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CHARACTERIZATION OF THE ASSEMBLY OF HUMAN CYTOMEGALOVIRUS gH/gL/gO and gH/gL/UL128-131 COMPLEXES AND STUDIES ON THEIR FUNCTIONS DURING VIRUS ENTRY AND TROPISM

By

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Dissertation

presented in partial fulfillment of the requirements
for the degree of

Ph.D.
in Cellular, Molecular and Microbial Biology

The University of Montana
Missoula, MT

May 2016

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Abstract Title: Characterization of the assembly of Human Cytomegalovirus gH/gL/gO and gH/gL/UL128-131 complexes and studies on their functions during virus entry and tropism

Chairperson: Dr. Jesse Hay
Research Advisor: Dr. Brent J Ryckman

Human cytomegalovirus (HCMV) is a human pathogen that can cause severe diseases in immunocompromised individuals, and also is a leading cause for congenital infection, making it a major public health concern. Currently, there is no effective vaccine available and antiviral treatment is often associated with problems, like drug toxicity, and drug resistance. Intervention in the virus entry process during the replication cycle could serve as a useful therapeutic strategy. The overall aims of the research in this dissertation are to characterize the roles of HCMV two gH/gL glycoprotein complexes during virus entry and tropism, and to study the molecular basis for the regulation of the assembly of those two complexes. The work has revealed that gH/gL/gO complex promotes virus fusion into all cell types whereas gH/gL/UL128-131 complex provides a non-fusion but necessary function for virus entry into select cell types. Importantly, the work also demonstrated that different HCMV strains vary dramatically in the relative abundance of those two gH/gL complexes on the virion envelope, and that could have a fundamental impact on virus efficiency of entry. The regulation of the assembly of those two complexes is likely influenced by multiple viral factors. This work will help us better understand the molecular biology of how HCMV initiates infection of different cell types, and will aid in the development of antiviral strategies in the future.

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ACKNOWLEDGEMENTS

I would like to express my most sincere thanks to my research mentor Dr. Brent Ryckman. I feel extremely grateful for having the opportunity to learn from such a talented and brilliant scientist. During the seven years of study in Ryckman lab, Brent has always been patient and dedicated in mentoring and training of me. The tremendous amount of time that he spent helping me in the lab has made me into the scientist who I am today. He is the person who helped me realized my dream and raised me up to more than I can be. His enthusiasm in science and persistent pursuit of ignorance will always be an inspiration that I would look up to in the future.

I also have a lot of thanks to my committee members Dr. J. Stephen Lodmell, Dr. Jesse Hay, Dr. Bruce Bowler, and Dr. Jack Nunberg for their guidance and advice regarding my projects and dissertation. Their effort and time are deeply appreciated. I feel fortunate to complete my Ph.D. study in such a positive environment that they contributed to create.

I want to thank my lab members for their support as well. Being thousands of miles away from my families and friends in China and studying abroad by myself sometimes can be hard, luckily enough I have such a “lab-family” in Missoula and I am grateful for the care and happiness they gave to me. Here I would like to specially thank Dr. Jean-Marc Lanchy for his generosity of sharing his scientific knowledge and expertise whenever I am seeking for a good source of discussion or help. He also taught me lab techniques that contributed greatly to the research work in this dissertation.

Last but not least, my deepest gratitude goes to my parents Xianghua Zhou and Xiumei Li for their unconditional love and support. There are not enough words to describe how thankful I am to both of them. Dad and Mom, thank you for always being there for me, no matter laughter and tears, ups and downs. Thank you for working so hard to provide me with the equal education opportunity as other children, which I know was not easy during the time I grew up in China and sometimes even meant you had to make sacrifice. Thank you for everything and I would work hard to make you proud.

CHAPTER 1. INTRODUCTION

Human cytomegalovirus (HCMV) is an enveloped, double-stranded DNA virus, a prototypic member of beta-herpesvirus family. Like all the other herpesviruses, HCMV is widely spread in human population. Although primary infection in healthy individuals is normally subclinical, persistent and latent infection will be established for a lifetime. In immunocompromised individuals, like AIDS patients and solid-organ or bone marrow transplant recipients, HCMV infection or reactivation can lead to severe diseases, causing high morbidity and mortality. HCMV is also a leading cause for congenital birth defects and can lead to serious neurological sequelae in children. The different disease manifestations correlate well with the virus ability to establish infection in a wide range of cells in human body. This dissertation is focused on the molecular mechanisms of HCMV entry into different cell types. The work has improved our understanding of the mechanisms about HCMV entry and tropism, which may help direct vaccine design that can be applied to HCMV susceptible individuals in the future.

Overview of HCMV pathogenesis

HCMV epidemiology

HCMV is also known as human herpes virus 5 (HHV5). There are eight human herpesviruses, which are herpes simplex virus 1 (HSV-1, HHV1), herpes simplex virus 2 (HSV-2, HHV2), varicella zoster virus (VZV, HHV3), Epstein-Barr virus (EBV, HHV-4), HHV6, HHV7 and Kaposi's Sarcoma virus (KSHV, HHV8). HSV-1, HSV-2 and VZV belong to alpha-herpesvirus subfamily; HCMV and HHV6/7 belong to beta-

herpesvirus subfamily; EBV and KSHV belong to gamma-herpesvirus subfamily. The pathogenesis features of all the human herpesviruses are compared in Table 1-1.

Table 1-1. Pathogenesis comparison of human herpesviruses.

		Transmission	Prevalence	Diseases	Vaccine
Alpha-	HSV-1 (HHV-1)	Intimate oral contact	80%	Oral lesion	N/A
	HSV-2 (HHV-2)	Intimate genital contact	80%	Genital lesion	N/A
	VZV (HHV-3)	Aerosol; contact with virus in varicella or zoster lesions	95%	Chicken pox (primary infection); Shingles (reactivation).	Licensed
Beta-	HCMV (HHV-5)	Contact with body fluids (saliva, tears, breast milk, semen, blood, urine, cervical secretions)	95%	Retinitis, encephalitis, hepatitis, transplant rejection in immunocompromised individuals; congenital disease	N/A
	HHV6A/ 6B	Oral contact (saliva)	90%	Exanthema; transplant rejection	N/A
	HHV-7	Oral contact (saliva)	90%	Exanthema; transplant rejection	N/A
Gamma-	EBV (HHV-4)	Generally by oral contact (saliva); could also be genital transmitted by blood transfusion and organ/bone marrow transplantation.	90%	Mononucleosis; B, T, NK-cell tumor; epithelial tumors	N/A
	KSHV (HHV-8)	Sexual contact; Salivary transmission is also likely	5-50% (Africa; Mediterranean sea)	B cell tumors; endothelial tumors	N/A

*Data and information shown in the table above is summarized from (1).

HCMV primary infection is usually not associated with overt symptoms, although some adults may experience mild mononucleosis-like symptoms including fever, fatigue or malaise. Following primary infection, HCMV, like all the other herpesviruses, establishes a life-long persistent or latent infection. Reactivation happens periodically and reinfection with multiple HCMV strains may also occur (2–7). HCMV is shed in various body fluids, particularly in saliva and urine. The person-to-person transmission usually happens via close contact with body fluids, blood transfusion or organ transplantation. HCMV is widespread, ranging from 20% to 95% in the population, depending on the geographic locations, ethnicity and socioeconomic status (SES). Primary infection can be acquired as a fetus, a neonate, a child or an adult. It was estimated that the seroprevalence (as measured by the presence of HCMV-specific IgG in the serum) of HCMV was 36.3% among 6-11 years old, 49.3% among 20-29 years old and 90.8% in those above 80 years old in the United States (8, 9). Interestingly, several publications have showed that the uptake of HCMV was much faster and occurred in a much younger group in the low SES groups than in the high SES groups (Figure. 1-1)(10, 11). The reason for this differential is less clear. It is possible that factors, such as over-crowded living place, inadequate sanitation and different maternal-infant feeding habits, could influence the chance of HCMV acquisition in the young age.

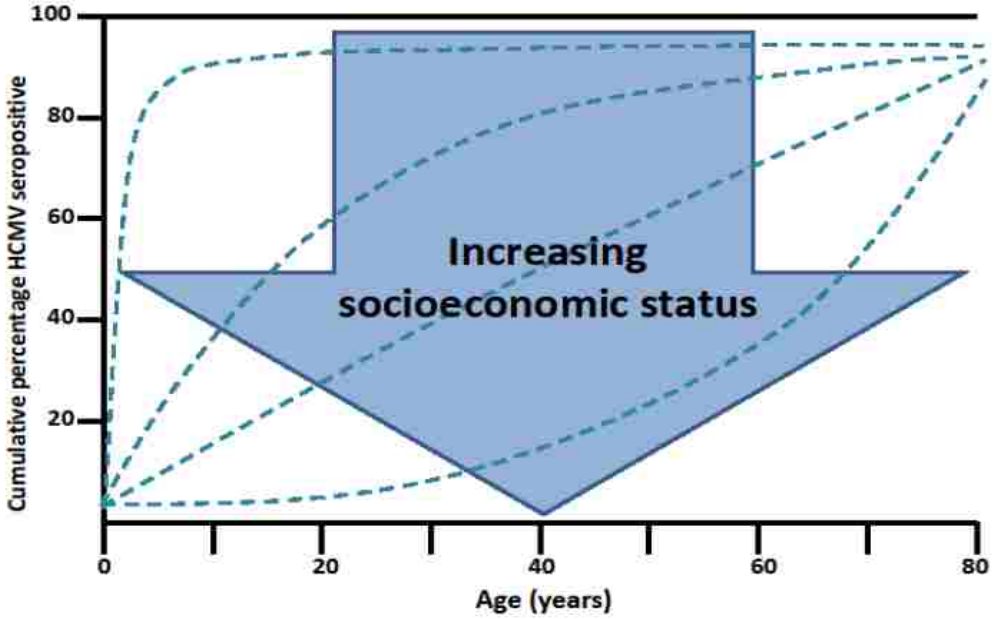

Figure 1-1 Model for the relationship between socioeconomic status and the cumulative HCMV seroprevalence by age.
(Modified from (10)).

HCMV pathogenesis in immunocompromised individuals

HCMV is often referred to as an "opportunistic pathogen", which mainly reflects the fact that this virus causes overt disease manifestations when host immune system is weakened or immature. In HIV/AIDS patients, whose immune system suffer severe infliction from loss of CD4 T cells, reactivation or reinfection by HCMV can cause serious diseases, including retinitis, pneumonitis, gastrointestinal diseases, and encephalitis, with retinitis accounting for 85% of the cases (12). Antiretroviral drugs that maintain the blood CD4 T cells counts above 100 cells/mm^3 exhibit efficient prophylaxis treatment for HCMV retinitis in AIDS patients, confirming the notion that sufficient immune response is an absolute requirement for controlling HCMV infection.

For solid-organ or bone marrow transplant recipients, who have been on immunosuppression drugs, HCMV reactivation or reinfection is common, ranging from 20~70% during the first year post transplantation. The infection is often associated with diseases including pneumonia, retinitis, hepatitis and encephalitis (13). Moreover, HCMV likely contributes to the graft failure in hematopoietic stem cell transplantation by inducing bone marrow hypoplasia (14). Also, in the case of solid-organ transplantation, HCMV infection is associated with both acute and chronic graft rejections (15). It was thought that HCMV-mediated injury and inflammation response can lead to the state of transplant vascular sclerosis, which eventually results in graft failure due to ischemia. Antiviral drugs that inhibit HCMV DNA replication, like ganciclovir or its analogs, can delay the graft rejection and improve the patient outcomes related to HCMV infection (16).

HCMV congenital infection

HCMV is the leading cause for congenital infections and birth defects in the United States, affecting from 0.2% to as high as 6% of all live births (17, 18). It was estimated that each year in the United States, about 40,000 children are born with congenital HCMV infection, resulting in an estimated 400 deaths and leaving 8,000 children with permanent neurological sequelae, such as hearing loss, vision loss, mental retardation or movement disabilities (19–21). Annual economic costs of caring for those affected children were estimated at $1-$2 billion in the United States (19). Moreover, while HCMV-associated diseases in AIDS or transplant patients can be prevented, controlled or cured by antiviral drugs, HCMV-associated

congenital injuries are usually irreversible even with antiviral therapy. These huge economic burdens and severe health problems highlight the priority of HCMV vaccine development (22, 23).

Congenital HCMV infection is caused by vertical transmission of the virus from mother to fetus through placenta, as a result of the following situations: maternal primary infection, reinfection with different strains, or reactivation of latent virus (24–28). Women who experience primary infection during pregnancy have a transmission rate about 30~40%, which is higher compared to women who experience non-primary infection (i.e., reinfection or reactivation) (18, 29). Moreover, maternal primary infection is more likely to result in severe sequelae in children born with HCMV congenital infections (9, 18). These observations indicate that the preexisting maternal immunity provides some, although incomplete protection to fetus from HCMV. This "incomplete maternal protection" might be due to the limit of the preexisting immunity to control the reinfection conducted by new strains of the virus or the reactivation of the existing virus.

HCMV pathogenesis in immunocompetent individuals

As aforementioned, HCMV infection in healthy individuals is generally asymptomatic, due to a competent immune system that controls the virus replication efficiently. And it may also reflect the fact that HCMV is an ancient virus evolved from a progenitor more than 100 million year ago (30, 31), and this long-time coevolution with human hosts leads to a fine-balanced coexistence status. Therefore, HCMV is generally thought to be an innocuous human pathogen.

However, recent studies have raised the concern for HCMV pathogenesis even in healthy individuals. First, for healthy seropositive individuals who have been critically ill because of burns, septic shock or heart disease, reactivation of HCMV happens at 30% rate, leading to viremia and in some cases, increasing mortality (32). In addition, HCMV persistent infection seems to contribute to the dysfunction and down-regulation of immune system exhibited in elderly individuals (12, 33). Moreover, there is evidence suggesting that HCMV infection might be associated with some human autoimmune diseases, although the causative linkage is still lacking to date (34). HCMV may also be involved in the development and acceleration of atherosclerosis through a chronic inflammatory process (35, 36). Although there are still a lot of controversies in the literature about the negative role of HCMV in affecting healthy individuals, this virus might be an underestimated pathogen and may associate with more diseases than what is previously acknowledged.

Overview of HCMV biology

HCMV virion structure

HCMV double-stranded linear DNA genome is encased inside of an icosahedral capsid, which is surrounded by a less structured layer of viral proteins, called tegument. These components are enclosed in a lipid bilayer envelope that is studded by a number of viral glycoproteins (Figure. 1-2). Mature virion size is about 200~300 nanometer (nm) in diameter.

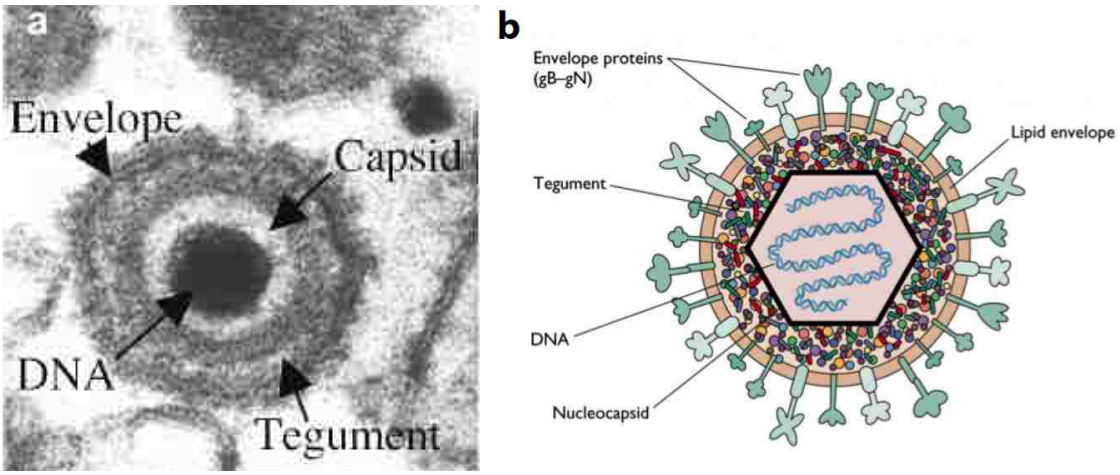

Figure 1-2 HCMV virion structure.
a) Electron microscopy picture (37). b) Schematic picture (38).

1) Genome

Compared to other human herpesviruses, HCMV contains the biggest genome of >230Kb (39). Due to high packing density, HCMV genome is estimated to be at a liquid-crystalline state inside of the capsid (40). The coding capacity of HCMV is big compared to other herpesviruses, with more than 200 predicted open reading frames (ORFs) (39, 41–43). The genome is composed of the unique long (UL) and unique short (US) segments, which are flanked by terminal repeated sequences TRL (*ab*) and TRS (*ca*) on one end and internal repeated sequences IRL (*b'a'*) (lab strain contains IRL while clinical strain does not) and IRS (*a'c'*) on the other end, resulting in a total TRL-UL-(IRL)-IRS-US-TRS configuration (Figure. 1-3). HCMV genes are named with a prefix based on the segments where they are located and are numbered sequentially (39), for instance, ORF 74 in UL region is named UL74, and the protein encoded by this ORF is named pUL74.

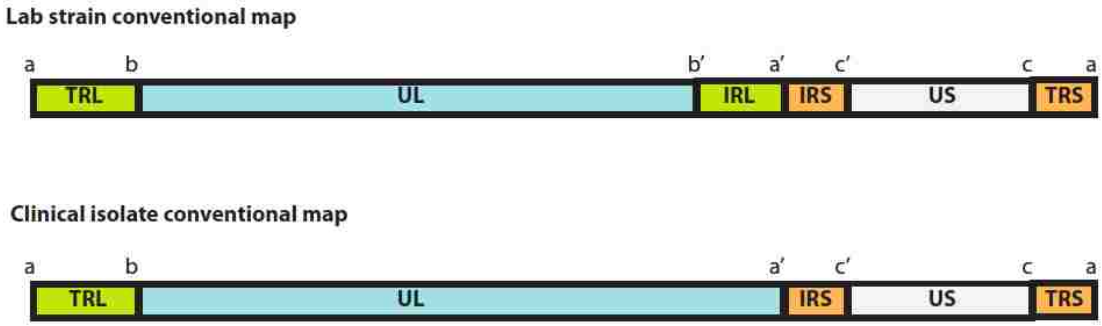

Figure 1-3 Schematic genome organization of HCMV.
The letters within each segment depicts the following features: terminal repeat long (TRL), unique long (UL), internal repeat long (IRL), internal repeat short (IRS), unique short (US), and terminal repeat short (TRS) (modified from (41)).

