect the viral DNA synthesis in early stages of infection, but rather later stages of infection, mainly the assembly.

4.5 Staurosporine modulated the Golgi structure in infected cells

Early researches on the AC showed that Golgi markers were re-modulated in HCMV infected cells (Sanchez et al., 200a, b; Azzeh et al., 2006; Das et al., 2007). These researches had shown that WGA Golgi and trans Golgi markers lost their thread like structure and localized to the trim of the AC structure. Staurosporine inhibition altered WGA and the trans Golgi in infected cells, but kept its localization to the AC. Furthermore, this work adds that cis Golgi markers were also re-modeled, but do not lose the co-localization to the AC. The vacuoles were also clearly seen in trans and cis Golgi indicating the involvement of both in vacuole building evoked in the AC for viral trafficking purposes.

4.6 The AC is Golgi derived and very robust structure

Using Golgi destruction reagent; BFA, both, the cellular Golgi marker GOLPH4/TGN46 and the viral AC marker pp28 lost their typical compact "bulb"-like appearance to a destructed structure showing staining throughout the cytoplasm. However, the AC structure seems to be very robust to serve the HCMV virus perfectly, since the center of the AC was still intact in most Golgi destruction experiments made in this work. Although not tested here, but maybe adding BFA at earlier time point during infection, i.e. 48-60 hpi, may give more information about the building of AC complex by trans and cis Golgi markers.

4.7 Expression of viral teguments protein during staurosporine inhibition

Expression of pp65 was dramatically affected by staurosporine inhibition, while pp28 was not. The viral tegument pp65 is the most abundant tegument protein, but pp28 should also suffer from inhibition. In comparison to pp28, which is a true late protein detected only in the cytoplasm, pp65 is an early late protein detected in the nucleus at early stages of infection within the first 24 hpi. There are three facts, which may explain this finding. First, despite performing most IF experiments at 96 hpi, some cells showed pp65 staining in the nucleus in staurosporine inhibited infected cells. Second viral load analysis support this finding, since it detects the precise amount of another abundant protein, the major glycoprotein gB. Third, pp65 is highly phosphorylated and although it was not documented elsewhere, we propose that serine/threonine kinase activity may play a role in pp65 phosphorylation. Phosphorylation of pp65 is essential for its activity and function during HCMV life cycle.

Kamil and Coen (Kamil and Coen, 2007) has documented that pp65 Co-IP with UL97, we propose this is due to the serine threonine phosphorylation activity of UL97. Since our results shown here document that staurosporine may interact directly or indirectly with UL97 to promote its inhibition activity, both, deletion of UL97 or its inhibition by staurosporine affected the overall protein expression of pp65.

4.8 Cellular Serine/threonine kinase is affected in infected staurosporine inhibited cells

The result shown in figure 3.24 revealed that the 28 kDa band was detectable in all lysates, however the 55 kDa band was clearly distinguishable in the AD169 (wt-HCMV) lysate mainly. The 55 kDa band was very weak in the other lysates, indicating that it may be the portion of the cellular serine/threonine kinase affected mostly by the inhibitor. This result was not surprising since inhibition of serine/threonine should result in defects of serine/threonine expression somehow. Earlier researches showed that serine/threonine expression was increased in HCMV infected cells. Apparently, this increase is reversed during inhibition using staurosporine leading to reduction of serine/threonine activity. Furthermore, the infection with Δ UL97 also showed reduction of serine/threonine activity similar to that in staurosporine inhibited wt-HCMV. This may not be a critical finding; since the UL97 is a serine/threonine kinase and its absence most probably affect the overall serine/threonine kinase activity in the cell. These data actually coincide with the published researches on the increasing activity of serine/threonine kinases during HCMV infection.

Taken together this work is the first to show evidence for a specific role of cellular serine/threonine kinases in HCMV infection.

Finally, the data presented here give a new insight into the possible involvement of a new, yet unknown, kinase inhibitor in HCMV assembly that may be a basis of a new trend in the antiviral drug development.

5. References

1. **Adler, S. P.** 1988. Cytomegalovirus transmission among children in day care, their mothers and caretakers. *Pediatr Infect Dis J* **7**:279-85.
2. **Adler, S. P., S. A. Plotkin, E. Gonczol, M. Cadoz, C. Meric, J. B. Wang, P. Dellamonica, A. M. Best, J. Zahradnik, S. Pincus, K. Berencsi, W. I. Cox, and Z. Gyulai.** 1999. A canarypox vector expressing cytomegalovirus (CMV) glycoprotein B primes for antibody responses to a live attenuated CMV vaccine (Towne). *J Infect Dis* **180**:843-6.
3. **Adlish, J. D., R. S. Lahijani, and S. C. St Jeor.** 1990. Identification of a putative cell receptor for human cytomegalovirus. *Virology* **176**:337-45.
4. **Ahn, K., A. Gruhler, B. Galocha, T. R. Jones, E. J. Wiertz, H. L. Ploegh, P. A. Peterson, Y. Yang, and K. Fruh.** 1997. The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity* **6**:613-21.
5. **Al Kuraya, K., A. K. Siraj, P. Bavi, N. Al-Jomah, H. El-Solh, A. Ezzat, F. Al-Dayel, A. Belgaumi, A. Al-Kofide, R. Sabbah, S. Sheikh, S. Amr, R. Simon, and G. Sauter.** 2006. High throughput tissue microarray analysis of FHIT expression in diffuse large cell B-cell lymphoma from Saudi Arabia. *Mod Pathol* **19**:1124-9.
6. **AuCoin, D. P., G. B. Smith, C. D. Meiering, and E. S. Mocarski.** 2006. Betaherpesvirus-conserved cytomegalovirus tegument protein ppUL32 (pp150) controls cytoplasmic events during virion maturation. *J Virol* **80**:8199-210.
7. **Azzeh, M., A. Honigman, A. Taraboulos, A. Rouvinski, and D. G. Wolf.** 2006. Structural changes in human cytomegalovirus cytoplasmic assembly sites in the absence of UL97 kinase activity. *Virology* **354**:69-79.
8. **Bain, M., and J. Sinclair.** 2007. The S phase of the cell cycle and its perturbation by human cytomegalovirus. *Rev Med Virol* **17**:423-34.
9. **Balcarek, K. B., R. Bagley, G. A. Cloud, and R. F. Pass.** 1990. Cytomegalovirus infection among employees of a children's hospital. No evidence for increased risk associated with patient care. *JAMA* **263**:840-4.
10. **Baldick, C. J., Jr., and T. Shenk.** 1996. Proteins associated with purified human cytomegalovirus particles. *J Virol* **70**:6097-105.
11. **Benko, D. M., R. S. Haltiwanger, G. W. Hart, and W. Gibson.** 1988. Virion basic phosphoprotein from human cytomegalovirus contains O-linked N-acetylglucosamine. *Proc Natl Acad Sci U S A* **85**:2573-7.
12. **Beral, V., T. A. Peterman, R. L. Berkelman, and H. W. Jaffe.** 1990. Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? *Lancet* **335**:123-8.