2) Capsid

HCMV capsid is about 130 nm in diameter (44). There are four main component proteins: major capsid protein (MCP; pUL86), minor capsid protein (mCP; pUL85), minor capsid binding protein (mC-BP; pUL46), and smallest capsid protein (SCP; pUL48.5). MCP is the most abundant component in capsid (45–49). The 162 capsomeres (150 hexons plus 12 pentons) are formed by MCP, which are located at the vertices of the T=16 icosahedral symmetry. Heterotrimers formed by two mCP and one mC-BP comprise of the 320 triplexes located in between the capsomeres (Figure. 1-4) (44, 50–52). SCP decorates each MCP hexon in the mature capsid and stabilizes the capsid structure through interaction with tegument proteins (53). One of the penton is shared with or occupied by a portal complex (formed by pUL104), through which the genome enters or leaves the capsid (54).

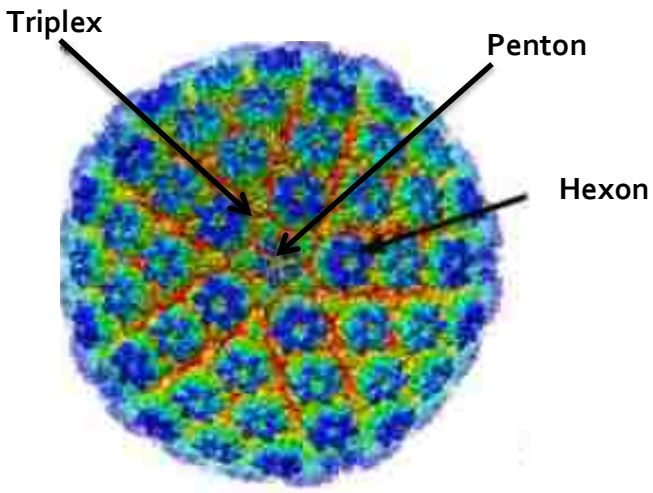

Figure 1-4 Structure of HCMV capsid in a T-16 symmetry.
The arrows point to penton, hexon and triplex (modified from (51)).

3) Tegument

HCMV tegument is an approximately 60-100 nm thick unstructured layer of proteins (50, 55). It contains the most abundant proteins in the virion (49, 56). Most of the viral tegument proteins are phosphorylated, thus they are usually named with the prefix pp, for phosphoprotein. Since no common signal sequences were found to specifically direct proteins into tegument, the packaging mechanisms of tegument are less clear. However, phosphorylation, interactions with viral capsid or the C-terminal tail of envelope glycoproteins might facilitate the incorporation process. Besides viral proteins, about 70 cellular proteins were found in the HCMV virions, most of which were probably in the layer of tegument (49). Moreover, viral or cellular RNA molecules are also packaged into tegument. The significance and functions of those cellular proteins or RNAs are still largely unknown.

It has been appreciated for a long time that HCMV tegument proteins could involve in the early stages of the infection, thus they are packaged within the virion as a "tool box" to ensure their presence upon a new round of infection before the de novo proteins synthesis. For example, upon entry, viral tegument protein pUL47 facilitate genome-containing capsid moving along microtubules to the nuclear pore through interactions with the capsid protein, and might also help the subsequential DNA release into the nucleus (57). In addition, viral tegument proteins could also play a critical role in gene expression. For example, after viral DNA is released into nucleus, a cellular protein Daxx is recruited to major immediate early promoters to repress viral gene transcription. Tegument protein pp71, encoded by UL82, gets inside of the nucleus independently from the capsid and induces the proteasome degradation of Daxx, thus activating viral immediate-early (IE) gene expression (58, 59). If this process occurs successfully, HCMV proceeds into lytic infection, however, if pp71 gets trapped in the cytoplasm and Daxx represses IE gene expression, latent infection would be established (56). Furthermore, viral tegument protein also influences late stages of the infection, e.g. assembly and egress. pp150, encoded by UL32, likely directs capsid to the assembly compartments for its packaging into the mature viral particle (60).

Last but not least, viral tegument proteins could modulate host cell antiviral immune response. pp65, gene product of UL83, is the most abundant protein in the virion (49). While the gene is totally dispensable for virus replication in tissue culture, the product of it is very important for virus immune evasion. pp65 achieves this goal through several mechanisms, including: 1) it mediates the phosphorylation

of viral IE proteins to block the recognition of IE proteins by MHC class I molecules (61); 2) it downregulates the surface presentation of MHC class II molecules by targeting them to lysosomal degradation (62); and 3) it attenuates the interferon response (63). Since pp65 plays such an important role in counteracting host immune response, it is probably not surprising that it is a major antigen target for cytotoxic T lymphocytes (64).

In conclusion, HCMV viral proteins residing in the tegument layer are involved in a wide variety of events during viral replication cycle. Thus a better understanding of their functions will benefit the intervention strategies against HCMV in the future.

4) Envelope

Herpesvirus acquires its viral envelope through an envelopment-deenvelopment-reenvelopment process. Viral capsid acquires the primary envelope through budding into the inner nuclear membrane, loses that envelope while exiting the outer nuclear membrane. Once getting into the cytoplasm, viral capsid acquires the secondary envelope (which is the final envelope) through budding into the Golgi-derived assembly apparatus membrane. HCMV potentially encodes 75 membrane-associated proteins, at least 25 of which are found on the envelope (41, 49). Most of the envelope proteins are glycosylated. Thus, they are often named with a prefix g, for glycoprotein. The functions of most of those glycoproteins are still elusive, but the hypothesis is that they mainly participate in the initiation of an infection.

Early studies have identified three families of glycoprotein complexes on HCMV envelope (designated as gC-I, gC-II, and gC-III) (65, 66). The functions and compositions of those complexes are becoming clearer now: gC-I is the homotrimeric complexes of gB. gB, encoded by UL55, is the fusion protein that mediates the membrane fusion between viral envelope and cellular membrane (67). Besides acting as a fusogen, gB is also thought to initiate HCMV absorption onto the target cell through interactions with cell surface proteins, like heparan sulfate proteoglycans (HSPGs) (68, 69). gC-II is the heterodimeric complex formed by gM disulfide-linked to gN (70). gM/gN, encoded by UL100 and UL73 respectively, are the most abundant component on the virion envelope (49). gM/gN are essential for virus replication, and studies suggest that the complex likely participate in both viral initial attachment and also assembly and egress processes (71, 72). gC-III is the disulfide-linked gH/gL/gO complex (73–76). Recently, another gH/gL complex was identified, gH/gL/UL128-131 (UL128-131 are small proteins encoded by UL128-131 genes) (77–80). Both gH/gL complexes are very important for HCMV entry. The characterization of their functions is one of the research focuses of this dissertation, thus more detail will be described or discussed in the following chapters.

Viral envelope glycoproteins are the main immunogens. Anti-gB, anti-gM, anti-gN, anti-gH, anti-gL and anti-UL128-131 neutralizing antibodies have all been described (81–85). Among all the glycoproteins listed above, gB, gH/gL, gM and UL128-131 are highly conserved among all strains of HCMV, while gN and gO are highly variable in amino acid sequences (86–89). The significance of this sequence diversity is less clear, but might reflect an immune evasion role of these proteins.

Besides viral glycoproteins, HCMV envelope also contains numbers of cellular proteins, such as CD46, CD55, CD59 and annexin II (90–93). CD46, CD55, and CD59 are the host-encoded complement regulatory proteins. The incorporation of these proteins into virion envelope might be a smart way of the virus to prevent complement-mediated lysis (91). Annexin II is a ubiquitous host protein distributed on cell surface or on the intracellular membranes. It is involved in a lot of cell processes, including cell mobility, endocytosis, and exocytosis. Annexin II might enhance virus binding to phospholipid membrane, but it is not found to influence HCMV entry into fibroblast cells (94, 95). Another possibility is that annexin II, as part of the virion, might play a role in modulating host cell responses during viral replication.

HCMV replication cycle

1) Attachment and entry

Virus replication cycle begins by attachment and entry process, which is initiated by the random collision between virus particle and the target cell, followed by tethering of viral particle to cell surface and then entering of the virus into the cell. Studies have shown that HCMV attachment is initiated by low-affinity binding of viral gB and gM/gN with cell surface heparan sulfate proteoglycans (HSPGs) (96, 97), this step helps to concentrate viral particles on cell surface, thus may increase the chance that viral particles could interact with downstream receptors exhibiting lower avidity. A subsequent quick switch to a more stable docking occurs by interaction between gB and the epidermal growth factor receptor (EGFR), platelet

derived growth factor receptor (PDGFR-alpha) or integrin α2β1, α6β1, αVβ3 (98–100). Although more recent studies didn't support their roles as HCMV entry receptors, the interaction with them probably further facilitates virus attachment (101, 102).

Depending on the cell type that HCMV encounters, entry could happen directly at plasma membrane or from within the endosome (Figure. 1-5). Regardless of either entry pathway, the critical event is viral envelope fusion with cellular membranes, which is mediated by the "core fusion machinery" comprised of gB and gH/gL. After membrane fusion, HCMV deposits its viral components into the cytoplasm. Genome-containing capsid, facilitated by some tegument proteins, is transported to nuclear pores along microtubules and DNA genome is released into nucleus afterwards (Figure. 1-5). At the same time, other tegument proteins diffuse to nucleus independently or stay in different subcellular locations to either regulate viral gene expression or inhibit host cell anti-viral immune response.

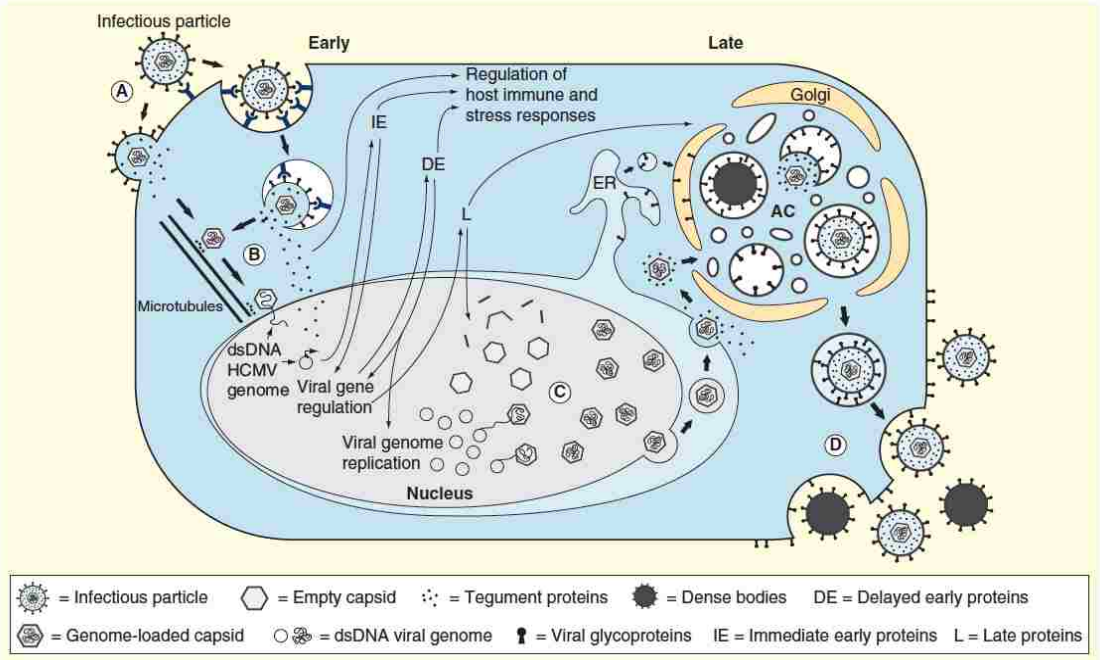

Figure 1-5 Schematic of HCMV replication cycle.
(Modified from (103)). Detailed description of the diagram is given in the text.

2) Gene expression

The expression of HCMV viral proteins is finely regulated, and falls into a cascade of events, which are divided into three main temporal classes: immediate early (IE), delayed early (DE) and late (L) (Figure. 1-5). IE genes are immediately expressed following nuclear entry, in absence of any de novo protein synthesis. Their expression can be detected within a few hours in HCMV infected cells (104). IE genes are transcribed by RNA polymerase II of the host cell under the control of major immediate early promoter (MIEP). The most abundant IE proteins are IE1-72 kDa (pUL123), and IE2-86 kDa (pUL122) (39, 104). IE1-72 kDa is necessary for efficient viral replication after low MOI infection (105). IE2-86 kDa is critical for viral DE genes expression and it can negatively regulate the transcription of IE1 and IE2 genes (106, 107). Together they act as trans-activators to stimulate the transcription of various viral and host genes, which are important for the efficiency

of viral replication (108). The progression of IE gene expression is very important in deciding virus fate of productive infection versus latent infection.

Expression of DE genes depends on IE gene expression. The products of DE genes are often directly involved in viral DNA replication (e.g., DNA polymerase) or priming the cellular environment ready for viral DNA replication (e.g., up-regulation of cellular genes exhibited in S-phase). And the kinetics of DE gene expression varies from as early as after IE gene expression to as late as before viral DNA synthesis.

Expression of L genes is largely dependent on viral DNA replication. There are two groups of L genes: leaky-late and true-late genes. Leaky-late genes are expressed at very low level at early times and the levels are tremendously increased at late times. True-late genes are exclusively after, and dependent on viral DNA replication. L genes are expressed about 24-48 hours after infection, the products of which are often structure proteins that are required for viral assembly and egress.

3) Gene replication

Soon after deposited into the nucleus, HCMV linear DNA genome is rapidly circularized and serves as the template for transcription and replication. The initiation position of DNA replication resides at a defined region on the genome, the so-called lytic origins of replication, *oriLyt*. The *oriLyt* for HCMV is identified in the middle of the UL segment, upstream of the gene (UL57) that encodes the single-stranded DNA binding protein (109, 110). Approximately at 16 hours post infection, DNA synthesis takes place in the vicinity of *oriLyt* as soon as essential viral proteins are present. The core DNA replication proteins include helicase-primase complex (formed by pUL105, pUL70 and pUL102), DNA polymerase (pUL54), polymerase

accessory protein (pUL44) and single-stranded DNA binding protein (pUL57). Another protein, gene product of UL84, is also essential. pUL84 is thought to trans-activate a responsive promoter within the *oriLyt* through interaction with IE2-86KDa, the process of which might partly initiate DNA replication (111). Moreover, study also showed that pUL84 has UTPase activity, which may help generate energy needed for helicase activity in the initiation of DNA replication (112).

The initiation of DNA replication for alpha-herpesviruses is thought to involve an origin-binding protein (OBP), which binds to specific origin sequences to provide the initial unwinding spot with its intrinsic helicase activity. Since HCMV lacks the homologue to OBP, the mechanism of initiation is less clear. One interesting model is that RNA-DNA hybrid formed during transcription may yield an open region that could be used as the initial spot. This hypothesis is in concert with the trans-activator property of the replication essential protein pUL84. An alternative but not mutually exclusive possibility is that HCMV could make use of cellular initiation machinery.

While alpha-herpesvirus DNA replication is believed to begin with a bidirectional theta-like mechanism, followed by a unidirectional rolling-circle mechanism at later stage (1), HCMV likely exclusively relies on the rolling-circle model, producing several thousands copies of concatemeric genomic DNA per cell (1).

4) Assembly and egress

a) Assembly and egress in nucleus

The first step in HCMV assembly and egress involves capsid assembly and genome packaging in the nucleus (Figure. 1-5). Capsid assembly initiates with the formation of a scaffold that could "crystalize" the capsid. There are two scaffolding proteins identified for HCMV, pUL80 and pUL80.5 (113, 114). Both proteins can interact with MCP through carboxyl terminus and lead to the translocation of MCP from cytoplasm to nucleus. The amino terminus of pUL80 and pUL80.5 promotes self-interactions, resulting in the generation of MCP multimers and formation of hexons and pentons (115, 116). At the same time, mCP and mc-BP translocate into nucleus to form triplexes. Hexons and pentons, together with triplexes, form an immature capsid shell. Next step is genome packaging. Two conserved sequence motifs, *pac-1* and *pac-2,* are located at each end of the concatemeric HCMV DNA sequence. Viral terminase complex comprised of pUL56 and pUL89 recognizes *pac-1* and *pac-2* and cleaves DNA into genome-length units (117, 118). Before DNA packaging, scaffolding protein pUL80 and pUL80.5 is disassociated from MCP by a protelytic cleavage at the carboxyl terminus. Then, insertion of DNA genome into capsid occurs through the portal formed by pUL104, coupled with the extrusion of the scaffolding protein remainders. It is worth noting that capsid formation and DNA packaging do not necessarily accompany each other. In fact, there are three types of viral capsid that could form: 1) A-capsid is an empty capsid lacking genome, which is a result of failure in DNA packaging; 2) B-capsid is an immature, scaffold containing capsids that lacks genome, probably due to unsuccessful proteolytic

digestion of scaffolding proteins; and 3) C-capsid is the mature, genome-containing capsid. Only C capsid will generate infectious viral particles. Although A-, B-capsids are not infectious, they might affect viral replication cycle somehow.

Nucleocapsid will then go through a process known as nuclear egress or primary envelopment. Nuclear lamina, a network of proteins underlying the nucleoplasmic side of the inner nuclear membrane (INM) is the first obstacle. Disruption of the nuclear lamina is essential to gain access to the inner nuclear membrane (INM). HCMV cleverly finds a way to achieve that by mimicking the phosphorylation-mediated depolymerizaion of nuclear lamina occurring during cellular mitosis. Virus encodes a nuclear egress complex (NEC), comprised of pUL50 and pUL53. pUL50 is a INM protein while pUL53 is a nucleoplasmic protein, which is brought to INM by binding with pUL50 (42, 119). HCMV NEC recruits cellular kinase PKC, as well as a viral kinase pUL97, to phosphorylate and dissolve nuclear lamina (120–122). The disruption of nuclear lamina allows the nucleocapsid budding into INM, resulting in an enveloped nucleocapsid in the lumen of nuclear membrane. A subsequent deenvelopment process occurs by fusion with the outer nuclear membrane (ONM) and leads to the delivery of the nucleocapsid into cytoplasm. It has been suggested that there is a quality control during nuclear egress to preferentially allow the genome-containing capsid budding through INM (123, 124). To support this hypothesis, C-capsid is found to be the predominant type of capsid present in the cytoplasm. Some tegument proteins are acquired during primary envelopment,, probably due to interactions with capsid proteins.

b) Assembly and egress in cytoplasm

Nucleocapsid acquires its final tegument and envelope by budding through the cytoplasmic assembly compartment (AC), the process of which is also called secondary envelopment (Figure. 1-5). AC is a juxtanuclear body formed by extensively reorganization of cellular secretory organelles (e.g., endoplasmic reticulum (ER), Golgi, trans-Golgi and endosomes). The formation of AC is a unique characteristic of HCMV infected cells during the late stage of infection. It is not well understood how nucleocapsid is directed to AC after nuclear egress, but tegument proteins are suggested to play an important role in it. Moreover, the putative intimate connection between nucleus and AC, achieved by HCMV-mediated remodeling of nucleus shape and formation of membranous channel on the nucleus-AC interface, might also facilitate nucleocapsid trafficking to AC (125). Some of the tegument proteins and almost all envelope glycoproteins are located in AC, which are finally incorporated into mature viral particle when nucleocapsid buds into the endosomal and/or trans-Golgi derived membranes. Enveloped viral particle is secreted to the extracellular environment by cellular exocytic-associated pathways. Together with mature viral particle, infected cells also produce noninfectious enveloped particles (NIEPs) and dense bodies (DBs) (126). NIEPs are viral particles that lack genome (i.e., formed by A- and B- capsid). DBs are viral particles that contain tegument proteins (mainly pp65) instead of capsid (therefore also lacking genome), thus they are noninfectious as well. The relative proportions of NIEPs and DBs to infectious virus particles may vary due to differences in genetic background of the virus and/or in cell types where progenies are produced. Although being

replication defective, it is possible that NIEPs and DBs could act like "decoy" to wear out host immune response (e.g., the humoral antibody attack), thus giving infectious particles higher chance to survive and establish infection in a new cell.

HCMV latent infection

HCMV infection of a host for the first time is considered as the "primary infection". Primary infection generally has two outcomes: 1) the virus enters the lytic replication cycle, where viral gene expression and replication successfully proceed, leading to production and release of infectious progeny virions; 2) Alternatively, under some conditions or in certain cell types, the virus enters a latent infection state, where the viral gene expression is largely limited and progeny production is shut off. The current consensus is that HCMV is never cleared away after primary infection and it coexists with the host through latency for a lifetime, with reactivation happening periodically. While shedding of the virions from body fluids peaks at a few months after primary infection, persistent virus shedding at low levels could be detected for years, and intermittently virus shedding due to reactivation continues for the rest of the life, all of which could contribute to the horizontal transmission of the virus. Surprisingly, a large portion (up to 40%) of an individual's T cell repertoire is directed against epitopes on HCMV viral proteins that are expressed at all stages of replication cycle (64), suggesting a continual exposure of host immunity to HCMV antigen. This observation implies there is a relatively huge immune burden added from HCMV due to persistent infection and/or frequent reactivation from latency.

Latency is a common feature of herpesvirus family. While alpha-herpesviruses and gamma-herpesviruses establish latency mainly in neurons and lymphocytes, respectively, beta-herpesviruses establish latency in myeloid lineage cells (Table. 1-2). CD34+ myeloid progenitor cells and CD14+ monocytes are the main latency sites for HCMV (127–131). The prevailing view for establishment of latency and triggering of reactivation is that HCMV major immediate early promoter (MIEP) is silenced by cellular transcriptional repressor in those myeloid cells, and the repression of MIEP hampers the IE gene expression thus prevents lytic infection, leading to the establishment of latency. During latency, HCMV genome exists in the nucleus like an episome, coupled with limited transcription of specific viral genes, which are thought to be important for latency maintenance. Following differentiation of myeloid cells into macrophages or dendritic cells, the cellular environment changes in a way that the repression on MIEP is relieved, which induces IE gene expression and reactivation of the virus (reviewed in (132, 133)).

Table 1-2. Comparison of host range, cell tropism and site of latency among human herpesviruses.

		Host range	Cell tropism	Site of latency
Alpha-	HSV-1 (HHV-1)	Human (In lab: *mice, rabbits, guinea pigs, zebrafish*)	Epithelial cells; Neurons	Neurons (Sensory ganglia, mainly in head and neck)
	HSV-2 (HHV-2)	Human (In lab: *mice, rabbits, guinea pigs, zebrafish*)	Epithelial cells; Neurons	Neurons (Sensory ganglia)
	VZV (HHV-3)	Human	Epithelial cells; Neurons; Lymphocytes (T cells)	Neurons (Sensory ganglia, all over the body)
Beta-	HCMV (HHV-5)	Human	All cell types	Monocyte lineage
	HHV6A/ 6B	Human	Lymphocytes (T cells); Neurons	Monocyte lineage
	HHV-7	Human	Lymphocytes (T cells)	Monocyte lineage
Gamma-	EBV (HHV-4)	Human	Epithelial cells; Lymphocytes (B cells)	Lymphocytes (Memory B cells)
	KSHV (HHV-8)	Human	Lymphocytes (B cells and T cells); Monocytes; Endothelial cells	Lymphocytes (T cells)

HCMV genetic diversity

When considering about HCMV genetic diversity, cautions need to be taken with some important terminologies. An "isolate" refers to HCMV recovered from a human specimen and passaged in tissue culture for a limited number of times. Generally an isolate is not plaque purified, suggesting that several genomically different viruses might present in one isolate. A "strain" refers to a passaged derivative of an isolate

acquired by plaque purification. Strains of HCMV differ in the pattern of viral DNA fragments generated by restriction enzyme digestion, which is later confirmed with genome-wide sequencing techniques. A "variant" refers to a derivative of a strain that expresses a phenotypic or genotypic characteristic different from the parental strain (134).

About third-quarter of HCMV genome shows polymorphisms, while one-quarter is highly conserved. The most conserved regions of the genome are localized to genes encoding DNA-processing enzymes, capsid, and tegument proteins, while the variable regions are genes encoding envelope glycoproteins and immune evasion proteins (135, 136). Several variable regions of the genome have been selected to define distinct genotype based on the clustering of polymorphisms, including genes that encode for glycoprotein gB (5 genotypes), gH (2 genotypes), gN (7 genotypes), and gO (8 genotypes) (87, 89, 137, 138).

It has been appreciated for decades that HCMV exhibits significant levels of genetic diversity between individuals (inter-host diversity) (139–141). However, more recent research work has showed that this virus also exhibited high level of genetic diversity within a single individual (intra-host diversity) (reviewed in (142)). Quite surprisingly, this intra-host diversity could be comparable to what has been observed for RNA viruses, a benchmark of highly diverse viral populations (7). Then the intriguing question is what source contributes to the high levels of HCMV intra-host diversity. Currently, there is no clear answer to explain the observed diversity, though several hypotheses could be proposed: 1) High levels of replication during primary infection could contribute to the generation of de novo mutations in each

host, albeit the total numbers of mutations may be limited by the proof reading of HCMV DNA polymerase. 2) Reinfections may offer a way for introducing diversity into virus intra-host population. Indeed, mixed infection of multiple strains of HCMV happens in a wide variety of hosts, including immunocompetent, immunocomprised and congenital infected individuals (2–7). 3) Recombination and natural selections within a host could also alter the pattern of HCMV intra-host diversity.

The high levels of HCMV intra-host diversity probably would help us rethink the evolution of the virus within a host. Given the high level of genetic diversity, HCMV should be considered more as a "swarm"-heterogeneous population. In line with this notion, recent work also showed that different genotypes were detected from different compartments within the body (e.g., plasma and urine) (143), suggesting that during virus dissemination to distal compartments, the viral population can rapidly evolve, either due to natural selection or stochastic mutations, and can lead to populations of virus colonized in distal organs or tissues genetically different from the ones present in peripheral blood. Meanwhile, research also suggests that HCMV is genetically stable over time, as shown by that while mixed genotypes were detected in patients, the compositions of the mixed populations remains nearly constant over time (144, 145). Thus, HCMV can be diverse but yet stable within its host. The intra-host diversity may provide a mechanism for the virus to gain overall fitness by rapidly adapting to the changing environment, like new host organ/tissue, immune surveillance, and antiviral therapy. However, once settled down, the virus population tends to remain stable probably due to its low mutation rate.

HCMV cell tropism and entry

HCMV *in vivo* cell tropism and viral dissemination

One of the fundamental questions in virology has been that why a given virus will particularly infect one host species but not the other, or at a cellular level, why a given virus will specifically infect one cell type while failing to infect others. The above-mentioned phenomena have been referred to as viral "host range" or "cell tropism". Natural infections with most herpesviruses are restricted to a single species, although some can infect other species experimentally. In general, alpha-herpesvirues have a relatively broader host range (excepting VZV) but restricted cell tropism. Beta-herpesviruses have a restricted host range but a wide cell tropism. Gamma-herpesviruses have both restricted host range and cell tropism (reviewed in (146)) (Table. 1-2). HCMV, a human-specific virus, is able to virtually infect all cell types in human body, including endothelial cells, epithelial cells, fibroblasts, smooth muscle cells, placental trophoblasts, hepatocyte, polymorphonuclear leukocyte (PMNL), monocyte, dendritic cells, macrophages, lymphocyte and neuronal cells (147–157). HCMV infection in PMNL, monocyte and lymphocyte is blocked at the IE gene expression stage, thus those cells are considered to be susceptible but not fully permissive to HCMV infection. It is worthwhile to point out that monocyte will turn into full permissiveness to HCMV when it is differentiated into macrophages or dendritic cells (154, 158). With this extremely wide range of cell tropism *in vivo*, HCMV can acquire systemic dissemination and cause pathology in multiple organs, e.g., lung, kidney, liver, spleen, intestine, heart, brain and bone marrow (159). Several cell types, of significant roles in viral dissemination, will be described below.

Epithelial cell

Epithelial cell, lining the major cavities of the body, could be the first cell that HCMV infects if the transmission route is through oral or genital contact. It is the main target of HCMV in lung, gastrointestinal tract, kidney, and secretory glands (147, 160). Since epithelial cell is permissive for HCMV infection, it mainly involves in virus shedding into body fluids. In addition, infected epithelial cells have been reported to detach from the basal membrane, thus might contribute to infectivity in body fluids as well (159).

Endothelial cell

Endothelial cell, lining the inner wall of blood and lymphatic vessel, could be the first cell that HCMV infects if transmission happens by blood transfusion. This cell type is believed to be involved in HCMV haematogenous dissemination for the following two reasons: 1) endothelial cell is permissive for HCMV infection and 2) infected endothelial cells may detach from vessel wall and circulate in peripheral blood (160, 161). Transmission of the virus from infected endothelial cells to leukocytes has been reported in tissue culture model (162). Moreover, evidence also suggests that endothelial cell can also be a site for HCMV latent infection (reviewed in (163)).

Monocyte/Macrophage

Monocyte is also found to be the key contributor for HCMV haematogenous dissemination. Although complete replication cycle is blocked, HCMV could promote monocyte migration into organ tissues and ensuing its differentiation into the permissive macrophage cell. Infected macrophage could then migrate into bone

marrow and infect myeloid progenitor cells, where latency is usually established (164).

Taken together, a putative model for HCMV *in vivo* dissemination is that epithelial cells or endothelial cells are first infected by HCMV at mucosal epithelium or in the blood vessel. HCMV then replicates and spreads to monocytes in the peripheral blood by some unresolved mechanisms, where HCMV enters a temporary latent state. Infection by HCMV increases monocyte migration into different organs, and differentiation into permissive macrophages. Infected macrophages could either enter bone marrow to establish latency in CD34+ myeloid progenitor cells or keep migrating and infect mucosal epithelial cells at a different site, where virus shedding and transmission take place.

HCMV *in vitro* cell tropism and associated adaptive genetic changes

Historically, HCMV *in vitro* isolation and propagation has been heavily dependent on primary human fibroblast cultures (e.g., primary human foreskin fibroblasts, HFF) because those cells supported high titer replications of HCMV. Based on the extent of passage on fibroblast cultures, strains of HCMV were conventionally divided into two categories: "lab strain" (high-passaged) and "clinical strain" (low-passaged). Two prototypic lab strains are AD169 and Towne. Strain AD169, isolated from the adenoid culture of a 7-year-old girl, was developed as an attenuated vaccine candidate by extensively propagation on fibroblasts and was then used by laboratories worldwide for over 50 years (165); Strain Towne, isolated from the urine of a congenitally infected infant, was also developed as an attenuated

vaccine candidate by passaging on fibroblasts for over 128 times (166). In contrast, strains like Toledo, isolated from the urine of a congenitally infected child (167), and TR, an ocular isolate from an AIDS patient with retinitis (168) are considered clinical strains due to their more limited propagations on fibroblast cells.

Lab strains and clinical strains exhibit different competencies in tropism. Lab strains are able to replicate on fibroblasts with high efficiency, but show restricted tropisms by failing to infect epithelial cells, endothelial cell, and some types of leukocytes (134, 169, 170). Clinical strains show broader cell tropism and replicate in cells types in addition to fibroblast cells (171), although unexpectedly, the clinical strain Toledo was later found to be negative in endothelial cells tropism (170, 172). Accordingly, the tissue culture tropism model for HCMV involves fibroblasts versus endothelial/epithelial cells, and other cell types in the body tend to fall into this dichotomy.

Initial genome comparative studies of lab strains AD169, Towne, and clinical strain Toledo found that both lab strains A169 and Towne had suffered dramatic genetic deletions, missing 15, and 13 Kb sequence from the right end of the UL region respectively (173). Perhaps in order to keep intact genome length, both AD169 and Towne also exhibit genetic rearrangement by replacing the missing region with an inverted version (*b'a' or also called IRL*) of the terminal repeated block (*ab or also called TRL*), which is otherwise lacking in clinical strains (Figure. 1-3) (41). Thus the missing region is designated the UL/b', which is predicted to encode 19 ORFs (UL133-151) or 14 ORFs (UL133-146) in AD169 or Towne respectively and many of those ORFs are predicted to be glycoproteins (41, 173).

Some of the ORFs missing in lab strains are displayed in an inverted orientation in clinical strain Toledo as compared to other clinical strains (41, 134). UL128-131 gene locus on the UL/b' was later shown to be indispensable for virus infection in epithelial/endothelial cells and transmission to leukocytes (78). This was confirmed by genome-wide sequencing showing that AD169 contains a single nucleotide insertion in UL131; Towne contains a frameshift mutation in UL130, and Toledo contains an inverted site disrupting UL128 (41, 78, 174, 175). Therefore the so-called clinical strain Toledo behaves more like lab strain with respect to the mutations acquired in the UL/b'.

Recently, Dargan *et al.* demonstrated that genetic changes inevitably occurred when clinical viruses were serially passaged in tissue cultures, including epithelial and endothelial cells (176). These authors passaged HCMV on fibroblasts, epithelial and endothelial cells in parallel and showed that in all three cultures, mutations were first selected in gene RL13, and then in genes UL128-131 (specifically associated with fibroblasts as described above for AD169, Towne and Toledo), and in some cases, eventually in UL/b' region (as what happened to AD169 and Towne). RL13 encodes a glycoprotein that tracks to the virion assembly site, suggesting it might be a virion envelope protein (177). Although the function of the RL13 protein is currently unknown, the rapid negative selection against it suggests that it is likely a potent inhibitor for HCMV *in vitro* replication and the loss of function in RL13 may also contribute, at least in part, to the less cell-associated phenotype observed in lab strains (177–180).

The overall picture is that HCMV strains that have been propagated *in vitro* tend to harbor mutation(s) resulted from selection pressure in all cell culture systems. And the appearance of the mutants is not only inevitable but also prone to emerge at early stages of the passage (43, 176). Therefore, even the less propagated "clinical strain" could contain mutations that we are not aware of, thus might not truly represent its clinical ancestor. Recently Wilkinson *et al.* suggested a more adequate terminology system, where HCMV strains are divided into "low-passage" and "high-passage" groups, with low-passage strains being more genome intact than high-passage strains (181).

Bacterial Artificial Chromosome (BAC) (*E. coli.* fertility plasmid) cloning technology was introduced to preserve HCMV genome from further mutations and also allowed for intentional mutagenesis manipulation of the virus genome (182, 183). Virus can be recovered from transfection of permissive cells with BAC. High-passage strains AD169 and Towne as well as low-passage strains TR, TB40/E, Merlin (ME), PH all have been cloned into BACs (41, 177, 184), and are used for research in this dissertation.

HCMV entry and membrane fusion event

Viral tropism can be determined at any replication step, beginning with the entry into cells and ending with the production of progeny. It has been well appreciated that viral tropism can be greatly influenced by virus entry. Different cell types vary a lot in their physical morphologies as well as surface protein, carbohydrate and lipid compositions. Thus virus entry is largely governed by how

the virus particle overcomes the barriers on cell surface and accomplishes interactions with their receptors. The broad cell tropism of HCMV reflects its flexibility at entry, suggesting that the virus might either bind to a ubiquitous receptor shared with all cell types or a number of specific ones on different cell types. On the other hand, HCMV broad tropism correlates well with its envelope complexity. This virus is remarkable because it contains more than 20 different kinds of glycoproteins on the envelope. It's almost like HCMV has a chain of "keys" to readily open different "locks".

As an enveloped virus, entry has to happen through membrane fusion event between viral envelope and cellular membranes. Depending on the cell types that the virus encounters, the site of fusion can be at either the plasma membrane or from within the endosomes. Moreover, the types of endosomes can vary depending on what kind of endocytosis that the virus triggers, thus the requirement for low-pH can vary as well. For HCMV, it has been reported that the virus enters fibroblasts through pH-independent fusion at the plasma membrane. Although more recent research suggested that the virus utilizes macropinocytosis pathway to enter fibroblasts in a pH-independent manner. Meanwhile, entry into epithelial/endothelial cells requires endocytosis and depends on low-pH (79, 185, 186).