13. **Bershadsky, A. D., and A. H. Futerman.** 1994. Disruption of the Golgi apparatus by brefeldin A blocks cell polarization and inhibits directed cell migration. *Proc Natl Acad Sci U S A* **91**:5686-9.
14. **Bhavanandan, V. P., and A. W. Katlic.** 1979. The interaction of wheat germ agglutinin with sialoglycoproteins. The role of sialic acid. *J Biol Chem* **254**:4000-8.
15. **Black, F. L.** 1975. Infectious diseases in primitive societies. *Science* **187**:515-8.
16. **Blajchman, M. A., M. Goldman, J. J. Freedman, and G. D. Sher.** 2001. Proceedings of a consensus conference: prevention of post-transfusion CMV in the era of universal leukoreduction. *Transfus Med Rev* **15**:1-20.
17. **Bogner, E., M. Reschke, B. Reis, T. Mockenhaupt, and K. Radsak.** 1993. Identification of the gene product encoded by ORF UL56 of the human cytomegalovirus genome. *Virology* **196**:290-3.
18. **Bold, S., M. Ohlin, W. Garten, and K. Radsak.** 1996. Structural domains involved in human cytomegalovirus glycoprotein B-mediated cell-cell fusion. *J Gen Virol* **77 (Pt 9)**:2297-302.
19. **Bonkowsky, H. L., R. V. Lee, and G. Klatskin.** 1975. Acute granulomatous hepatitis. Occurrence in cytomegalovirus mononucleosis. *JAMA* **233**:1284-8.
20. **Borthwick, A. D.** 2005. Design of transactam HCMV protease inhibitors as potent antivirals. *Med Res Rev* **25**:427-52.
21. **Boshoff, C., T. F. Schulz, M. M. Kennedy, A. K. Graham, C. Fisher, A. Thomas, J. O. McGee, R. A. Weiss, and J. J. O'Leary.** 1995. Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. *Nat Med* **1**:1274-8.
22. **Bradshaw, P. A., M. R. Duran-Guarino, S. Perkins, J. I. Rowe, J. Fernandez, K. E. Fry, G. R. Reyes, L. Young, and S. K. Foung.** 1994. Localization of antigenic sites on human cytomegalovirus virion structural proteins encoded by UL48 and UL56. *Virology* **205**:321-8.
23. **Bresnahan, W. A., E. A. Thompson, and T. Albrecht.** 1997. Human cytomegalovirus infection results in altered Cdk2 subcellular localization. *J Gen Virol* **78 (Pt 8)**:1993-7.
24. **Britt, W. J., M. Jarvis, J. Y. Seo, D. Drummond, and J. Nelson.** 2004. Rapid genetic engineering of human cytomegalovirus by using a lambda phage linear recombination system: demonstration that pp28 (UL99) is essential for production of infectious virus. *J Virol* **78**:539-43.
25. **Britt, W. J., and M. Mach.** 1996. Human cytomegalovirus glycoproteins. *Intervirology* **39**:401-12.

26. **Camozzi, D., S. Pignatelli, C. Valvo, G. Lattanzi, C. Capanni, P. Dal Monte, and M. P. Landini.** 2008. Remodelling of the nuclear lamina during human cytomegalovirus infection: role of the viral proteins pUL50 and pUL53. *J Gen Virol* **89**:731-40.
27. **Chandler, S. H., K. K. Holmes, B. B. Wentworth, L. T. Gutman, P. J. Wiesner, E. R. Alexander, and H. H. Handsfield.** 1985. The epidemiology of cytomegaloviral infection in women attending a sexually transmitted disease clinic. *J Infect Dis* **152**:597-605.
28. **Chang, Y., E. Cesarman, M. S. Pessin, F. Lee, J. Culpepper, D. M. Knowles, and P. S. Moore.** 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**:1865-9.
29. **Chen, D. H., H. Jiang, M. Lee, F. Liu, and Z. H. Zhou.** 1999. Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus. *Virology* **260**:10-6.
30. **Chen, Z., E. Knutson, A. Kurosky, and T. Albrecht.** 2001. Degradation of p21cip1 in cells productively infected with human cytomegalovirus. *J Virol* **75**:3613-25.
31. **Chijiwa, T., A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, and H. Hidaka.** 1990. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem* **265**:5267-72.
32. **Compton, T., and A. Feire.** 2007. Early events in human cytomegalovirus infection.
33. **Compton, T., D. M. Nowlin, and N. R. Cooper.** 1993. Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. *Virology* **193**:834-41.
34. **Corey, L., H. G. Adams, Z. A. Brown, and K. K. Holmes.** 1983. Genital herpes simplex virus infections: clinical manifestations, course, and complications. *Ann Intern Med* **98**:958-72.
35. **Couldwell, W. T., D. R. Hinton, S. K. He, T. C. Chen, I. Sebat, M. H. Weiss, and R. E. Law.** 1994. Protein-Kinase-C Inhibitors Induce Apoptosis in Human-Malignant Glioma Cell-Lines. *Febs Letters* **345**:43-46.
36. **Crawford, D. H., K. F. Macsween, C. D. Higgins, R. Thomas, K. McAulay, H. Williams, N. Harrison, S. Reid, M. Conacher, J. Douglas, and A. J. Swerdlow.** 2006. A cohort study among university students: identification of risk factors for Epstein-Barr virus seroconversion and infectious mononucleosis. *Clin Infect Dis* **43**:276-82.
37. **Dal Monte, P., S. Pignatelli, N. Zini, N. M. Maraldi, E. Perret, M. C. Prevost, and M. P. Landini.** 2002. Analysis of intracellular and intraviral localization of the human cytomegalovirus UL53 protein. *J Gen Virol* **83**:1005-12.