Although thermodynamically favorable, fusion of two membranes must overcome a repulsive "hydration force", which increases steeply as the two membranes come closer than 20 angstrom (187). Because of that, membrane fusion requires a source of free energy to bring them together, for example, a chemical

called polyethylene glycol (PEG) could induce membrane fusion by dehydration of the inter-membrane-space to overcome the repulsive "hydration force" and bring apposing membranes closer than 20 angstrom. Essentially, enveloped viruses encode a "fusogen" to achieve this end, which is defined as viral fusion protein. Viral fusion protein is held in a "prefusion conformation" on the virion envelope by constraints that either come from another part of the same protein or from another viral protein. Usually two events have to happen before fusion protein conformational change: 1) "priming" of fusion protein to make the transition possible, e.g., proteolytic cleavage; 2) "triggering" of fusion protein to initiate the transition, e.g., ligand binding. The ligand can be a receptor (e.g., receptor-mediated fusion of HIV virus) or a proton (e.g., low-pH induced fusion of influenza virus). Upon the triggering, the constraints that hold the fusion protein in the "prefusion conformation" is released, leading to a dramatic conformational change and exposure of the hydrophobic "fusion peptide" or "fusion loop", which then inserts into the apposing cellular membrane. This state of the fusion protein is "metastable", and favors to fold back to the more energetic stable "postfusion conformation". Thus, the collapse of the intermediate conformation draws two membranes together (review in (188)).

Fusion proteins fall into three categories. (1) Class I fusion proteins, represented by influenza virus hemagglutinin (HA) or HIV-1 gp160, are mainly composed of alpha-helices and contain a "fusion peptide" located closed to the N-terminus. The postfusion structure of it is a trimeric alpha-helical coiled coil. (2) Class II fusion proteins, represented by flavivirus envelope protein E, are mostly

comprised of beta-sheets. Unlike class I fusion proteins being perpendicular to viral envelope, class II fusion proteins form a parallel lattice on the envelope. Upon triggering, the proteins flip up to insert internal "fusion loop" to cellular membrane. (3) Class III fusion proteins, represented by vesicular stomatitis virus glycoprotein G (VSV-G), combine the feature of both class I and class II. They contain a central trimeric coiled coil domain just like class I fusion proteins in their postfusion state, but other domains are mostly made up by beta-sheets, and the "fusion loop" is buried internally, typical feature of class II fusion proteins (reviewed in (188)).

HCMV core fusion machinery and accessory glycoproteins

All herpesviruses share conserved core fusion machinery comprised of gB and gH/gL complexes (146, 189, 190). All three glycoproteins are essential for virus entry, for example, HCMV gB-null, gH-null and gL-null mutants all fail to infect cells unless treated with the chemical fusogen PEG, suggesting that the defect is at fusion (191–193).

HCMV gB (pUL55) is a 160~170 kDa transmembrane protein. It contains a cleavage motif R-X-K/R-R recognized by intracellular endoprotease furin and is cleaved into a 116 kDa N-terminal fragment and a 55 kDa C-terminal fragment, which are hold together by disulfide bonds. The proteolytic cleavage probably occurs in the TGN-derived assembly compartment during virus maturation, thus gB incorporated into virion envelope is a disulfide-linked complex of the two fragments (194, 195). Several gB homologues across the three herpesvirus families share this cleavage feature, including gB of pseudorabies virus (PRV), VZV, EBV, KSHV, and

other betaherpesviruses (e.g., HHV-6 and HHV-7) (196–201). In contrast, gB of some members of herpesviruses do not harbor the cleavage site, including HSV-1 and HSV-2 (202, 203). The influence of gB cleavage on virus replication has been variable depending on different viruses. There are reports showing that for HCMV, PRV and VZV, the cleavage site of gB is dispensable for virus growth in tissue culture, while for EBV, the proteolytic cleavage seems to be required for gB function in cell-cell fusion (197, 204–206). The fact that cleavage of gB in HCMV is not necessarily needed during replication suggests that this furin cleavage is not required for the activation of fusion potential, as in the case of influenza virus HA or HIV gp160. Moreover, posttranslational glycosylation adds both N-linked and O-linked polysaccharides to gB, contributing to one third of the total molecular weight (122, 207, 208). Recent research found that antigenic sites that elicit neutralizing antibodies are more glycosylated than those eliciting non-neutralizing antibodies, suggesting that the glycans might help shielding the neutralizing epitopes on gB (67). Crystal structure of HCMV gB ectodomain has been resolved and has revealed structural homology to HSV-1 and EBV gB, all of which resemble the structure of class III fusion protein VSV-G (67, 209, 210). However, unlike VSV-G being necessary and sufficient in mediating membrane fusion, herpesvirus gB is not sufficient on its own and it needs a partner gH/gL.

HCMV gH (pUL75) is a 97-kDa transmembrane protein, and gL (pUL115) is a 37-kDa soluble protein. Together they form a disulfide-linked heterodimer (211, 212). Crystal structures have revealed the overall similar but slightly different structures between HSV and EBV gH/gL. HSV gH/gL adopts a boot-like

configuration whereas EBV gH/gL is more linear and elongated. In both cases, gH/gL heterodimer is intimately integrated with each other, with gL binding to the N-terminal of gH, suggesting the two proteins require one another for proper folding (213, 214). Although HCMV gH/gL crystal structure has not been resolved yet, cryo-EM analysis suggested a more similar structure to HSV gH/gL (215). HCMV gB and gH/gL together are necessary and sufficient to mediate cell-cell fusion in a transient expression system (216), with gH/gL provides an essential regulatory role for gB's fusion function. However, the mechanisms by which gH/gL regulates gB-fusion are still largely unknown.

Besides the core fusion machinery, each herpesvirus employs accessory proteins to regulate the action of the gB and gH/gL. Accessory proteins usually interact with gH/gL transiently or stably, and influence viral tropism through receptor binding. HSV encodes gD to bind to cell surface receptors like herpesvirus entry mediator (HVEM), nectin, or heparan sulfate (217). Upon receptor binding, gD experiences conformational changes, which leads to transient interaction with gH/gL, followed by gB-mediated fusion (218–220). EBV encodes gp42, which forms a trimeric complex with gH/gL. Thus EBV gH/gL exists in two forms, gH/gL/gp42 or unbound gH/gL. gH/gL/gp42 promotes virus entry into B cells through interaction between gp42 and MHC II molecules on B cell surface. gH/gL promotes virus entry into epithelial cells through interaction between gH/gL and specific integrins on epithelial cell surface. Binding of gp42 blocks gH/gL interaction with integrins, thus gp42 acts like a tropism switch adaptor (74, 221–224). HCMV gH/gL can be bound by gO or the small set of proteins UL128-131, forming gH/gL/gO or

gH/gL/UL128-131 complex. Both complexes could influence HCMV entry and tropism.

HCMV gH/gL/UL128-131 and gH/gL/gO complexes in entry and tropism

gH/gL/UL128-131

The gene locus of UL128, UL130, and UL131 (collectively designated the UL128 locus, UL128L) encodes three small soluble proteins UL128 (15 kDa), UL130 (35 kDa), and UL131 (18 kDa). It has been shown that an upstream promoter drives a long transcript that expresses UL131, UL130 and UL128, at the same time, there might be a UL128 specific promoter overlapping the UL131-UL130 region to independently express UL128 (78, 175). Both UL130 and UL131 are modified by N-linked glycosylations, while UL128 are not (80, 225, 226). UL128 and UL130 have also been predicted to contain chemokine domains. UL128 potentially has a CC chemokine domain near the N terminus, while UL130 might contain a central CXC chemokine domain (78, 175, 226). Whether those chemokine domains functions *in vivo* has not been convincingly determined yet.

UL128 is covalently bound to gH/gL through disulfide linkage to gL, while UL130/UL131 are disulfide linked to each other but are noncovalently bound to gH/gL (Figure. 1-6). Together UL128-131 proteins bind to gH/gL, forming a pentameric gH/gL/UL128-131 complex. Incomplete complexes lacking any of the UL128-131 proteins would not be transported to assembly compartment (AC) to be incorporated into the mature virion envelope (77–80, 215, 225, 227). UL128-131 sequences from different HCMV strains are highly conserved (>92.3% at nucleotide

level and >91.1% at amino acid level) (11, 228), suggesting the pentameric complex likely provides an important function for HCMV replication.

The consensus now is that gH/gL/UL128-131 is necessary for HCMV entry into endothelial/epithelial cells, but is dispensable on fibroblasts. Several lines of evidences support this conclusion: (1) Natural selection on fibroblasts leads to spontaneous loss-of-function mutations within UL128L. Historically, HCMV was mainly propagated on fibroblasts, and as a result, the high-passage viruses were associated with restricted tropism on epithelial and endothelial cells. The high-passage strains AD169 and Towne were found to contain a single nucleotide insertion in UL131, and a frameshift mutation in UL130 respectively. Relatively less passaged strain Toledo contains an inverted UL/b' region, with the site of inversion within UL128 (41, 43, 78, 173–175). More recently Dargan *et al.* took isolates of HCMV from clinical samples and intentionally passaged them on various tissue cultures, and found that mutations in UL128L inevitably happened and were exclusively limited to fibroblasts (176). (2) Reversions of the natural epithelial/endothelial loss-of–tropism mutants are all associated with reversal in UL128L. Similarly, when UL131 is intentionally repaired, AD169 recovers infectivity on endothelial/epithelial cells. (3) Introductions of targeted deletions into UL128L result in loss of endothelial/epithelial tropism. (4) Trans-complementation with individual UL128, UL130, and UL131 could also partially rescue endothelial/epithelial tropism (41, 77–79, 225). Furthermore, antibodies against UL128, UL130, or UL131 efficiently blocks virus entry into endothelial/epithelial

cells, but not fibroblasts (77), confirming that gH/gL/UL128-131 is required for HCMV entry into endothelial/epithelial cells but not fibroblasts.

Trans-expression of gH, gL, UL128-131 renders epithelial cell resistant to HCMV infection, indicating gH/gL/UL128-131 facilitates HCMV entry into epithelial cells through interactions with a specific, yet unknown, receptor (229). This putative receptor interaction likely triggers a signaling cascade (e.g., Src/paxillin pathway), which leads to internalization of viral particle through endocytosis (230). Subsequent virus entry happens by fusion from within the endosome in a low-pH dependent manner (79). Interestingly, viruses lacking gH/gL/UL128-131 can still be efficiently internalized into endothelial/epithelial cells, although productive infection is not observed (79, 231), suggesting that subsequent fusion from within the endosome is not allowed. This implies two possibilities: 1) gH/gL/UL128-131 is also required for the subsequent fusion event, or 2) when lacking the "right" gH/gL/UL128-131-specific endocytosis, virus gets internalized through a "wrong" signaling pathway, from which escape into cytoplasm via membrane fusion is simply impossible. If the first possibility were true, it would suggest that HCMV internalization on epithelial cells is promiscuous and not entirely dependent on gH/gL/UL128-131. And gH/gL/UL128-131 is required specifically for the fusion events, indicating that gH/gL/UL128-131 is sufficient for entry into epithelial/endothelial cells. However, this view is challenged by gO-null mutant phenotype (descrided later in gH/gL/gO section), thus favors the second possibility. In line with this, Vanarsdall *et al.* reported that while wild type HCMV can be internalized into epithelial cells through endocytosis (i.e., macropinocytosis)

followed by low-pH fusion with endosome, gH/gL/UL128-131-null virus can be internalized as well, although via a different pathway (i.e., dynamin-dependent endocytosis), however virus particles were not capable of fusion with endosome, and were probably degraded in lysosome instead (102).

gH/gL/gO

gO is an approximately 55 kDa protein encoded by UL74 gene. Posttranslational modifications, including N-link glycosylation, result in gO glycoforms ranging from 100~130 kDa. gO is among the most diverse proteins encoded by HCMV. Phylogenetic analysis showed that there are at least eight families of gO in nature, varying by 10~30% at amino acid level (89). gO forms a complex with gH/gL through a disulfide linkage to gL (Figure. 1- 6) (73, 74, 76, 215, 232). The binding of gO promotes gH/gL trafficking from ER to Golgi-derived assembly compartment, resulting in the incorporation of gH/gL/gO complex into virion envelope (232, 233).

The functions of gH/gL/gO have been mostly studied by deletion mutants. gO-null mutants on the background of several strains have exhibited a "small plaque" phenotype on fibroblasts. Similar phenotype is also observed with the deletion mutants of gO homologues in murine cytomegalovirus and guinea pig cytomegalovirus (42, 234–237). It has been suggested that the "small plaque" phenotype is due to less production of cell-free progeny virions, as a result of deficiency in secondary envelopment (235, 236). However, a more recent study by Wille *et al.* demonstrated that the gO-null virus released comparable amount of progeny virions as wild type virus, arguing that gO-null mutant was not defective in secondary envelopment and egress, instead the "small plaque" phenotype was

probably due to poor infectivity of the progeny. In line with this hypothesis, cell-free gO-null viruses fail to enter fibroblasts (238), indicating gH/gL/gO is required for entry into fibroblasts. Trans-expression of gH/gL/gO renders fibroblasts resistant to virus infection (239), further suggesting that gH/gL/gO probably engages specific receptor on fibroblasts to mediate virus entry. Phenotypes of gO-null virus on epithelial/endothelial cells have not been looked carefully until a more recent analysis of gO-null virus published by Wille *et al*. Compared to wild type virus, the mutant virus contained drastically less total gH/gL, probably due to loss of gO, but had increasing amount of gH/gL/UL128-131. However the mutant virus failed to enter endothelial/epithelial cells in the presence of elevated level of gH/gL/UL128-131 (238). Thus, it seems unlikely that gH/gL/UL128-131 is sufficient for virus entry into endothelial/epithelial cells; instead infection of epithelial/endothelial cells requires both gH/gL/gO and gH/gL/UL128-131 complexes. Taken together, gO-null mutagenesis studies seem to suggest that gH/gL/gO is required for HCMV entry into both fibroblasts and epithelial/endothelial cells.

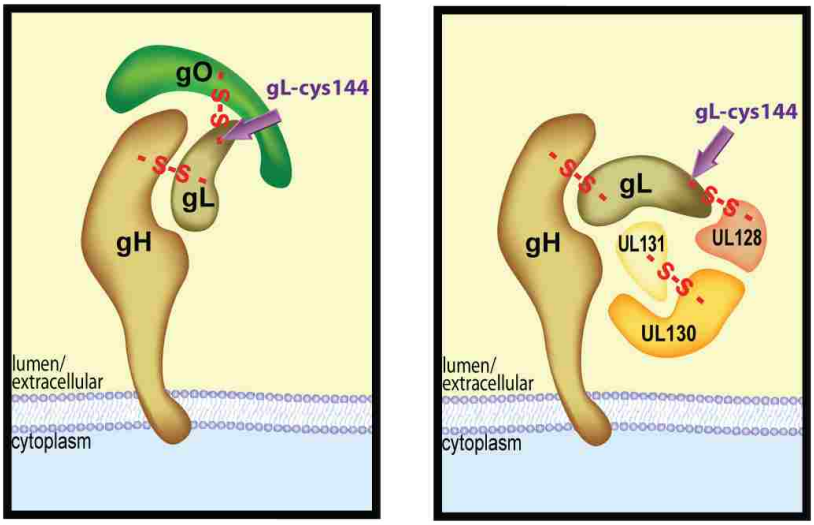

Figure 1-6 Schematic of gH/gL/gO and gH/gL/UL128-131 complexes.
S-S represents disulfide linkage. Most of the disulfide bonds are not resolved, except recently characterized gO-gL-cys144 in gH/gL/gO, and UL128-gL-cys144 in gH/gL/UL128-131.

Focus of the dissertation

The central focus of this dissertation is to study the entry mechanisms of HCMV into different cell types. Interaction between gH/gL and the fusion protein gB is a conserved feature of the entry mechanism for all herpesviruses. HCMV gH/gL can be bound by gO, or by the set of proteins UL128, UL130, and UL131, forming gH/gL/gO and gH/gL/UL128-131 complexes in the virion envelope.

Studies in Chapter 2 showed that gO and UL128-131 competed for binding to gH/gL, and that the ratio of the two complexes in the virion envelope can vary dramatically among strains. This result was quite surprising and provided an opportunity to address the functions and mechanisms of the gH/gL complexes. The prevailing view has been that gH/gL/gO promotes gB-fusion on fibroblast cells while gH/gL/UL128-131 does the same thing on epithelial/endothelial cells. This model is supported by the phenotype of UL128-131 deletion mutants, where the

mutants fail to infect epithelial/endothelial cells but can infect fibroblast cells as efficiently as wild type viruses. However, analysis of gO deletion mutants seems to be inconsistent with this model. gO mutants fail to enter any cell type, suggesting that gH/gL/UL128-131 is not sufficient on epithelial/endothelial cells, and entry into that cell type requires both complexes. However, further analysis is largely limited due to the severe growth defect of the gO mutants and the lack of a complementation system. Studies in Chapter 3 bypassed this problem by utilizing viruses that contain different amount of gH/gL/gO and gH/gL/UL128-131 on the virion envelope, and showed that entry into of both fibroblasts and epithelial cells was positively correlated with the abundance of gH/gL/gO, but not with gH/gL/UL128-131, indicating a new model where gH/gL/gO provides the conserved herpesvirus "gH/gL entry function" of promoting gB fusion for entry into all cell types, whereas gH/gL/UL128-131 provides a distinct, yet necessary function on select cell types (i.e. epithelial/endothelial cells) (Figure. 1-7). Studies in Chapter 4 underpinned multiple viral factors involving in the regulation of HCMV gH/gL complexes assembly.

In conclusion, these findings stressed the importance of until now underappreciated HCMV strain variation in the level of gH/gL glycoprotein complexes. The resultant distinct function model highlighted that the fusion-competent complex gH/gL/gO could serve as a potential vaccine target, and the non-fusion complex gH/gL/UL128-131 may provide an unique opportunity to characterize the fundamental mechanisms by which gH/gL regulates gB-fusion.