38. **Das, S., and P. E. Pellett.** 2007. Members of the HCMV US12 family of predicted heptaspanning membrane proteins have unique intracellular distributions, including association with the cytoplasmic virion assembly complex. *Virology* **361**:263-73.
39. **Das, S., A. Vasanji, and P. E. Pellett.** 2007. Three-dimensional structure of the human cytomegalovirus cytoplasmic virion assembly complex includes a reoriented secretory apparatus. *J Virol* **81**:11861-9.
40. **Davis, M. G., and E. S. Huang.** 1985. Nucleotide sequence of a human cytomegalovirus DNA fragment encoding a 67-kilodalton phosphorylated viral protein. *J Virol* **56**:7-11.
41. **Deardourff, S. L., F. A. Deture, D. M. Drylie, Y. Centifano, and H. Kaufman.** 1974. Association between herpes hominis type 2 and the male genitourinary tract. *J Urol* **112**:126-7.
42. **Demmler, G. J., M. Schydlower, and R. M. Lampe.** 1985. Texas, teenagers, and CMV. *J Infect Dis* **152**:1350.
43. **Druker, B. J., M. Talpaz, D. J. Resta, B. Peng, E. Buchdunger, J. M. Ford, N. B. Lydon, H. Kantarjian, R. Capdeville, S. Ohno-Jones, and C. L. Sawyers.** 2001. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* **344**:1031-7.
44. **Eddleston, M., S. Peacock, M. Juniper, and D. A. Warrell.** 1997. Severe cytomegalovirus infection in immunocompetent patients. *Clin Infect Dis* **24**:52-6.
45. **Ensoli, B., S. Nakamura, S. Z. Salahuddin, P. Biberfeld, L. Larsson, B. Beaver, F. Wong-Staal, and R. C. Gallo.** 1989. AIDS-Kaposi's sarcoma-derived cells express cytokines with autocrine and paracrine growth effects. *Science* **243**:223-6.
46. **Evans, A. S., J. Wanat, and J. C. Niederman.** 1983. Failure to demonstrate concomitant antibody changes to viral antigens other than Epstein-Barr virus (EBV) during or after infectious mononucleosis. *Yale J Biol Med* **56**:203-9.
47. **Evers, D. L., G. Komazin, R. G. Ptak, D. Shin, B. T. Emmer, L. B. Townsend, and J. C. Drach.** 2004. Inhibition of human cytomegalovirus replication by benzimidazole nucleosides involves three distinct mechanisms. *Antimicrob Agents Chemother* **48**:3918-27.
48. **Feire, A. L., R. M. Roy, K. Manley, and T. Compton.** 2010. The glycoprotein B disintegrin-like domain binds beta 1 integrin to mediate cytomegalovirus entry. *J Virol* **84**:10026-37.
49. **Fortunato, E. A., A. K. McElroy, I. Sanchez, and D. H. Spector.** 2000. Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. *Trends Microbiol* **8**:111-9.

50. **Fowler, K. B., S. Stagno, R. F. Pass, W. J. Britt, T. J. Boll, and C. A. Alford.** 1992. The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. *N Engl J Med* **326**:663-7.
51. **Gaddy, C. E., D. S. Wong, A. Markowitz-Shulman, and A. M. Colberg-Poley.** 2010. Regulation of the subcellular distribution of key cellular RNA-processing factors during permissive human cytomegalovirus infection. *J Gen Virol* **91**:1547-59.
52. **Gerber, P., S. Lucas, M. Nonoyama, E. Perlin, and L. I. Goldstein.** 1972. Oral excretion of Epstein-Barr virus by healthy subjects and patients with infectious mononucleosis. *Lancet* **2**:988-9.
53. **Ghoreschi, K., A. Laurence, and J. J. O'Shea.** 2009. Selectivity and therapeutic inhibition of kinases: to be or not to be? *Nat Immunol* **10**:356-60.
54. **Gibson, W., M. K. Baxter, and K. S. Clopper.** 1996a. Cytomegalovirus "missing" capsid protein identified as heat-aggregable product of human cytomegalovirus UL46. *J Virol* **70**:7454-61.
55. **Gibson, W., K. S. Clopper, W. J. Britt, and M. K. Baxter.** 1996b. Human cytomegalovirus (HCMV) smallest capsid protein identified as product of short open reading frame located between HCMV UL48 and UL49. *J Virol* **70**:5680-3.
56. **Glass, D. B.** 1983. Differential responses of cyclic GMP-dependent and cyclic AMP-dependent protein kinases to synthetic peptide inhibitors. *Biochem J* **213**:159-64.
57. **Glezen, W. P., G. W. Fernald, and J. A. Lohr.** 1975. Acute respiratory disease of university students with special reference to the etiologic role of Herpesvirus hominis. *Am J Epidemiol* **101**:111-21.
58. **Goldner, T., G. Hewlett, N. Ettischer, H. Ruebsamen-Schaeff, H. Zimmermann, and P. Lischka.** 2011. The novel Anti-Cytomegalovirus Compound AIC246 inhibits HCMV Replication through a Specific Antiviral Mechanism that involves the viral Terminase. *J Virol*.
59. **Gompels, U. A., J. Nicholas, G. Lawrence, M. Jones, B. J. Thomson, M. E. Martin, S. Efstathiou, M. Craxton, and H. A. Macaulay.** 1995. The DNA sequence of human herpesvirus-6: structure, coding content, and genome evolution. *Virology* **209**:29-51.
60. **Gratacap-Cavallier, B., J. L. Bosson, P. Morand, N. Dutertre, B. Chanzy, P. S. Jouk, C. Vandekerckhove, P. Cart-Lamy, and J. M. Seigneurin.** 1998. Cytomegalovirus seroprevalence in French pregnant women: parity and place of birth as major predictive factors. *Eur J Epidemiol* **14**:147-52.
61. **Gratama JW, O. M., Klein G,Ernberg I.** 1990. EBNA size polymorphism can be used to trace Epstein-Barr virus spread within families. *J Virol*. 1990 Oct;64(10):4703-8.

62. **Hakki, M., and A. P. Geballe.** 2008. Cellular serine/threonine phosphatase activity during human cytomegalovirus infection. *Virology* **380**:255-63.
63. **Hamirally, S., J. P. Kamil, Y. M. Ndassa-Colday, A. J. Lin, W. J. Jahng, M. C. Baek, S. Noton, L. A. Silva, M. Simpson-Holley, D. M. Knipe, D. E. Golan, J. A. Marto, and D. M. Coen.** 2009. Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress. *PLoS Pathog* **5**:e1000275.
64. **Hanks, S. K., and T. Hunter.** 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J* **9**:576-96.
65. **Hecker, M., D. Qiu, K. Marquardt, G. Bein, and H. Hackstein.** 2004. Continuous cytomegalovirus seroconversion in a large group of healthy blood donors. *Vox Sang* **86**:41-4.
66. **Heise, M. T., M. Connick, and H. W. t. Virgin.** 1998. Murine cytomegalovirus inhibits interferon gamma-induced antigen presentation to CD4 T cells by macrophages via regulation of expression of major histocompatibility complex class II-associated genes. *J Exp Med* **187**:1037-46.
67. **Henle, G., W. Henle, P. Clifford, V. Diehl, G. W. Kafuko, B. G. Kirya, G. Klein, R. H. Morrow, G. M. Munube, P. Pike, P. M. Tukei, and J. L. Ziegler.** 1969. Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. *J Natl Cancer Inst* **43**:1147-57.
68. **Hirota, K., K. Muraguchi, N. Watabe, M. Okumura, M. Kozu, K. Takahashi, Y. Machida, Y. Funayama, T. Oshima, and Y. Numazaki.** 1992. Prospective study on maternal, intrauterine, and perinatal infections with cytomegalovirus in Japan during 1976-1990. *J Med Virol* **37**:303-6.
69. **Hizel, S., S. Parker, and U. Onde.** 1999. Seroprevalence of cytomegalovirus infection among children and females in Ankara, Turkey, 1995. *Pediatr Int* **41**:506-9.
70. **Homman-Loudiyi, M., K. Hultenby, W. Britt, and C. Soderberg-Naucler.** 2003. Envelopment of human cytomegalovirus occurs by budding into Golgi-derived vacuole compartments positive for gB, Rab 3, trans-golgi network 46, and mannosidase II. *J Virol* **77**:3191-203.
71. **Hume, A. J., J. S. Finkel, J. P. Kamil, D. M. Coen, M. R. Culbertson, and R. F. Kalejta.** 2008. Phosphorylation of retinoblastoma protein by viral protein with cyclin-dependent kinase function. *Science* **320**:797-9.
72. **Hutfield, D. C.** 1966. History of herpes genitalis. *Br J Vener Dis* **42**:263-8.
73. **Isaacson, M. K., A. L. Feire, and T. Compton.** 2007. Epidermal growth factor receptor is not required for human cytomegalovirus entry or signaling. *J Virol* **81**:6241-7.

74. **Jahn, G., B. C. Scholl, B. Traupe, and B. Fleckenstein.** 1987. The two major structural phosphoproteins (pp65 and pp150) of human cytomegalovirus and their antigenic properties. *J Gen Virol* **68** (Pt 5):1327-37.
75. **Jeffrey I. Cohen , S. E. s., Ann m Arvin R. Whitley.** 2007. *Varicella-Zoster Virus Replication, Pathogenesis, and Management*, 5th edition ed. Lippincott Williams & Wilkins.
76. **Jenson, H. B.** 2000. Acute complications of Epstein-Barr virus infectious mononucleosis. *Curr Opin Pediatr* **12**:263-8.
77. **Johnson, R. A., X. Wang, X. L. Ma, S. M. Huong, and E. S. Huang.** 2001. Human cytomegalovirus up-regulates the phosphatidylinositol 3-kinase (PI3-K) pathway: inhibition of PI3-K activity inhibits viral replication and virus-induced signaling. *J Virol* **75**:6022-32.
78. **Jones, N. L., J. C. Lewis, and B. A. Kilpatrick.** 1986. Cytoskeletal disruption during human cytomegalovirus infection of human lung fibroblasts. *Eur J Cell Biol* **41**:304-12.
79. **Jones, T. R., and S. W. Lee.** 2004. An acidic cluster of human cytomegalovirus UL99 tegument protein is required for trafficking and function. *J Virol* **78**:1488-502.
80. **Josey, W. E., A. J. Nahmias, and Z. M. Naib.** 1972. The epidemiology of Type 2 (genital) herpes simplex virus infection. *Obstet Gynecol Surv* **27**:suppl:295-302.
81. **Josey, W. E., A. J. Nahmias, Z. M. Naib, P. M. Utley, W. J. McKenzie, and M. T. Coleman.** 1966. Genital herpes simplex infection in the female. *Am J Obstet Gynecol* **96**:493-501.
82. **Kalinyak, J. E., G. Fleagle, and J. J. Docherty.** 1977. Incidence and distribution of herpes simplex virus types 1 and 2 from genital lesions in college women. *J Med Virol* **1**:175-81.
83. **Kamil, J. P., and D. M. Coen.** 2007. Human cytomegalovirus protein kinase UL97 forms a complex with the tegument phosphoprotein pp65. *J Virol* **81**:10659-68.
84. **Kapasi, A. J., and D. H. Spector.** 2008. Inhibition of the cyclin-dependent kinases at the beginning of human cytomegalovirus infection specifically alters the levels and localization of the RNA polymerase II carboxyl-terminal domain kinases cdk9 and cdk7 at the viral transcriptosome. *J Virol* **82**:394-407.
85. **Kaposi , M.** 1872. Idiopathisches multiples pigmentsarkom her haut. *Arch Dermat Shypilol*.
86. **Kari, B., and R. Gehrz.** 1992. A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope. *J Virol* **66**:1761-4.