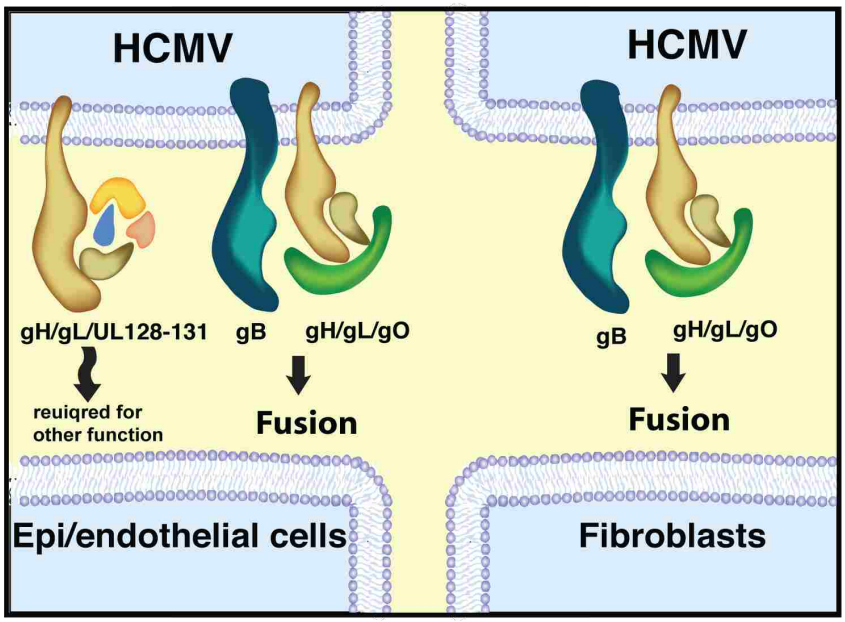

Figure 1-7 HCMV distinct-function tropism model.
gH/gL/gO provides the core-fusion function by promoting gB-fusion on all cell types, whereas gH/gL/UL128-131 broadens virus tropism by providing a non-fusion, yet necessary function for entry into select cell types, like endothelial/ epithelial cells.

CHAPTER 2. COMPARATIVE ANALYSIS OF gO ISOFORMS REVEALS THAT STRAINS OF HUMAN CYTOMEGELOVIRUS DIFFER IN THE RATIO OF gH/gL/gO AND gH/gL/UL128-131 IN THE VIRION ENVELOPE

This chapter is a modified version of the manuscript published in Journal of Virology in September 2013; 87(17), 9680-9690

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen that contributes to significant disease throughout the world (16, 240–242). Infections of immunocompetent adults are generally mild, but like other herpesviruses, HCMV establishes a persistent, or latent infection. Immunosuppression resulting from HIV infection or post-transplantation chemotherapy can allow reactivation of latent HCMV, leading to a wide range of pathologies, including gastroenteritis, encephalitis, retinitis, pneumonitis, and in the case of transplant recipients, graft rejection. In pregnant women, HCMV can cross the placental barrier, and is associated with several types of birth defects. The pleomorphic manifestation of HCMV-associated disease likely relates to the ability of the virus to infect a diverse spectrum of cell types in the human body, including epithelial and endothelial cells, fibroblasts, monocyte/macrophages, dendritic cells, hepatocytes, neurons and leukocytes (147, 148). The broad cell tropism of HCMV may reflect the abundance of distinct glycoprotein complexes in the virion envelope. Computer predictions and mass spectrometry analysis of purified virions have suggested that HCMV envelope may contain more than 20 different glycoproteins (41, 49).

Homologues of gB and gH/gL are found in all herpesviruses, and they represent the core entry machinery for the family. Extensive studies of herpes simplex virus (HSV) and Epstein - Barr virus (EBV) have shown that gB is the fusion protein, and that the gH/gL provides a regulatory role, likely requiring direct interaction with gB (209, 210, 213, 214, 219). In addition, each of the different herpesviruses encodes glycoproteins that appear to modify, or regulate the activity of the gB-gH/gL core

fusion machinery. For example HSV, EBV, and human herpesvirus 6 (HHV-6) encode gD, gp42 and gQ1/gQ2 respectively. Each of these glycoproteins interacts transiently, or stably with gH/gL, and each engages cell surface proteins as entry-mediating receptors (reviewed in (189, 243)) (244). For EBV, the accessory protein gp42 has a well-characterized effect on tropism. Unmodified gH/gL can bind to integrin molecules to mediate entry into epithelial cells (222, 223). When bound to gH/gL, gp42 likely blocks engagement of integrin by gH/gL, and instead gp42 engages MHC II molecules on the surfaces of B cells, thus acting like an adaptor to switch viral tropism from epithelial cells to B cells (214, 245, 246). Importantly, entry of EBV into epithelial or B cells requires either gH/gL (epithelial cells) or gH/gL/gp42 (B cells), not both (221, 247). This indicates that, given the proper cell receptors, either complex can make the necessary interactions with gB to mediate fusion.

Because entry into all cell types requires membrane fusion mediared by gB, the regulation of gB through direct interactions may be considered the core entry function of the herpesvirus gH/gL. In a reduced system involving expression of HCMV proteins by replication-defective adenovirus (Ad) vectors, gH/gL and gB were sufficient to induce cell-cell fusion, indicating that the HCMV gH/gL provides the direct interaction surfaces involved in the regulation of gB fusion activity (216). However, like EBV, HCMV encodes a set of proteins that bind to gH/gL, i.e., gO and the set of UL128, UL130, and UL131 proteins (73, 74, 77, 227). Trafficking of HCMV gH/gL from the endoplasmic reticulum (ER) to the Golgi-derived viral envelopment compartment depends on interactions with either the UL128-131 or gO, but the

specific effects of these accessory proteins on the entry-mediating function(s) of gH/gL are not well understood (80, 233).

UL128 binds to gH/gL through disulfide bond, while UL130/UL131 are not (215). The binding of UL128-131 proteins promotes gH/gL trafficking to trans-Golgi derived assembly compartment, leading to a pentameric complex gH/gL/UL128-131 present in virion envelope (80). UL128-131 deleion mutants failed to enter epithelial or endothelial cells, but replicated well in primary fibroblasts (77–79, 248), and epithelial cells expressing gH/gL/UL128-131 from replication-defective adenovirus (Ad) vectors were resistant to infection with HCMV (229). These data suggest that the gH/gL/UL128-131 facilitates entry by engaging molecules on the surfaces of specific cell types.

The role of gO as an interaction partner of HCMV gH/gL is complicated by the fact that it is among the most diverse proteins encoded by the virus. Rasmussen *et al*. performed a phylogenetic analysis of the predicted gO sequences of more than 40 clinical isolates of HCMV, and found eight groups, or families (89). Within each group, the gO amino acid sequences were 98-100% identical, whereas differences between groups ranged from about 10-30%. Stanton *et al.* analyzed HCMV from clinical specimens collected from donors over 10 years, and found gO sequences that fit within the families described by Rassmussen *et al.* Remarkably, the gO sequences within individual donors were stable over time (145). Similar results were also reported by Gorzer *et al.* (249). The tight grouping of gO sequences into families, and their relative stability suggests that they represent alleles of the same

gene, fixed in nature. Thus, the gO proteins encoded by different strains or isolates of HCMV may be considered different gO isoforms.

Only limited isoforms of gO have been studied in detail. Huber *et al.* first characterized gO as an approximately 55 kDa protein encoded by the UL74 gene of the fibroblast-adapted strain AD 169 (AD) (75). Posttranslational modifications, including extensive N-linked glycosylation, result in gO glycoforms that range from about 100 to 130 kDa, and interactions with gH/gL involve disulfide bonds such that a trimer of gH/gL/gO is present in the AD virion envelope (73, 74, 76). More recent analyses of the low-passage clinical strain TR failed to detect gO in the virion envelope, and several lines of evidence supported the conclusion that TRgO might act as a true chaperone, promoting trafficking of gH/gL lacking UL128-131 from the ER to the Golgi-derived envelopment compartment, and then disassociating from the complex, leaving unmodified gH/gL in the virion envelope (233). Because HCMV strains encoding other gO isoforms were not analyzed, it was not clear if the observed difference between AD, and TR reflected the diversity of gO, or the fibroblast-adaptation of AD. Regardless, gO mutants on the background of several strains have exhibited extensive replication defects in a wider range of cell types than were observed for UL128-131 mutants (42, 234, 235, 238). Wille *et al*. found that a gO null mutant TR, released normal numbers of DNA-containing viral particles to the culture supernatants, but these particles were defective for entry into fibroblasts. Surprisingly, gO-null TR was also unable to enter epithelial or endothelial cells, despite elevated levels of the pentameric gH/gL/UL128-131 complex in the virion envelope. In sum, these observations suggest a model in

which there are different requirements for entry of HCMV into different cells types. Entry into every cell type likely requires interactions between a gH/gL molecule and the fusion protein gB. The failure of gO null TR to enter any cell type suggests that this function is performed by gH/gL/gO or gH/gL, and not the pentameric gH/gL/UL128-131. This is markedly different from the model of EBV tropism, in which either gH/gL or gH/gL/gp42 is sufficient as a "core gH/gL complex" for entry in epithelial or B cells, respectively.

The aim of the studies reported this chapter was to further explore gO function(s) through comparative analysis of a wider set of gO isoforms. Experiments involving reconstruction of gH/gL – gO interactions by replication-defective adenovirus (Ad) vector expression suggested that despite the sequence diversity, all isoforms of gO form stable disulfide-linked trimers with gH/gL. Analyses of extracellular HCMV virions indicated that unlike previously reported, all HCMV strains, including TR, likely contain gH/gL/gO and gH/gL/UL128-131, and little if any unbound gH/gL. Furthermore, strains of HCMV differ dramatically in the ratio of gH/gL/gO to gH/gL/UL128-131 incorporated into the virion envelope, which may relate to the pleomorphic tropism, and clinical pathology observed in HCMV infections.

MATERIALS AND METHODS

Cell lines. Primary human foreskin fibroblasts (HFF; LifeTechnologies), and U373-MG human microglial cells (American Type Culture Collection; Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; LifeTechnologies)

supplemented with 12%, heat-inactivated fetal bovine serum (FBS; HyClone), or 10% bovine growth serum (BGS; HyClone). 239(IQ) cells (Microbix, Toronto, Canada) were grown in minimum essential medium (MEM; LifeTechnologies) plus 10% FBS. HFFtet cells, which express the tetracycline repressor protein, were provided by Richard Stanton (Cardiff Univ., Cardiff, UK), and were propagated in DMEM supplemented with 12% FBS (177).

Human cytomegalovirus (HCMV). All HCMV were derived from bacterial artificial chromosome (BAC) clones. BAC clone TR was provided by Jay Nelson (Oregon Health and Sciences Univ., Portland OR, USA.) (41). BAC clone TB40/e (BAC-4;TB) was provided by Christian Sinzger (Univ. of Tübingen, Tübingen, Germany) (184). BAC clones PH, and AD169 (AD) were provided by Tom Shenk (Princeton Univ., Princeton, NJ, USA) (41). BAC clone Merlin (ME) (pAL1393), which harbors tetracycline operator sequences within the transcriptional promoter of UL130, and UL131, was provided by Richard Stanton (Cardiff Univ., Cardiff, UK) (177). Infectious HCMV was recovered by electroporation of BAC DNA into MRC-5 fibroblasts as described by Wille *et al.* (238). HCMV stocks were produced by infecting HFF (or HFFtet for ME) using 0.1 plaque-forming units (PFU) per cell for 10-16 days. Cell-associated virus was prepared by sonic disruption of infected cells, and clearing large cellular debris by centrifugation at 1500 x g for 10 min. Extracellular virus was concentrated from culture supernatants by centrifugation at 50,000 x g for 1 h, and pellets were resuspended in DMEM plus 10% FBS. PFU were determined by plaque assay, or by TCID50 on replicate HFF cultures.

Phylogenetic and sequence alignment analyses. DNA sequences of the UL74 genes of clinical HCMV isolates, and HCMV BAC clones have been reported along with Genbank accession numbers (41, 43, 89). Alignment, and phylogenetic analyses were performed using the default parameters of the online MAFFT (v.7) tool offered by the Computational Biology Research Center (CBRC), National Institute of Advanced Industrial Science and Technology (AIST), Japan.

Antibodies. Monoclonal antibodies (MAb) specific for HCMV gH (14-4b) were provided by Bill Britt (Univ. of Alabama, Birmingham, AL) (250). Anti-HA MAb were obtained from Sigma Aldrich (St Louis, MO, USA). Anti-UL128 MAb, 4B10 was provided by Tom Shenk (77). Rabbit, polyclonal anti-peptide antibodies directed against HCMV gH, gL, TRgO, UL130 and UL131 were provided by David Johnson (Oregon Health and Sciences Univ., Portland OR, USA) (80, 233). Additional rabbit polyclonal antisera were raised against synthetic peptides corresponding to residues 258-277 of MEgO, 250-269 of TBgO, 252-271 of ADgO, and 248-267 of TNgO. Peptide synthesis, rabbit immunization, and serum collection was performed by GenScript (Piscataway, NJ, USA.).

Replication defective Ad vectors. Nonreplicating (E1-) Ad vectors for expression of HCMV TR gH, gL, and gO, MEgO, TBgO, ADgO and TNgO were generating using a commercial (Microbix) modification of the method of Matthews et al. (251). Briefly, HCMV genes (gH: UL75, gL: UL115, gO: UL74) were synthesized, and codon optimized by GeneArt/Invitrogen (Regensburg, Germany). Coding sequence corresponding to the influenza HA epitope was added, in frame with the C-terminus of UL74 constructs. HCMV genes were ligated into shuttle plasmid

pDC316 (io) (Microbix), and shuttle plasmids were transfected into 293IQ cells along with the Ad genomic plasmid, pBHGloxΔE1, 3Cre (Microbix). Cre-lox recombination resulted in Ad vectors that were subsequently propagated on 293IQ cells, which express the lac repressor protein. The lac repressor protein binds to lac operator sequences between the promoter, and the HCMV transgene, reducing expression of the transgene in 293IQ cells. Ad vector PFU counts were determined using 293IQ cells. Multiplicities of infection (MOI) for experiments on U373 or HFF cells were determined empirically for each Ad vector to give appropriate expression, and ranged from 3 - 30 PFU/cell. There was little production of Ad proteins, and little or no cytopathic effect was observed.

Radiolabeling of Ad vector transduced cells, and immunoprecipitation (IP) analysis of expressed proteins. Cells were transduced with Ad vectors for 12-24 hours, washed extensively in labeling medium (DMEM lacking methionine and cysteine) then incubated in this medium for 45 min at 37 C. Cells were then incubated for 5 min in labeling medium supplemented with [^{35}S]-methionine/cysteine (500-1000 μCi/ml; Perkin Elmer). Radioactivity was chased by incubating cells in DMEM containing 10-fold excess nonradioactive methionine and cysteine for indicated times. Cell proteins were extracted with 1% Triton X-100, 0.5% sodium deoxycholate in 20mM tris (pH 6.8), 100 mM NaCl; (TBS-TX/DOC), supplemented with 0.5% bovine serum albumin (BSA), and 1mM phenylmethylsulfonyl fluoride (PMSF). Extracts were clarified by centrifugation at 16000 x g for 30 min, and incubation with protein A-agarose beads (Invitrogen) for

1 – 2 h. Proteins were IP'ed by the addition of MAb, or rabbit polyclonal antiserum as indicated for 2 h followed by protein A-agarose for an additional 2 h. Agarose beads were collected by centrifugation, and washed 3 times in TBS-TX/DOC. For endo H and PNGase F treatment, IP'ed proteins bound to protein A-agarose were incubated with endo H or PNGase F in the buffer specified by the enzyme manufacturer (New England Biolabs). Proteins were eluted from protein A-agarose by boiling in 2% SDS, and 2% 2-mercaptoethanol, and separated by electrophoresis using SDS-polyacrylamide gels. For sequential IP experiments, proteins were eluted from protein A-agarose beads by incubation in 2% SDS, 30mM dithiothreitol (DTT), in 100 mM tris (pH 7.4), 100mM NaCl at 95 C for 5min. The samples were diluted 35-fold with solution of 50 mM tris (pH 7.4), 300mM NaCl, 10mM iodoacetamide, 1%TX100, 0.5% DOC, 5mM EDTA, 0.02% sodium azide. Then, the secondary IP antibody was added, and samples analyzed as described above.

Western blot analysis of Ad vector - or HCMV- infected cells, and HCMV extracellular virus particles. HFF cells were transduced with Ad vectors for 12-24 days, and proteins were extracted in TBS-TX/DOC. For HCMV experiments, HFF or HFFtet cells were infected with an MOI of 3 of different strains of HCMV. At 5-10 days post infection (depending on the strain) proteins were extracted from infected cells, and extracellular virus collected from the culture supernatant by centrifugation as described in (233) in the same volume of TBS-TX/DOC. Extracts were clarified by centrifugation at 16000 x g for 30 min, and adjusted to 2% SDS with, or without 2% 2-ME, and boiled for 10 min. Proteins were separated by SDS-PAGE and transferred to polyvinylidine fluoride membranes (Whatman) in a buffer

containing 10mM $NaHCO_3$ and 3mM Na_2CO_3 (pH 9.9), plus 10% methanol. Transferred proteins were probed with rabbit polyclonal antibodies, or MAbs specific for HCMV proteins, or the HA epitope, followed by horseradish peroxidase-conjugated secondary antibodies, and chemiluminescence was detected using a Fujifilm LAS-3000 luminescent image analyzer.

RESULTS

Diversity of HCMV gO among laboratory strains, and clinical isolates of HCMV. A meta-analysis of the gO sequences reported by Rasmussen *et al.* was performed using modern phylogeny software, and included several commonly studied low-passage, "clinical" BAC-cloned strains of HCMV that were not available at the time of the prior studies (89) (Figure. 2-1). The gO isoforms encoded by HCMV TR, Merlin (ME), TB40/e (TB) and PH fit well into the groups described by Rasmussen *et al.* TRgO, MEgO, and TBgO were each identical to at least one of the other sequences from the same group, whereas PHgO differs from its closest family members, (540, 1176, and 1736) by two amino acid substitutions (G280V and S398P). Strains AD169 (AD), and Towne (TN) were extensively propagated in cultured fibroblasts, and are known to harbor many genetic mutations, deletions and rearrangements compared to the low-passage strains, such as TR, ME, TB and PH (41, 43, 173).