87. **Kaul, D. R., S. Stoelben, E. Cober, T. Ojo, E. Sandusky, P. Lischka, H. Zimmermann, and H. Rubsamen-Schaeff.** 2011. First report of successful treatment of multidrug-resistant cytomegalovirus disease with the novel anti-CMV compound AIC246. *Am J Transplant* **11**:1079-84.
88. **Kaye, J. F., U. A. Gompels, and A. C. Minson.** 1992. Glycoprotein H of human cytomegalovirus (HCMV) forms a stable complex with the HCMV UL115 gene product. *J Gen Virol* **73 (Pt 10)**:2693-8.
89. **Keay, S., and B. Baldwin.** 1991. Anti-idiotypic antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. *J Virol* **65**:5124-8.
90. **Keay, S., and B. R. Baldwin.** 1996. Evidence for the role of cell protein phosphorylation in human cytomegalovirus/host cell fusion. *J Gen Virol* **77 (Pt 10)**:2597-604.
91. **Keller, M. A., and E. R. Stiehm.** 2000. Passive immunity in prevention and treatment of infectious diseases. *Clin Microbiol Rev* **13**:602-14.
92. **Khanna, R., S. R. Burrows, P. M. Steigerwald-Mullen, S. A. Thomson, M. G. Kurilla, and D. J. Moss.** 1995. Isolation of cytotoxic T lymphocytes from healthy seropositive individuals specific for peptide epitopes from Epstein-Barr virus nuclear antigen 1: implications for viral persistence and tumor surveillance. *Virology* **214**:633-7.
93. **Kim, S., S. S. Yu, I. S. Lee, S. Ohno, J. Yim, and H. S. Kang.** 1999. Human cytomegalovirus IE1 protein activates AP-1 through a cellular protein kinase(s). *J Gen Virol* **80 (Pt 4)**:961-9.
94. **Krosky, P. M., M. C. Baek, and D. M. Coen.** 2003a. The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress. *J Virol* **77**:905-14.
95. **Krosky, P. M., M. C. Baek, W. J. Jahng, I. Barrera, R. J. Harvey, K. K. Biron, D. M. Coen, and P. B. Sethna.** 2003b. The human cytomegalovirus UL44 protein is a substrate for the UL97 protein kinase. *J Virol* **77**:7720-7.
96. **Krosky, P. M., M. R. Underwood, S. R. Turk, K. W. Feng, R. K. Jain, R. G. Ptak, A. C. Westerman, K. K. Biron, L. B. Townsend, and J. C. Drach.** 1998. Resistance of human cytomegalovirus to benzimidazole ribonucleosides maps to two open reading frames: UL89 and UL56. *J Virol* **72**:4721-8.
97. **Landini, G., G. P. Mission, and P. I. Murray.** 1992. Fractal properties of herpes simplex dendritic keratitis. *Cornea* **11**:510-4.
98. **Lischka, P., G. Hewlett, T. Wunberg, J. Baumeister, D. Paulsen, T. Goldner, H. Rubsamen-Schaeff, and H. Zimmermann.** 2010. In vitro and in vivo activities of the novel anticytomegalovirus compound AIC246. *Antimicrob Agents Chemother* **54**:1290-7.

99. **Liu, X., J. Yuan, A. W. Wu, P. W. McGonagill, C. S. Galle, and J. L. Meier.** 2010. Phorbol ester-induced human cytomegalovirus major immediate-early (MIE) enhancer activation through PKC-delta, CREB, and NF-kappaB desilences MIE gene expression in quiescently infected human pluripotent NTera2 cells. *J Virol* **84**:8495-508.
100. **Lu, H., and S. Thomas.** 2004. Maribavir (ViroPharma). *Curr Opin Investig Drugs* **5**:898-906.
101. **Manning, G., D. B. Whyte, R. Martinez, T. Hunter, and S. Sudarsanam.** 2002. The protein kinase complement of the human genome. *Science* **298**:1912-34.
102. **Marschall, M., S. Feichtinger, and J. Milbradt.** 2011. Regulatory roles of protein kinases in cytomegalovirus replication. *Adv Virus Res* **80**:69-101.
103. **Marschall, M., A. Marzi, P. aus dem Siepen, R. Jochmann, M. Kalmer, S. Auerochs, P. Lischka, M. Leis, and T. Stamminger.** 2005. Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina. *J Biol Chem* **280**:33357-67.
104. **Martiny-Baron, G., M. G. Kazanietz, H. Mischak, P. M. Blumberg, G. Kochs, H. Hug, D. Marme, and C. Schachtele.** 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* **268**:9194-7.
105. **Matthews, R. E. F.** 1979. Classification and nomenclature of viruses.
106. **McArdle, J., X. L. Schafer, and J. Munger.** 2011. Inhibition of calmodulin-dependent kinase kinase blocks human cytomegalovirus-induced glycolytic activation and severely attenuates production of viral progeny. *J Virol* **85**:705-14.
107. **McGavran, M. H., and M. G. Smith.** 1965. Ultrastructural, Cytochemical, and Microchemical Observations on Cytomegalovirus (Salivary Gland Virus) Infection of Human Cells in Tissue Culture. *Exp Mol Pathol* **76**:1-10.
108. **McMillan, J. A., L. B. Weiner, A. M. Higgins, and V. J. Lamparella.** 1993. Pharyngitis associated with herpes simplex virus in college students. *Pediatr Infect Dis J* **12**:280-4.
109. **Mehta, S. K., R. P. Stowe, A. H. Feiveson, S. K. Tying, and D. L. Pierson.** 2000. Reactivation and shedding of cytomegalovirus in astronauts during spaceflight. *J Infect Dis* **182**:1761-4.
110. **Mettenleiter, T. C.** 2004. Budding events in herpesvirus morphogenesis. *Virus Res* **106**:167-80.
111. **Mettenleiter, T. C., B. G. Klupp, and H. Granzow.** 2006. Herpesvirus assembly: a tale of two membranes. *Current Opinion in Microbiology* **9**:423-429.
112. **Meyer, T., U. Regenass, D. Fabbro, E. Alteri, J. Rosel, M. Muller, G. Caravatti, and A. Matter.** 1989. A derivative of staurosporine (CGP 41 251) shows selectivity for

- protein kinase C inhibition and in vitro anti-proliferative as well as in vivo anti-tumor activity. *Int J Cancer* **43**:851-6.
113. **Milbradt, J., R. Webel, S. Auerochs, H. Sticht, and M. Marschall.** 2010. Novel mode of phosphorylation-triggered reorganization of the nuclear lamina during nuclear egress of human cytomegalovirus. *J Biol Chem* **285**:13979-89.
 114. **Miller, C. S., S. A. Avdiushko, R. J. Kryscio, R. J. Danaher, and R. J. Jacob.** 2005. Effect of prophylactic valacyclovir on the presence of human herpesvirus DNA in saliva of healthy individuals after dental treatment. *J Clin Microbiol* **43**:2173-80.
 115. **Milne, R. S., D. A. Paterson, and J. C. Booth.** 1998. Human cytomegalovirus glycoprotein H/glycoprotein L complex modulates fusion-from-without. *J Gen Virol* **79** (Pt 4):855-65.
 116. **Mocarski E. , S. t., Pass R.,.** 2007. *Cytomegaloviruses*, 5th ed. Lippincott Williams and Wilkins.
 117. **Mocarski, E. S., M. Bonyhadi, S. Salimi, J. M. McCune, and H. Kaneshima.** 1993. Human cytomegalovirus in a SCID-hu mouse: thymic epithelial cells are prominent targets of viral replication. *Proc Natl Acad Sci U S A* **90**:104-8.
 118. **Morrison, J. A., M. L. Gulley, R. Pathmanathan, and N. Raab-Traub.** 2004. Differential signaling pathways are activated in the Epstein-Barr virus-associated malignancies nasopharyngeal carcinoma and Hodgkin lymphoma. *Cancer Res* **64**:5251-60.
 119. **Murph, J. R., J. F. Bale, Jr., J. C. Murray, M. F. Stinski, and S. Perlman.** 1986. Cytomegalovirus transmission in a Midwest day care center: possible relationship to child care practices. *J Pediatr* **109**:35-9.
 120. **Murray, P. R., K. S. Rosenthal, and M. A. Pfaller.** 2002. *Medical Microbiology*. Mosby.
 121. **Nahmias, A. J., W. R. Dowdle, Z. M. Naib, A. Highsmith, R. W. Harwell, and W. E. Josey.** 1968. Relation of pock size on chorioallantoic membrane to antigenic type of herpesvirus hominis. *Proc Soc Exp Biol Med* **127**:1022-8.
 122. **Natali, A., P. Valcavi, M. C. Medici, E. Dieci, S. Montali, and C. Chezzi.** 1997. Cytomegalovirus infection in an Italian population: antibody prevalence, virus excretion and maternal transmission. *New Microbiol* **20**:123-33.
 123. **Navarro, L., K. Mowen, S. Rodems, B. Weaver, N. Reich, D. Spector, and M. David.** 1998. Cytomegalovirus activates interferon immediate-early response gene expression and an interferon regulatory factor 3-containing interferon-stimulated response element-binding complex. *Mol Cell Biol* **18**:3796-802.

124. **Neipel, F., J. C. Albrecht, and B. Fleckenstein.** 1997. Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? *J Virol* **71**:4187-92.
125. **Neipel, F., J. C. Albrecht, and B. Fleckenstein.** 1998. Human herpesvirus 8--the first human Rhadinovirus. *J Natl Cancer Inst Monogr*:73-7.
126. **Nguyen, N. L., A. N. Loveland, and W. Gibson.** 2008. Nuclear localization sequences in cytomegalovirus capsid assembly proteins (UL80 proteins) are required for virus production: inactivating NLS1, NLS2, or both affects replication to strikingly different extents. *J Virol* **82**:5381-9.
127. **Nicholas, J.** 1996. Determination and analysis of the complete nucleotide sequence of human herpesvirus. *J Virol* **70**:5975-89.
128. **Nicholas, J., V. R. Ruvolo, W. H. Burns, G. Sandford, X. Wan, D. Ciuffo, S. B. Hendrickson, H. G. Guo, G. S. Hayward, and M. S. Reitz.** 1997. Kaposi's sarcoma-associated human herpesvirus-8 encodes homologues of macrophage inflammatory protein-1 and interleukin-6. *Nat Med* **3**:287-92.
129. **Nilsson, K., G. Klein, W. Henle, and G. Henle.** 1971. The establishment of lymphoblastoid lines from adult and fetal human lymphoid tissue and its dependence on EBV. *Int J Cancer* **8**:443-50.
130. **Ogawa-Goto K, T. K., Gibson W, Moriishi E, Miura Y, Kurata T, Irie S, Sata T.** 2003. Microtubule network facilitates nuclear targeting of human cytomegalovirus capsid. *J Virol.* 2003 Aug;77(15):8541-7.
131. **Olson, L. C., E. L. Buescher, M. S. Artenstein, and P. D. Parkman.** 1967. Herpesvirus infections of the human central nervous system. *N Engl J Med* **277**:1271-7.
132. **Omura, S., Y. Iwai, A. Hirano, A. Nakagawa, J. Awaya, H. Tsuchiya, Y. Takahashi, and R. Masuma.** 1977. New Alkaloid Am-2282 of *Streptomyces* Origin Taxonomy, Fermentation, Isolation and Preliminary Characterization. *Journal of Antibiotics* **30**:275-282.
133. **Pachl, C., W. S. Probert, K. M. Hermsen, F. R. Masiarz, L. Rasmussen, T. C. Merigan, and R. R. Spaete.** 1989. The human cytomegalovirus strain Towne glycoprotein H gene encodes glycoprotein p86. *Virology* **169**:418-26.
134. **Parker, J. D., and J. E. Banatvala.** 1967. Herpes genitalis; clinical and virological studies. *Br J Vener Dis* **43**:212-6.
135. **Pass, R. F., A. M. Duliege, S. Boppana, R. Sekulovich, S. Percell, W. Britt, and R. L. Burke.** 1999. A subunit cytomegalovirus vaccine based on recombinant envelope glycoprotein B and a new adjuvant. *J Infect Dis* **180**:970-5.

136. **Pass, R. F., S. C. Hutto, D. W. Reynolds, and R. B. Polhill.** 1984. Increased frequency of cytomegalovirus infection in children in group day care. *Pediatrics* **74**:121-6.
137. **Pietropaolo, R., and T. Compton.** 1999. Interference with annexin II has no effect on entry of human cytomegalovirus into fibroblast cells. *J Gen Virol* **80 (Pt 7)**:1807-16.
138. **Prichard, M. N.** 2009. Function of human cytomegalovirus UL97 kinase in viral infection and its inhibition by maribavir. *Rev Med Virol* **19**:215-29.
139. **Prichard, M. N., W. J. Britt, S. L. Daily, C. B. Hartline, and E. R. Kern.** 2005. Human cytomegalovirus UL97 Kinase is required for the normal intranuclear distribution of pp65 and virion morphogenesis. *J Virol* **79**:15494-502.
140. **Rasmussen, L. E., R. M. Nelson, D. C. Kelsall, and T. C. Merigan.** 1984. Murine monoclonal antibody to a single protein neutralizes the infectivity of human cytomegalovirus. *Proc Natl Acad Sci U S A* **81**:876-80.
141. **Rawlinson, W. D., H. E. Farrell, and B. G. Barrell.** 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. *J Virol* **70**:8833-49.
142. **Rechter, S., G. M. Scott, J. Eickhoff, K. Zielke, S. Auerochs, R. Muller, T. Stamminger, W. D. Rawlinson, and M. Marschall.** 2009. Cyclin-dependent Kinases Phosphorylate the Cytomegalovirus RNA Export Protein pUL69 and Modulate Its Nuclear Localization and Activity. *J Biol Chem* **284**:8605-13.
143. **Reeves, W. C., L. Corey, H. G. Adams, L. A. Vontver, and K. K. Holmes.** 1981. Risk of recurrence after first episodes of genital herpes. Relation to HSV type and antibody response. *N Engl J Med* **305**:315-9.
144. **Revello, M. G., and G. Gerna.** 2002. Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant. *Clin Microbiol Rev* **15**:680-715.
145. **Ribbert, H.** 1904. Ueber protozoenartige Zellen in der Niere einessyphilitischen Neugeborenen und in der Parotis von Kindern. . *ZblAll Pathol*:945-948.
146. **Rifkind, D.** 1965. Cytomegalovirus infection after renal transplantation. *Arch Intern Med* **116**:554-8.
147. **Roby, C., and W. Gibson.** 1986. Characterization of phosphoproteins and protein kinase activity of virions, noninfectious enveloped particles, and dense bodies of human cytomegalovirus. *J Virol* **59**:714-27.
148. **Rodier, M. H., J. Berthonneau, A. Bourgoin, G. Giraudeau, G. Agius, C. Burucoa, A. Hekpazo, and J. L. Jacquemin.** 1995. Seroprevalences of Toxoplasma, malaria, rubella, cytomegalovirus, HIV and treponemal infections among pregnant women in Cotonou, Republic of Benin. *Acta Trop* **59**:271-7.