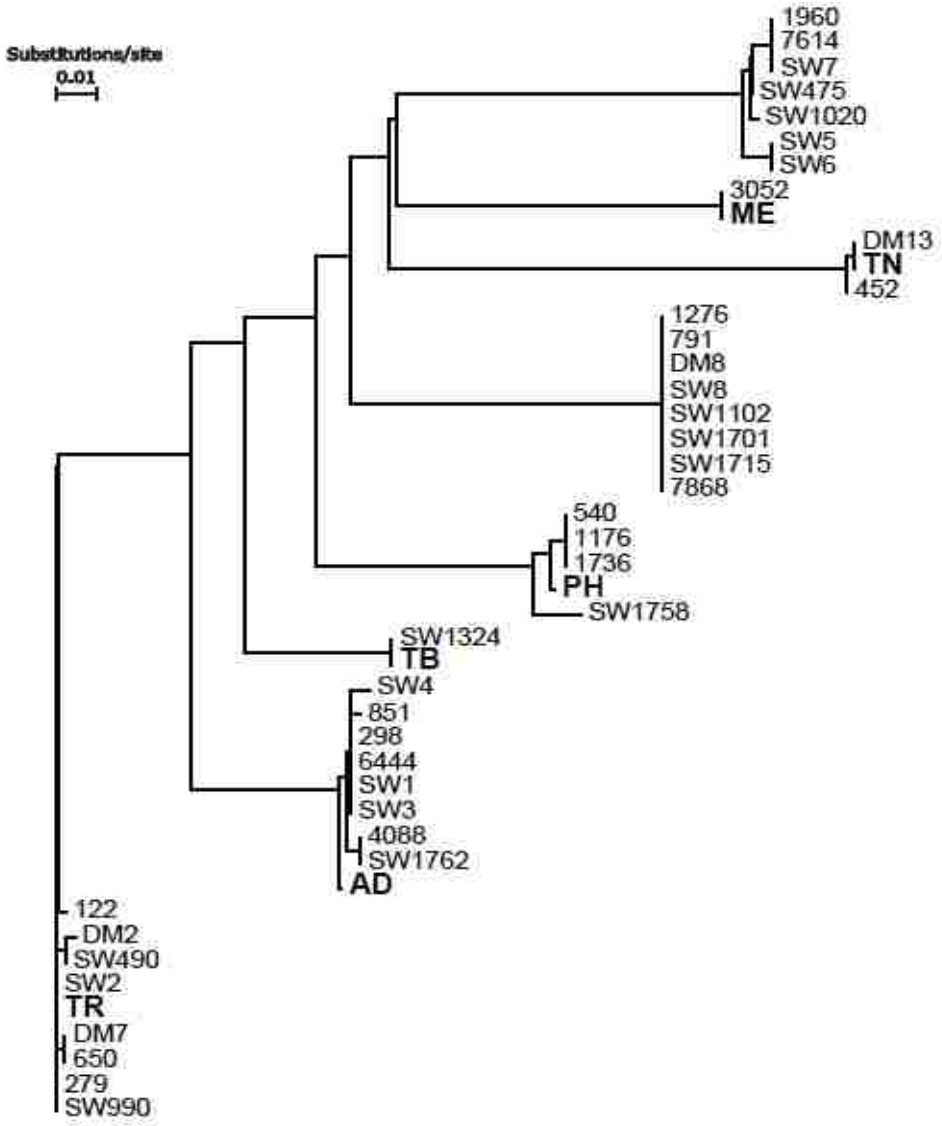

Figure 2-1 Phylogenetic relationships of predicted gO amino acid sequences encoded by lab strains and clinical isolates of HCMV.

HCMV BAC clones used in the current studies (bold) and clinical isolates described in Rasmussen *et al.* (89) were included, and the phylogeny was estimated using Neighbour-joining with the JTT codon model implemented in MAFFT v.7. Horizontal bar represents the expected number of amino acid substitutions per site according to the indicated scale.

However the gO sequence of TN is identical to that of the clinical isolate DM13, and ADgO differs from the gO of clinical isolates 298, 6444, SW1, and SW3 by only one

amino acid located within the predicted signal peptide. Thus, the gO isoforms encoded by these lab and clinical strains are reflective of the diversity of this protein in nature. It is important to note that given the limited number of sequences available, and the lack of understanding of gO function(s), no conclusions can be made concerning potential adaptive evolution of gO isoforms along lineages from a common ancestor. Despite this, there clearly exists extensive diversity in gO encoded by strains of HCMV in nature, and it is a compelling hypothesis that these differences may affect the function(s) of gO, and the biology of the virus.

Sequence alignments of representative gO isoforms revealed regions of high diversity, and regions of conservation (Figure. 2-2). Sequence diversity was found in the N-terminal 100 residues, of which approximately 70 are predicted to remain following cleavage of the signal peptide. The region corresponding to approximately 270-340 of the alignment also harbored notable diversity. Six conserved cysteine (cys) residues were identified at aligned positions 31, 152, 160, 178, 229, and 354. Cys 31 is located within the predicted signal peptide, suggesting that it is not relevant to the function of gO. The remaining five cys are also found in the gO homologues encoded by other beta-herpesviruses such as HHV-6, rhesus CMV, and murine CMV, suggesting the importance of disulfide bonds for the structure, and function of the gO interactions with gH/gL complex (252–254).

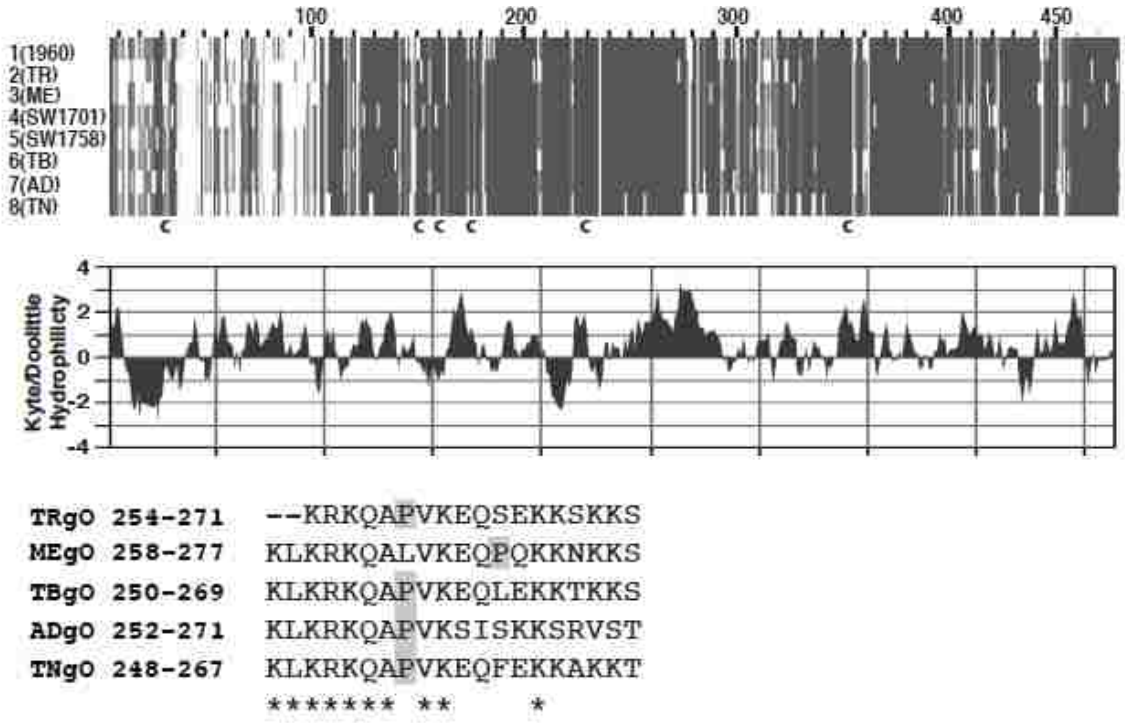

Figure 2-2 Comparisons of amino acid sequences of gO encoded by laboratory strains, and clinical isolates of HCMV.
Top: MAFFT alignment of one representative gO sequence from each of the eight groups indicated in Figure. 2-1. Darker shading indicates sequence conservation. The approximate location of 6 conserved cysteine residues is indicated. Middle: Kyte/Doolittle hydrophilicity analysis of TRgO. Bottom. Sequences of synthetic peptides used to raise anti-gO antibodies. Asterisks at the bottom indicate conserved residues, and shaded boxes highlight the position of a proline in each peptide sequence.

Construction of replication-defective Ad vectors to express gO isoforms. To study the effects of gO sequence diversity on interactions with gH/gL, we constructed Ad vectors to express the gO isoforms encoded by strains TR, ME, TB, AD, and TN, each with a C-terminal HA-epitope tag. These Ad vectors represent the HCMV strains for which there are published mutational analyses of gO, and/or represent the commonly studied fibroblast-adapted, and low-passage clinical strains (42, 234, 235, 238).

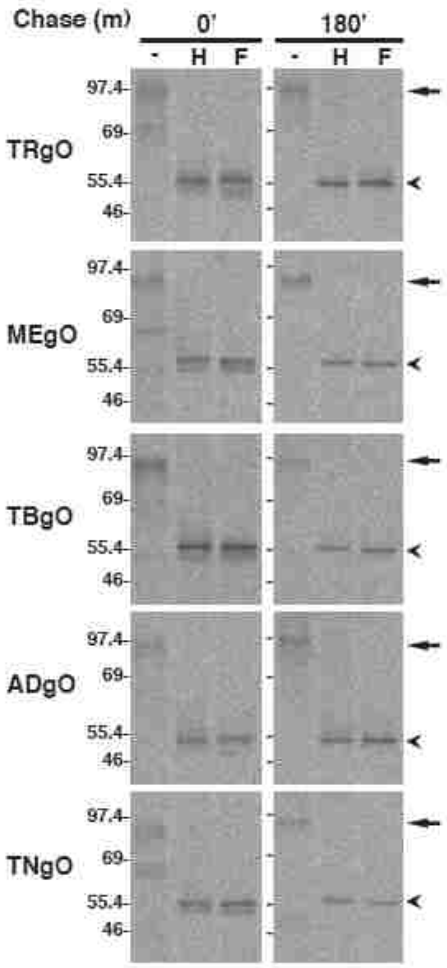

Figure 2-3 Expression of HCMV gO isoforms by replication-defective adenovirus (Ad) vectors.
U373 cells were transduced with Ad vectors expressing gO isoforms (HA-tagged) derived from HCMV TR (TRgO), Merlin (MEgO), TB40 (TBgO), AD169 (ADgO), and Towne (TNgO). Cells were labeled for 5 min with [35S] methionine/cysteine, and then the label was chased for 0 or 180 min. Proteins were immunoprecipitated with anti-HA antibodies, left untreated (-), or treated with endo H (H) or PNGaseF (F), and separated by SDS-PAGE (8%) under reducing conditions. Arrows, and arrowheads mark untreated, and deglycosylated forms of gO, respectively.

For initial characterization of gO isoforms, cells were transduced with Ad vectors, and proteins were pulse-radiolabeled, and then chased for 0 or 180 min. Cell extracts were then analyzed by immunoprecipitation (IP) with anti-HA

antibodies followed by treatment with endoglycosidase H (endo H), which removes the high-mannose N-linked glycans added to proteins in ER, or PNGase F, which can remove all types of N-linked glycans, including those that have been modified by enzymes present only in the Golgi complex. Proteins were then separated by SDS-PAGE under reducing conditions (Figure. 2-3). Each gO isoform migrated with an apparent molecular weight of approximately 100 kDa. Slight differences in mobility likely related to differences in polypeptide length among these isoforms (predicted gO amino acids, including signal peptide: TR; 462, ME; 472, TB; 464, AD; 466, and TN; 456), as well as differences in N-linked glycosylation, or other post translation modifications. Faster migrating bands were also observed in the untreated lanes of the 0 min chase samples. Since these bands were absent following glycosidase treatment, and were not apparent after 3 hours of chase, they were likely glycosylation intermediates of gO present in the cells after the pulse labeling. The N-linked glycans on each gO isoform remained sensitive to endo H treatment for at least 3 h of chase time, as indicated by the decrease in apparent molecular weight to approximately 55 kDa. These data indicate that gO isoforms share the characteristics of being highly modified with N-linked glycans, and being retained in the ER when expressed in the absence of other HCMV proteins.

Cross-reactivity of antibodies raised against distinct gO isoforms.

Kyte/Doolittle analysis of TRgO indicated one prominent hydrophilic stretch

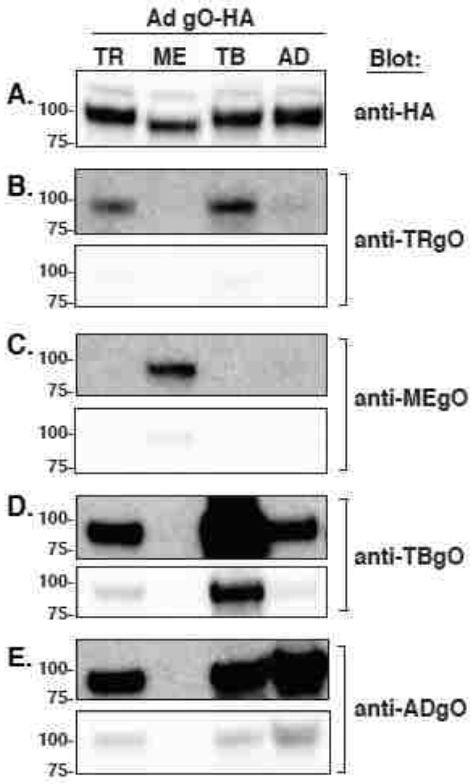

Figure 2-4 Western blot detection of gO isoforms by anti-peptide rabbit sera.
Primary human fibroblasts (HFF) were transduced with Ad vectors expressing the indicated gO isoforms (HA-tagged). Cell extracts were analyzed by western blot using anti-HA antibodies (A), or antibodies raised against peptides derived from gO of HCMV TR (A), Merlin (B), TB40 (C), or AD169 (E). For detection with anti-peptide antibodies (A – E), long, and short chemiluminescent detections (top and bottom, respectively) were performed in parallel, allowing comparison of signal intensities.

including amino acids 245-271 (Figure. 2-2). A synthetic peptide corresponding to these residues was used to raise anti-TRgO antibodies in rabbits that reacted well with TRgO, but not other gO isoforms (233). Kyte/Doolittle analysis of other gO isoforms indicated the presence of a similar hydrophilic region at the aligned location (data not shown). However, there are some amino acid differences in this

region that may explain the lack of cross-reactivity of the TRgO antiserum with other gO isoforms.

To allow more comprehensive comparative analyses of gO isoforms, antibodies were raised against peptides corresponding to the aligned hydrophilic region of the gO isoforms encoded by strains ME, TB, AD, and TN, and including conserved lysine, and leucine residues (Figure. 2-2). These antibodies were characterized by western blot analysis using extracts from cells transduced with Ad vectors expressing the HA-tagged gO isoforms (Figure. 2-4A). The previously characterized anti-TRgO antibodies cross-reacted with TBgO, but failed to detect MEgO or ADgO (Figure. 2-4B). Both the anti-TBgO, and anti-ADgO antibodies showed overall stronger reactivity, and unexpected cross-reactivity with other isoforms of gO, detecting TRgO better than the TRgO-specific antiserum (Figure. 2-4 compare panel B, with D and E). In contrast, anti-MEgO antibodies were highly specific for MEgO (Figure. 2-4C). The specificity of the anti-MEgO antibodies may be related to the unique position a proline residue in the aligned peptide sequences (Figure. 2-2). The TNgO peptide sequence is less hydrophilic than the others (data not shown). Consistent with this, antibodies raised against TNgO peptides failed to react with any isoform of gO, and other antibodies failed to detect TNgO (data not shown). Thus, strain TN was not included in the comparative analyses of gO in the HCMV envelope described below. However, the TBgO and ADgO antibodies detected the gO isoform expressed by the low-passage strain, PH (data not shown).

Reconstruction of gH/gL/gO complexes by Ad vector expression. Unlike gO, gH/gL is highly conserved among strains of HCMV, differing by <5% of amino

acids among strains, but it is possible that gO diversity reflects coevolution with specific gH/gL isoforms. To test whether or not distinct gO isoforms could bind to a common isoform of gH/gL, Ad vectors were used to express TRgH/gL along with the different gO isoforms. Cells were pulse-chase labeled, and analyzed by IP with anti-gH antibodies followed by treatment with endo H or PNGaseF, and separation by reducing SDS-PAGE (Figure. 2-5). In the absence of gO, gH/gL remained endo H sensitive for at least 3 h, indicative of ER retention. This was consistent with previous results (233). Coexpression of any of the gO isoforms resulted in conversion of the majority of the labeled gH/gL to an endo H resistant form, indicating transit from the ER to the trans-Golgi network (TGN). It was also of interest to determine whether gO isoforms remain bound to gH/gL during transit to the TGN. Previous analyses of extracellular AD virions demonstrated that some of the N-linked glycans on virion-associated gO remain (partially) endo H sensitive (i.e., high mannose content) despite transit through the TGN during virion morphogenesis (233, 255). This would result in comigration of post-Golgi gO with ER-associated gH, precluding direct observation of gO glycoforms in the experiment described in Figure. 2-5. Thus, a modified version of the experiment was performed in which pulse-chase labeled proteins were IP'ed with anti-gH antibodies, and heat denatured in the presence of SDS, and reducing agents. The SDS, and reducing agents in the samples were then diluted, and gO isoforms were analyzed by IP using anti-HA antibodies, treatment with endo H or PNGaseF, and separation by reducing SDS-PAGE (Figure. 2-6).

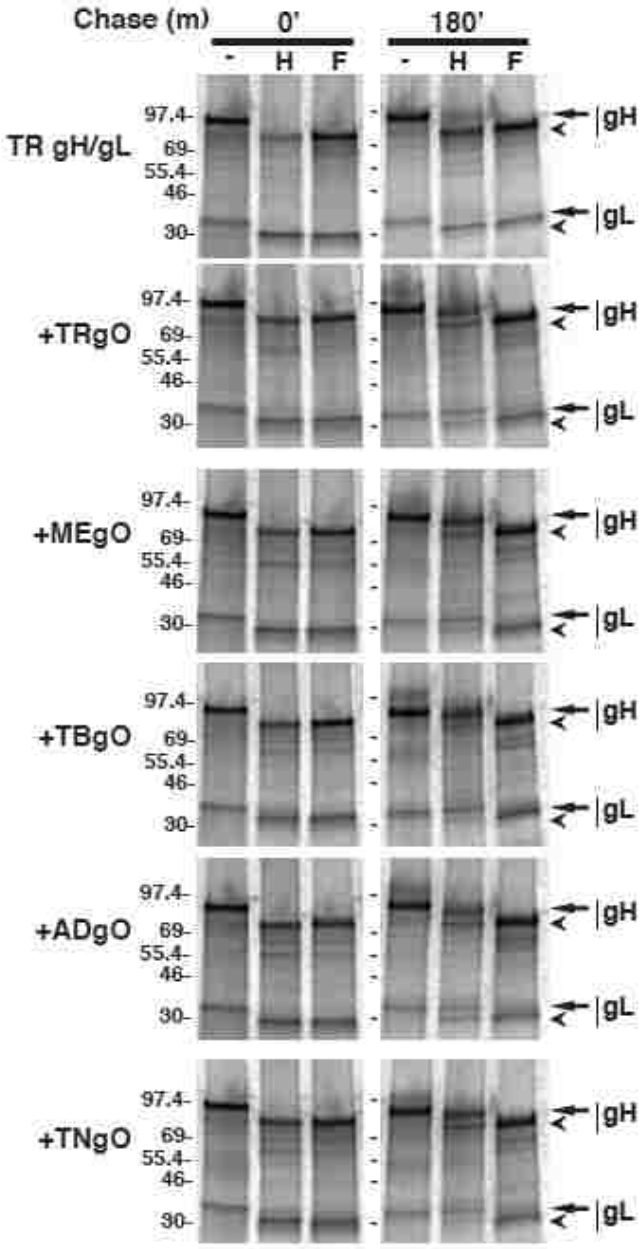

Figure 2-5 Effects of distinct gO isoforms on the intracellular trafficking of a common isoform of gH/gL.

U373 cells were transduced with Ad vectors expressing gH/gL (derived from TR) alone, or together with Ad vectors expressing gO isoforms derived from TR (TRgO), Merlin (MEgO), TB40 (TBgO), AD169 (ADgO), and Towne (TNgO). Cells were pulse-labeled with [35S] methionine/ cysteine, and then the label was chased for 0 or 180 min. Proteins were immunoprecipitated with anti-gH MAb 14-4b, left untreated (-), or treated with endo H (H) or PNGaseF (F), and separated by SDS-PAGE under reducing conditions. Arrows mark untreated, or resistant forms of gH and gL. Arrowheads mark deglycosylated forms of gH and gL.

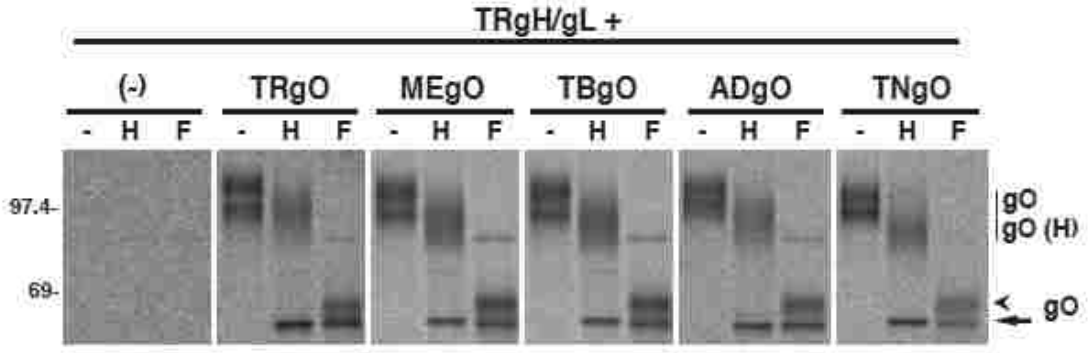

Figure 2-6 Detection of Golgi-associated glycoforms of gO isoforms during coexpression with gH/gL.
U373 cells were transduced with Ad vectors expressing gH/gL (derived from HCMV TR) alone, or together with Ad vectors expressing gO isoforms derived from TR (TRgO), Merlin (MEgO), TB40 (TBgO), AD169 (ADgO), and Towne (TNgO). Cells were labeled for 5 min with [35S] methionine/ cysteine, and then the label was chased for 180 min. Proteins were immunoprecipitated with anti-gH MAb 14-4b, denatured with SDS, and reducing agents, and then gO isoforms were immunoprecipitated with anti-HA antibodies, treated with endo H or PNGaseF as indicated, and analyzed by SDS-PAGE under reducing conditions. Diffuse bands corresponding to glycosylated gO, and endo H resistant gO glycoforms (gO(H)) are indicated with vertical lines. Forms of gO deglycosylated by endo H, or PNGaseF are indicted by the arrow, and the arrowhead.

In the absence of endo H, or PNGaseF treatment, each gO isoform resolved into two bands migrating at approximately 100~120 kDa. After endo H treatment, these bands were replaced with a diffuse band of approx. 80-90 kDa that likely represented gO glycoforms bearing mostly endo H resistant N-linked glycans (i.e., Golgi-associated), and a tighter band of approx. 55 kDa that likely represented glycoforms bearing exclusively endo H sensitive glycans (i.e., ER-associated). PNGase F treatment yielded a similar 55 kDa band, as well as a slightly more slowy migrating, more diffuse band, and a faint band at approximately 75-80 kDa. The presence of the latter band was inconsistent in these experiments, and given its

apparently molecular weight, it likely represented residual gH–gO interactions recaptured in the second IP reaction. The 55 kDa band produced by PNGase F likely represented the same fraction of gO that resolved at 55 kDa following endo H treatment (i.e. ER-associated), whereas the slower migrating species produced by PNGase F likely corresponded to the fraction of gO that was endo H resistant (Golgi-associated). This suggests the acquisition of modifications, in addition to N-linked oligosaccharides, such as O-linked glycans (76). Also, extracts from cells coexpressing gH/gL and any of the gO isoforms contained a proteins species that migrated at approximately 240-260 kDa under non-reducing conditions that were detected by both anti-gL antibodies (Figure. 2-7A), anti-HA (gO) antibodies (Figure. 2-7B) and anti-gH antibodies (data not shown). Together, these data strongly suggest that the assembly of all gH/gL/gO complexes involves disulfide interactions, and the conserved sequences of gO. However, it is important to note that these Ad vector expression experiments were not well suited to compare how efficiently each gO isoform interacts with gH/gL (i.e., binding affinity).

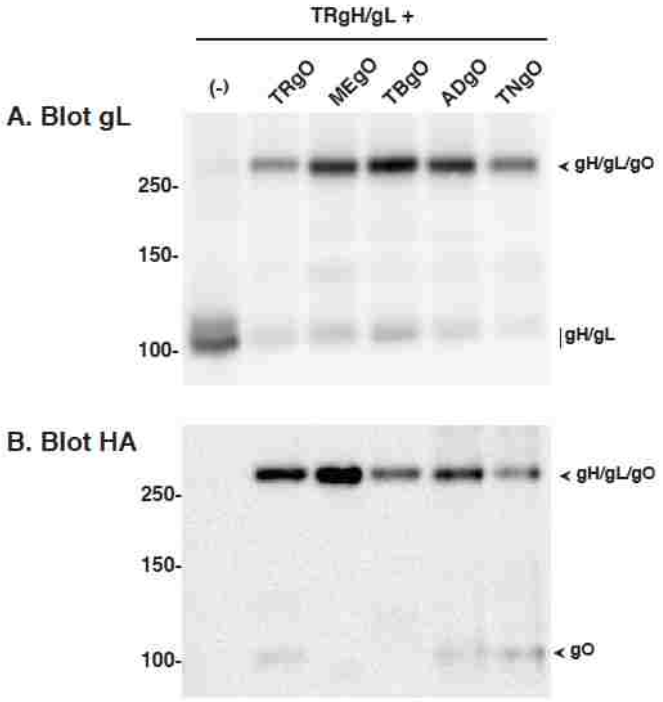

Figure 2-7 Analysis of disulfide bonds in the interactions between gH/gL and gO isoforms.
U373 cells were transduced with Ad vectors expressing gH/gL (derived from TR) alone, or together with Ad vectors expressing gO isoforms derived from TR (TRgO), Merlin (MEgO), TB40 (TBgO), AD169 (ADgO), and Towne (TNgO). Cell extracts were separated under non-reducing conditions and, analyzed by western blot using anti-gL (A), or HA (B) antibodies. The predicted migration of gH/gL/gO trimers, gH/gL dimers, and gO monomers are indicated to the right of both panels.

HCMV strains differ in the ratio of gH/gL/gO, and gH/gL/UL128-131 in the virion envelope. The results of the Ad vector expression experiments described above indicated that all isoforms of gO can form a disulfide-linked complex with gH/gL. Previous comparative analysis of gO from strains TR and AD did not address disulfide interactions (233). Thus, using the new gO antibodies described in Figure. 2-4, we analyzed cell-free TR, ME, TB, AD and PH virions, and infected cells by reducing, and non-reducing western blot (Figure. 2-8). Under reducing conditions,

and probing for gO, two bands were detected in TR-infected cell extracts, a prominent 100 kDa band, and a fainter, more diffuse band of approximately 120 kDa (Figure. 2-8A). These bands likely corresponded to different glycoforms of gO reflecting maturation of N-linked glycans, and other modifications acquired during transit though the Golgi-derived, virion assembly compartment. Consistent with the previous report, gO was not detected in extracts of extracellular TR virions under reducing conditions (Figure. 2-8A) (233). However, when analyzed under non-reducing conditions, a band was detected in both cell, and virion extracts that migrated slower than the 250 kDa marker, consistent with a gH/gL/gO complex (Figure. 2-8A). This species was also detected with anti-gL (Figure. 2-8B) and anti-gH (not shown) antibodies, confirming its identity as gH/gL/gO. Analysis of 4 other strains of HCMV, each encoding a different gO isoform, yielded similar results (Figure. 2-8A and B). Since these experiments involved detection of gO isoforms using different antibodies, and separate chemiluminescent detections, only the fraction of gO detected as gH/gL/gO versus gO monomer can be compared between strains. Note that on non-reducing gels, a greater fraction of the total gO migrated at the expected monomeric size in ME infected cells than in cells infected with other strains (Figure. 2-8A).

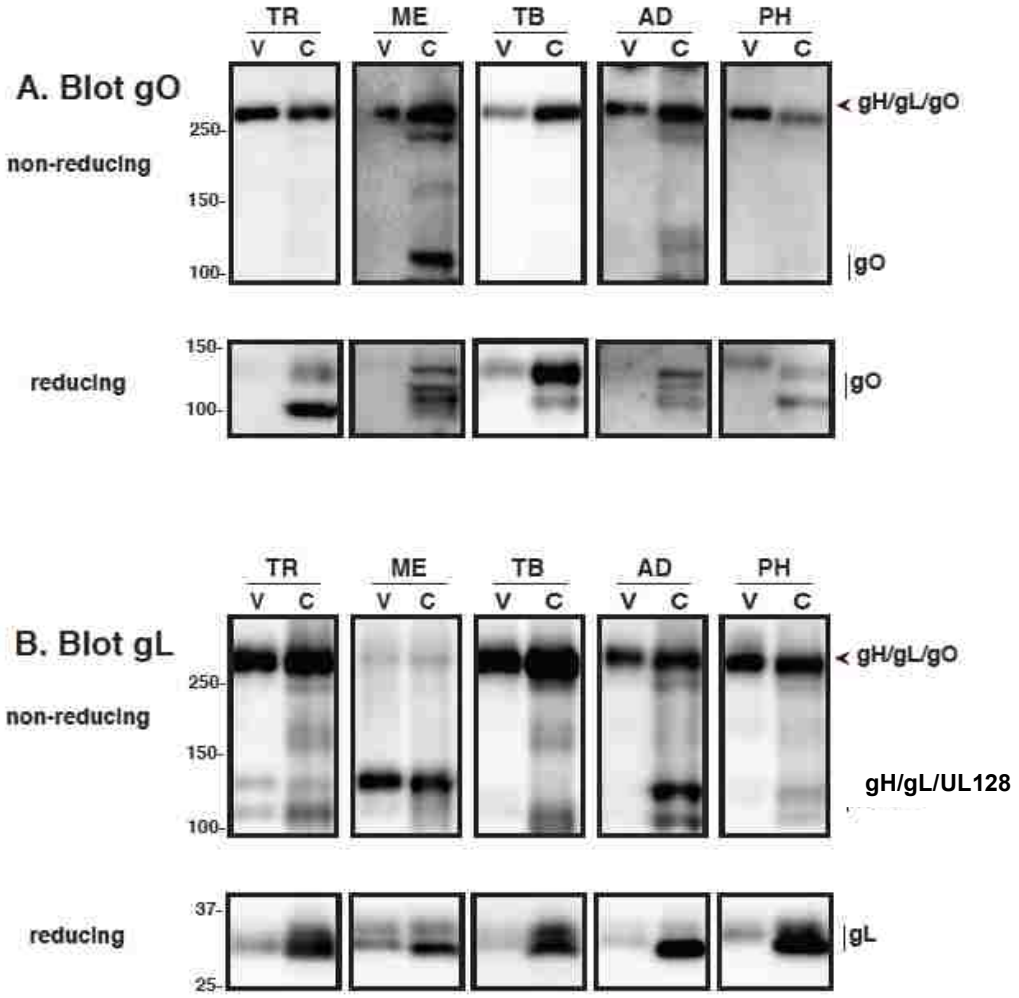

Figure 2-8 Comparative analysis of gH/gL/gO complexes from different strains of HCMV.

Extracts of HFF cells infected with BAC-derived HCMV TR, Merlin, TB40/e, AD169, or PH, or virions collected from the culture supernatant were separated by non-reducing or reducing SDS-PAGE transferred to PVDF membranes and probed with anti-gO (A) or anti-gL (B) antibodies. For gO blots, anti-TBgO antibodies were used for TR, TB and PH samples, anti-MEgO antibodies used for ME samples, and anti-ADgO antibodies were used for AD samples. Arrowheads mark bands corresponding to the disulfide-linked gH/gL/gO trimer, and vertical lines indicate bands that correspond to gO, gL or the disulfide-linked gH/gL/UL128.

The detection of gO in TR virions when bound to gH/gL, but not when liberated from the complex by reduction of disulfide bonds is difficult to explain since the anti-peptide epitopes are generally more accessible following reduction of disulfide bonds. It is notable that similar results were observed for MEgO, ADgO (Figure. 2-8A) and gL (Figure. 2-8B), but not TBgO and PHgO (Figure. 2-8A). While we cannot fully explain this phenomenon, the results of these experiments clearly indicate that the disulfide-linked gH/gL/gO complex is a conserved feature of the HCMV envelope.

Since gO and UL128 bind to gH/gL through disulfide bonds whereas UL130/131 associate with gH/gL through non-covalent interactions, (and non-covalent interactions will be disrupted on SDS-PAGE gel), under non-reducing condition, gH/gL/gO would still stay intact while UL130/UL131 dissociate from the the gH/gL/UL128-131 complex, leaving gH/gL/UL128 behind, which can be distinguished by different molecular weitght from gH/gL/gO. The analyses of virion, and infected cell extracts with anti-gL antibodies revealed a striking difference between ME, and other strains (Figure. 2-8B). For each of the strains TR, TB, AD, and PH, the majority of the gL detected in virions extracts under non-reducing conditions was associated with gH/gL/gO whereas for ME, most of the gL was detected at an apparent molecular weight consistent with gH/gL/UL128 heterodimers; approx. 130 kDa (Figure. 2-8B, top row). Similar results were obtained using anti-gH antibodies (not shown). In this context, these results suggest that TR, TB, AD and PH virions contain vastly more gH/gL/gO compared to gH/gL/UL128-131, whereas ME virions contain more gH/gL/UL128-131.

The ratio of gH/gL/gO and gH/gL/UL128-131 in the HCMV virion envelope is influenced by competition between gO, and the set of UL128-131 proteins for binding to gH/gL. To confirm that the apparent gH/gL/UL128 detected in the ME virion envelope was used to be gH/gL/UL128-131 complex, and to test the hypotheses that the ratio of gH/gL complexes in the virion envelope is influenced by competition between gO and UL128-131 proteins, the tetracycline (Tet) expression-repression system described by Stanton *et al.* was used (177). Briefly, ME is highly sensitive to mutational inactivation of the UL128-131 genes during serial propagation in cultured fibroblasts, making it difficult to maintain wild-type ME in culture (175, 176). To overcome this, Stanton *et al.* engineered a ME BAC with Tet operator sequences inserted within the promoter that regulates expression of UL130, and UL131, and a fibroblast cell line expressing the Tet repressor protein (HFFtet). During replication in HFFtet cells, transcription from the UL130/131 promoter, and consequently, assembly of the gH/gL/UL128-131 complex is suppressed, reducing selective pressure against UL128-131.