149. **Roizman, B., D. M. Knipe, and R. Whitley.** 2007. Herpes simplexviruses, 5th edition ed. Lippincott Williams & Wilkins.
150. **Ruegg, U. T., and G. M. Burgess.** 1989. Staurosporine, K-252 and Ucn-01 - Potent but Nonspecific Inhibitors of Protein-Kinases. *Trends in Pharmacological Sciences* **10**:218-220.
151. **Russo, J. J., R. A. Bohenzky, M. C. Chien, J. Chen, M. Yan, D. Maddalena, J. P. Parry, D. Peruzzi, I. S. Edelman, Y. Chang, and P. S. Moore.** 1996. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc Natl Acad Sci U S A* **93**:14862-7.
152. **Saitoh, M., T. Ishikawa, S. Matsushima, M. Naka, and H. Hidaka.** 1987. Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. *J Biol Chem* **262**:7796-801.
153. **Salvant, B. S., E. A. Fortunato, and D. H. Spector.** 1998. Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription. *J Virol* **72**:3729-41.
154. **Sanchez, V., C. L. Clark, J. Y. Yen, R. Dwarakanath, and D. H. Spector.** 2002. Viable human cytomegalovirus recombinant virus with an internal deletion of the IE2 86 gene affects late stages of viral replication. *J Virol* **76**:2973-89.
155. **Sanchez, V., K. D. Greis, E. Sztul, and W. J. Britt.** 2000a. Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly. *J Virol* **74**:975-86.
156. **Sanchez, V., J. A. Mahr, N. I. Orazio, and D. H. Spector.** 2007. Nuclear export of the human cytomegalovirus tegument protein pp65 requires cyclin-dependent kinase activity and the Crm1 exporter. *J Virol* **81**:11730-6.
157. **Sanchez, V., A. K. McElroy, and D. H. Spector.** 2003. Mechanisms governing maintenance of Cdk1/cyclin B1 kinase activity in cells infected with human cytomegalovirus. *J Virol* **77**:13214-24.
158. **Sanchez, V., A. K. McElroy, J. Yen, S. Tamrakar, C. L. Clark, R. A. Schwartz, and D. H. Spector.** 2004. Cyclin-dependent kinase activity is required at early times for accurate processing and accumulation of the human cytomegalovirus UL122-123 and UL37 immediate-early transcripts and at later times for virus production. *J Virol* **78**:11219-32.
159. **Sanchez, V., and D. H. Spector.** 2006. Cyclin-dependent kinase activity is required for efficient expression and posttranslational modification of human cytomegalovirus proteins and for production of extracellular particles. *J Virol* **80**:5886-96.

160. **Sanchez, V., E. Sztul, and W. J. Britt.** 2000b. Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. *J Virol* **74**:3842-51.
161. **Sanfilippo, A. M., V. Barrio, C. Kulp-Shorten, and J. P. Callen.** 2003. Common pediatric and adolescent skin conditions. *J Pediatr Adolesc Gynecol* **16**:269-83.
162. **Schleiss, M. R.** 2010. Can we build it better? Using BAC genetics to engineer more effective cytomegalovirus vaccines. *J Clin Invest* **120**:4192-7.
163. **Schleiss, M. R.** 2006. Role of breast milk in acquisition of cytomegalovirus infection: recent advances. *Current Opinion in Pediatrics* **18**:48-52.
164. **Seo, J. Y., and W. J. Britt.** 2006. Sequence requirements for localization of human cytomegalovirus tegument protein pp28 to the virus assembly compartment and for assembly of infectious virus. *J Virol* **80**:5611-26.
165. **Seynaeve, C. M., M. G. Kazanietz, P. M. Blumberg, E. A. Sausville, and P. J. Worland.** 1994. Differential inhibition of protein kinase C isozymes by UCN-01, a staurosporine analogue. *Mol Pharmacol* **45**:1207-14.
166. **Silva, M. C., Q. C. Yu, L. Enquist, and T. Shenk.** 2003. Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids. *J Virol* **77**:10594-605.
167. **Smith, J. W., S. P. Lowry, J. L. Melnick, and W. E. Rawls.** 1972. Antibodies to surface antigens of herpesvirus type 1- and type 2-infected cells among women with cervical cancer and control women. *Infect Immun* **5**:305-10.
168. **Sohn, Y. M., K. I. Park, C. Lee, D. G. Han, and W. Y. Lee.** 1992. Congenital cytomegalovirus infection in Korean population with very high prevalence of maternal immunity. *J Korean Med Sci* **7**:47-51.
169. **Somogyi, T., S. Michelson, and M. J. Masse.** 1990. Genomic location of a human cytomegalovirus protein with protein kinase activity (PK68). *Virology* **174**:276-85.
170. **Spaete, R. R., R. C. Gehrz, and M. P. Landini.** 1994. Human cytomegalovirus structural proteins. *J Gen Virol* **75 (Pt 12)**:3287-308.
171. **Spaete, R. R., K. Perot, P. I. Scott, J. A. Nelson, M. F. Stinski, and C. Pachl.** 1993. Coexpression of truncated human cytomegalovirus gH with the UL115 gene product or the truncated human fibroblast growth factor receptor results in transport of gH to the cell surface. *Virology* **193**:853-61.
172. **Spaete, R. R., R. M. Thayer, W. S. Probert, F. R. Masiarz, S. H. Chamberlain, L. Rasmussen, T. C. Merigan, and C. Pachl.** 1988. Human cytomegalovirus strain Towne glycoprotein B is processed by proteolytic cleavage. *Virology* **167**:207-25.

173. **Stagno, S., R. F. Pass, G. Cloud, W. J. Britt, R. E. Henderson, P. D. Walton, D. A. Veren, F. Page, and C. A. Alford.** 1986. Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. *JAMA* **256**:1904-8.
174. **Stanberry, L. R., S. L. Rosenthal, L. Mills, P. A. Succop, F. M. Biro, R. A. Morrow, and D. I. Bernstein.** 2004. Longitudinal risk of herpes simplex virus (HSV) type 1, HSV type 2, and cytomegalovirus infections among young adolescent girls. *Clin Infect Dis* **39**:1433-8.
175. **Stankus, S. J., M. Dlugopolski, and D. Packer.** 2000. Management of herpes zoster (shingles) and postherpetic neuralgia. *Am Fam Physician* **61**:2437-44, 2447-8.
176. **Staskus, K. A., W. Zhong, K. Gebhard, B. Herndier, H. Wang, R. Renne, J. Beneke, J. Pudney, D. J. Anderson, D. Ganem, and A. T. Haase.** 1997. Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. *J Virol* **71**:715-9.
177. **Sturzl, M., C. Blasig, A. Schreier, F. Neipel, C. Hohenadl, E. Cornali, G. Ascherl, S. Esser, N. H. Brockmeyer, M. Ekman, E. E. Kaaya, E. Tschachler, and P. Biberfeld.** 1997. Expression of HHV-8 latency-associated T0.7 RNA in spindle cells and endothelial cells of AIDS-associated, classical and African Kaposi's sarcoma. *Int J Cancer* **72**:68-71.
178. **Tartakoff, A. M., and P. Vassalli.** 1983. Lectin-binding sites as markers of Golgi subcompartments: proximal-to-distal maturation of oligosaccharides. *J Cell Biol* **97**:1243-8.
179. **Taylor, H. P., and N. R. Cooper.** 1990. The human cytomegalovirus receptor on fibroblasts is a 30-kilodalton membrane protein. *J Virol* **64**:2484-90.
180. **Thomas, M., S. Rechter, J. Milbradt, S. Auerochs, R. Muller, T. Stamminger, and M. Marschall.** 2009. Cytomegaloviral protein kinase pUL97 interacts with the nuclear mRNA export factor pUL69 to modulate its intranuclear localization and activity. *J Gen Virol* **90**:567-78.
181. **Thorne, J. E., D. A. Jabs, J. H. Kempen, J. T. Holbrook, C. Nichols, and C. L. Meinert.** 2006. Incidence of and risk factors for visual acuity loss among patients with AIDS and cytomegalovirus retinitis in the era of highly active antiretroviral therapy. *Ophthalmology* **113**:1432-40.
182. **Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, and et al.** 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266**:15771-81.
183. **Trus, B. L., W. Gibson, N. Cheng, and A. C. Steven.** 1999. Capsid structure of simian cytomegalovirus from cryoelectron microscopy: evidence for tegument attachment sites. *J Virol* **73**:2181-92.

184. **Tugizov, S., E. Maidji, and L. Pereira.** 1996. Role of apical and basolateral membranes in replication of human cytomegalovirus in polarized retinal pigment epithelial cells. *J Gen Virol* **77 (Pt 1):**61-74.
185. **Tugizov, S., E. Maidji, J. Xiao, Z. Zheng, and L. Pereira.** 1998. Human cytomegalovirus glycoprotein B contains autonomous determinants for vectorial targeting to apical membranes of polarized epithelial cells. *J Virol* **72:**7374-86.
186. **Vales-Gomez, M., M. Shiroishi, K. Maenaka, and H. T. Reyburn.** 2005. Genetic variability of the major histocompatibility complex class I homologue encoded by human cytomegalovirus leads to differential binding to the inhibitory receptor ILT2. *J Virol* **79:**2251-60.
187. **Vanarsdall, A. L., B. J. Ryckman, M. C. Chase, and D. C. Johnson.** 2008. Human cytomegalovirus glycoproteins gB and gH/gL mediate epithelial cell-cell fusion when expressed either in cis or in trans. *J Virol* **82:**11837-50.
188. **Vink, C., E. Beuken, and C. A. Bruggeman.** 2000. Complete DNA sequence of the rat cytomegalovirus genome. *J Virol* **74:**7656-65.
189. **Virtanen, I., P. Ekblom, P. Laurila, S. Nordling, K. O. Raivio, and P. Aula.** 1980. Characterization of storage material in cultured fibroblasts by specific lectin binding in lysosomal storage diseases. *Pediatr Res* **14:**1199-1203.
190. **Wang, X., D. Y. Huang, S. M. Huong, and E. S. Huang.** 2005. Integrin alphavbeta3 is a coreceptor for human cytomegalovirus. *Nat Med* **11:**515-21.
191. **Wang, X., S. M. Huong, M. L. Chiu, N. Raab-Traub, and E. S. Huang.** 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. *Nature* **424:**456-61.
192. **Wildy, P.** 1972. *The Herpesviruses.*
193. **Winston, D. J., J. A. Young, V. Pullarkat, G. A. Papanicolaou, R. Vij, E. Vance, G. J. Alangaden, R. F. Chemaly, F. Petersen, N. Chao, J. Klein, K. Sprague, S. A. Villano, and M. Boeckh.** 2008. Maribavir prophylaxis for prevention of cytomegalovirus infection in allogeneic stem cell transplant recipients: a multicenter, randomized, double-blind, placebo-controlled, dose-ranging study. *Blood* **111:**5403-10.
194. **Wolontis, S., and S. Jeansson.** 1977. Correlation of herpes simplex virus types 1 and 2 with clinical features of infection. *J Infect Dis* **135:**28-33.
195. **Wright, H. T., Jr., C. R. Goodheart, and A. Lielausis.** 1964. Human Cytomegalovirus. Morphology by Negative Staining. *Virology* **23:**419-24.
196. **Yeager, A. S., F. C. Grumet, E. B. Hafleigh, A. M. Arvin, J. S. Bradley, and C. G. Prober.** 1981. Prevention of transfusion-acquired cytomegalovirus infections in newborn infants. *J Pediatr* **98:**281-7.

197. **Yu, Y., and J. C. Alwine.** 2002. Human cytomegalovirus major immediate-early proteins and simian virus 40 large T antigen can inhibit apoptosis through activation of the phosphatidylinositide 3'-OH kinase pathway and the cellular kinase Akt. *J Virol* **76**:3731-8.
198. **Yue, C., K. L. Dodge, G. Weber, and B. M. Sanborn.** 1998. Phosphorylation of serine 1105 by protein kinase A inhibits phospholipase C β 3 stimulation by G α q. *J Biol Chem* **273**:18023-7.
199. **Zhong, W., H. Wang, B. Herndier, and D. Ganem.** 1996. Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. *Proc Natl Acad Sci U S A* **93**:6641-6.