Extracts of ME virions produced in HFF, and HFFtet cells were analyzed by reducing and non-reducing western blots (Figure. 2-9). Reducing blots indicated that virions produced by both cell types contained comparable amounts of total gL (Figure. 2-9A), and gB (not shown). On non-reducing gels, the majority of gL detected in ME virions produced by HFF cells was in the form of gH/gL/UL128, consistent with the results of the previous experiment (compare Figure. 2-8B and 2-9A). Strikingly, in virions produced under conditions of suppressed UL128-131 expression (HFFtet cells), the majority of the gL corresponded to the slower

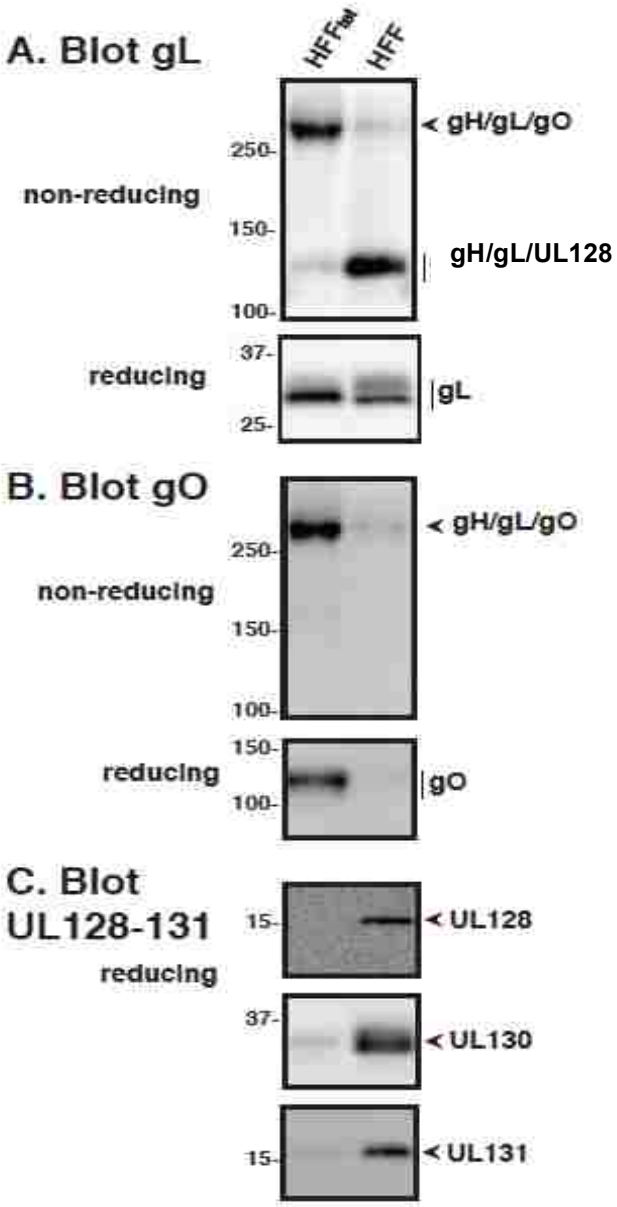

Figure 2-9 Suppression of UL128-131 expression enhances incorporation of gH/gL/gO into the Merlin virion envelope.

BAC-derived Merlin containing Tet operator sequences in the UL130/131 promoter was used to infect HFF cells, or HFF cells expressing the Tet repressor protein (HFFtet). Extracellular virions collected from culture supernatants were analyzed by reducing or and non-reducing western blot using (A) anti-gL, (B) anti-gO, or (C) anti-UL128, anti-UL130, or UL131 antibodies as indicated.

migrating gH/gL/gO complex (Figure. 2-9A). Consistent with this, ME virions produced from HFFtet cells contained more gO (Figure. 2-9B), and less UL128-131 proteins (Figure. 2-9C). Note that due to the increased virion-associated MEgO under conditions of UL128-131 suppression, much shorter chemiluminescent exposures were needed for detection of MEgO in these experiments than for those described in Figure. 2-8A. Since unbound gH/gL dimers are poorly transported out of the ER, and the presence of UL128-131 in the virion exactly correlates with the detection of gH/gL/UL128, these data strongly support the conclusion that the gH/gL/UL128 remainder detected in ME virions represented the gH/gL/UL128-131 complex, and the ratio of the two gH/gL complexes in the virion envelope is influenced by competition between gO and the UL128-131 proteins for binding to gH/gL.

DISCUSSION

Previous analyses suggested that some gO isoforms remain bound to gH/gL in the virion envelope, and others behave more like chaperones to promote assembly of unbound gH/gL dimers into the virion envelope (233). The chaperone model was based largely on the failure to detect gO in TR virions by reducing western blots. Here, we used non-reducing gel systems to compare several strains of HCMV. In all cases, gO clearly was detected as part of a disulfide-linked complex with gH/gL in the virion envelope.

The discrepancy between non-reducing and reducing western blots is puzzling. As noted, anti-peptide antibodies are generally thought to react better with proteins

that have been separated under denaturing-reducing conditions because continuous epitopes should be more accessible. It is notable that virion-associated ADgO was clearly detected in the reducing western blot experiments reported in Ryckman *et al.* (233), but not in the similar experiments reported here. This difference might be explained by the fact that in the previous studies, ADgO was detected with the polyclonal serum described by Huber *et al.* (75), which was raised against full length gO, whereas in the current studies, anti-peptide antibodies were used for detection of all gO isoforms. This observation points towards specific modification of gO, such as proteolysis, at or near the anti-peptide antibody target site (the conserved hydrophilic domain shown in Figure. 2-2). If this were the case, then perhaps the disulfide nature of the gH/gL/gO complex might preserve epitope recognition in western blot experiments. Potential modifications are likely coincident with virion morphogenesis, and release because intracellular gO was easily detected with the anti-peptide antibodies on reducing western blots. Also, since detection of some gO isoforms was less affected by disulfide reduction, sequence differences might affect these potential modifications. It is also important to note that a similar phenomenon was observed during analysis of gL, and it is interesting that the anti-gL antibodies used were also raised against synthetic peptides. In order to efficiently elicit antibodies, synthetic peptides are conjugated to a carrier protein (such as keyhole limpet hemocyanin; KLH) via a terminal cys residue that either represents a cys in the protein of interest, or one that is added to the synthetic peptide for the sole purpose of conjugation to the carrier. Thus, it is tempting to suggest that the presence of a cys in the peptides used to generate the antibodies

might contribute to the observed phenomenon. However, for all antibodies described, the cys used for conjugation was extraneous, and not present in the actual protein. Finally, it is possible that the observed effects relate to differences between cell-associated, and virion-associated proteins in the transfer to blotting membranes, and that these differences are less apparent during transfer of the intact gH/gL/gO complex. Again, this explanation would suggest posttranslational modifications of proteins coincident with virion release. Regardless, the analyses of TR, and other HCMV strains reported here clearly demonstrate that disulfide-linked gH/gL/gO complexes are a conserved feature of the HCMV virion envelope.

Regulation of gB can be considered the "core entry function" of the herpesvirus gH/gL. HCMV gH/gL, without UL128-131 or gO, can regulate gB-mediated cell-cell fusion, indicating that the necessary surfaces for gB interaction are comprised of amino acids contained within gH/gL alone (216). However, data presented here indicate that the HCMV envelope contains a mixture of gH/gL/UL128-131 and gH/gL/gO, and little or no unbound gH/gL. Thus, for HCMV, the core function involving interactions with gB must be provided by either the pentameric gH/gL/UL128-131, or the trimeric gH/gL/gO. EBV also has different types of gH/gL, unmodified gH/gL or a trimeric gH/gL/gp42, and either can perform this function depending on the cell type involved (221, 247). In contrast, the phenotypes of HCMV gO, and UL128-131 mutants strongly suggest that gH/gL/gO performs the core gB-interaction function during entry into all cell types, whereas gH/gL/UL128-131 serves a distinct, receptor-binding function that is an additional necessity for entry into specific cell types such as epithelial, endothelial, and dendritic cells, but

not others, such as fibroblasts or neurons (42, 77, 79, 156, 227, 234, 235, 238, 248, 256). The receptors for gH/gL/UL128-131 are unknown, but engagement of these molecules might trigger endocytosis, actin rearrangement or other cell surface functions that then allow for the universally required gH/gL/gO - gB interactions that result membrane fusion and entry. An important prediction of this model is that the surfaces on gH/gL that interact with gB are inaccessible on gH/gL/UL128-131, but are accessible on gH/gL/gO, and studies to test aspects of this model are in progress.

The significance of gO sequence diversity on the composition of the HCMV envelope, and the function of the gH/gL/gO complex adds a layer of complexity to models of HCMV entry and tropism that has not previously been addressed. In the analyses presented here, all isoforms of gO bound to a common isoform of gH/gL to form a disulfide-linked trimer that was transported from the ER to the TGN. This suggests that gH/gL/gO complexes share a similar structure involving the conserved sequences of gO. This conclusion is consistent with the model of gH/gL/gO providing a core entry function since all gO isoforms must leave critical surfaces on gH/gL available for interaction with gB. These results are also consistent with those of Rasmussen *et al.*, who investigated genetic linkages of polymorphic loci in clinical HCMV isolates, and found evidence of several combinations of gH/gL and gO isoforms (257).

The finding that virions of TR, TB, AD and PH virions contained vastly more gH/gL/gO than gH/gL/UL128-131, whereas ME contain more gH/gL/UL128-131 than gH/gL/gO was unexpected, and has a number of important implications. First,

the observation that UL128-131 suppression during ME replication dramatically shifted the ratio of gH/gL complexes in the virion envelope towards gH/gL/gO indicates that gO, and UL128-131 compete for binding of gH/gL to form the two complexes, and the relative abundance of unbound gO in ME infected cells suggests that MEgO may be less able to compete than other gO isoforms. Thus, the variable sequences of gO might affect the ratio of the core entry factor gH/gL/gO to the tropism factor gH/gL/UL128-131. However, these results do not exclude the possibility that differences in protein expression levels, or small sequence differences in gH/gL, or UL128-131 also contribute to strain variation. Wille *et al.* showed that a TR mutant that could not express gO produced virions that contained elevated amounts of gH/gL/UL128-131, but drastically lower total amounts of gH/gL, indicating that UL128-131 was unable to bind a significant fraction of the remaining gH/gL (238). This might indicate that TR expresses more gH/gL, or less UL128-131 than ME. Second, it is well appreciated that there is a selective pressure against UL128-131 during replication in fibroblast cultures, and that ME is especially sensitive to this selection (175–177). If the assembly of gH/gL/gO and gH/gL/UL128-131 come at the expense of one another, and if the gH/gL/UL128-131 cannot participate in the interactions with gB that are necessary for fusion, then mutation of UL128-131 would be expected to result in a greater fibroblast-replication fitness advantage for a virus like ME than for a virus like TR. This model predicts an evolutionary balancing act requiring the virus to express enough gH/gL/UL128-131 for broad tropism, while retaining enough gH/gL/gO to promote fusion on all cell types. Clearly, the amounts of each complex in the ME virion are

sufficient to allow infection of both fibroblasts, and epithelial/endothelial cells, but the relative efficiency (i.e. per virion) compared to other HCMV strains with different amounts of the complexes is difficult to directly measure. As noted above, it is possible that HCMV strains differ in the total amount of gH/gL, and this could either lessen, or magnify the importance of the ratio of the two complexes. Clearly suppression of UL128-131 enhances the replication of ME, but the effects of manipulation the expression of gO or gH/gL has not been addressed. Finally, It is tempting to speculate that adaptive evolution of HCMV tropism observed in culture may also occur *in vivo*. Infection of neuronal cells is likely independent of gH/gL/UL128-131 (156). Thus, it is conceivable that the ratio of the core entry factor (gH/gL/gO), and the tropism factor (gH/gL/UL128-131) might be subject to similar selective pressures in neuronal or, other tissues as has been observed in fibroblast cultures. If so, the gO genotype of HCMV might influence viral pathology, and the ability of the virus to spread to other hosts, which likely involves replication in epithelial and endothelial tissues.

Host cell characteristics may also influence the protein composition of the HCMV envelope. Wang *et al.* found that HCMV produced by epithelial cells entered epithelial cells though fusion at the plasma membrane rather than via fusion following endocytosis, as had been described for fibroblast-produced virus. They suggested that this correlated with an approximately 2-fold increase in the ratio of gH/gL/UL128-131 vs. gH/gL/gO in epithelial cell-produced virions (258). Scrivano *et al.* similarly analyzed HCMV virions produced by fibroblasts with those produced by endothelial cells, and suggested that both cell types produce virion populations

that are heterogeneous in the relative levels of gH/gL/gO and gH/gL/UL128-131 (259). The notion of heterogeneity in the amounts of gH/gL complexes incorporated into the HCMV envelope had been previously suggested by Li *et al.* (260). Our results linking gO diversity with the ratio of the two gH/gL complexes raises the question of whether all strains display similar envelope heterogeneity, or are equally sensitive to producer cell.

In summary, the data presented in this report clearly indicate that the gH/gL/gO complex is an envelope component of all strains of HCMV, and suggest that the sequence differences between gO isoforms may affect the ratio of gH/gL complexes incorporated in the virion envelope. The diverse sequences of gO may also affect other functions, and aspects of the gH/gL/gO complex. For example, Vanarsdall *et al.* provided evidence from receptor interference experiments that suggested gH/gL/gO might act as a receptor-binding complex, and the gO isoform might participate in these interactions or modulate the activity (239). Also, Jiang *et al*. recently suggested that the extensive array of N-linked glycans on gO might act to shield the virus from the effects of neutralizing antibodies (261). Research work in Chapter 4 studied the possible significances of gO diversity by analyzing inter-strain of gO recombinant HCMVs.

CHAPTER 3. CHARACTERIZATION OF HUMAN CYTOMEGALOVIRUS gH/gL COMPLEXES FUNCTION DURING VIRUS ENTRY AND TROPISM

This chapter is a modified version of the manuscript published in Journal of Virology in September 2015; 89(17), 8999-9009

INTRODUCTION

Primary infection of healthy adults by human cytomegalovirus (HCMV) is usually subclinical or mildly symptomatic, but leads to lifelong persistent or latent infection. Primary infection or reactivation of HCMV in immunocompromised hosts, such those infected with HIV and transplant recipients on anti-rejection chemotherapies, is associated with significant morbidity and mortality, and maternal transmission of HCMV to the developing fetus across the placenta can result in severe congenital birth defects (16, 21, 240). The diverse nature of HCMV-associated disease likely relates to the ability of the virus to infect many cell types *in vivo*, including epithelial and endothelial cells, fibroblasts, monocyte-macrophages, dendritic cells, hepatocytes, leukocytes and neurons (147, 148, 156). Much research effort has been focused on understanding the mechanisms by which HCMV initiates infection of different cell types (reviewed in (190)). Most studies have described an entry mechanism dichotomy between fibroblasts and epithelial/endothelial cells. Other relevant cell types, though more difficult to work with in the laboratory, likely fit to one side or the other of the fibroblasts – epithelial/endothelial entry dichotomy.

Entry of all herpesviruses requires membrane fusion, which is mediated by a conserved core fusion apparatus comprised of glycoprotein B (gB) and gH/gL. Extensive studies of herpes simplex virus (HSV) and Epstein-Barr virus (EBV) have shown that gB has characteristics of a class III viral fusion protein (209, 210, 213, 214). The necessary role of gH/gL in herpesvirus membrane fusion is unclear, but likely involves direct interaction between gH/gL and gB (218, 223, 262–265).

Herpesviruses also encode accessory glycoproteins such as HSV gD and EBV gp42, which regulate the core fusion apparatus through receptor-binding, and stable or transient interactions with gH/gL (reviewed in (189)).

HCMV shares the conserved features of herpesviruses entry, but there are some important unique aspects, especially related to cell tropism. Structural analyses of HCMV gB suggest general mechanistic similarities with other herpesvirus gB homologues (122) and the combination of HCMV gB and gH/gL is necessary, and sufficient for membrane fusion in cell-cell fusion experiments (216). The ectodomain of HCMV gH/gL can be bound by either gO, or proteins encoded by UL128, UL130 and UL131 (UL128-131), forming the gH/gL/gO, and gH/gL/UL128-131 complexes on the virion envelope (73, 74, 77, 227). Experiments reported in Chapter 2 indicated the following: 1) extracellular virions contain little, if any unbound gH/gL, 2) the ratio of gH/gL/gO to gH/gL/UL128-131 can vary dramatically between different HCMV strains, and 3) gO and UL128-131 compete for binding of gH/gL (232). The mutually exclusive nature of gH/gL/gO and gH/gL/UL128-131 formation is consistent with a recent structural analysis showing that gO, and the UL128 protein make a disulfide bond with the same cysteine residue of the gL polypeptide (215). The gH/gL/gO and gH/gL/UL128-131 complexes influence cell tropism at the level of entry, although the mechanisms are not well understood.

The roles of HCMV gH/gL complexes have mostly been studied with deletion mutant approaches. In general, mutants that lack gH/gL/UL128-131 replicate as well, or better than wild type strains on fibroblasts (77–79, 227). This indicates that

gB-mediated fusion with fibroblast membranes can be facilitated by gH/gL/gO. In contrast gH/gL/UL128-131 mutants fail to infect cell types such as epithelial, endothelial and monocyte/macrophages (77, 79, 227, 248, 266). These observations have generally been interpreted to mean that gB-mediated fusion with membranes of epithelial cells (and other cell types for which gH/gL/UL128-131 is necessary for HCMV entry) is facilitated by gH/gL/UL128-131. The resulting model suggests that both gH/gL/gO, and gH/gL/UL128-131 provide essentially the same function for entry into different cell types, i.e., promoting gB-mediated membrane fusion. This model was attractive due to analogy with the well-described model of EBV tropism (reviewed in (189)). EBV gH/gL can exist in the virion envelope as unmodified gH/gL, or bound by gp42. For entry into epithelial cells, gH/gL binds specific integrins, and promotes gB-mediated membrane fusion. The presence of gp42 on gH/gL blocks integrin binding, and instead gp42 binds MHC II, which facilitates entry into B-cell. Presumably, both gH/gL and gH/gL/gp42 promote gB-mediated membrane fusion through interactions involving "pro-fusion" surfaces of gH/gL, with gp42 acting as a receptor-adapter to switch cell tropism. While HCMV mutants lacking gH/gL/UL128-131 replicate well on fibroblasts, mutants lacking gH/gL/gO are severely replication impaired on all cell types (42, 234, 235, 238). In particular, Wille *et al.* showed that HCMV virions lacking gH/gL/gO were impaired at the fusion step of entry into fibroblasts, epithelial, and endothelial cells, despite containing elevated amounts of gH/gL/UL128-131 (238). Together, the results of deletion mutant approaches seem to suggest that gH/gL/gO is sufficient for entry into fibroblast, but that entry into epithelial/endothelial cells requires both gH/gL/gO

and, gH/gL/UL128-131.

Characterization of the mechanisms by which HCMV gH/gL/gO and gH/gL/UL128-131 facilitate cell type dependent entry using deletion mutants has been limited largely due to the severe replication defects associated with gO deletions, and the lack of an adequate complementation system. The studies described herein, capitalize on our previously reported observations that the amounts of gH/gL complexes in the HCMV virion envelope can vary dramatically among strains, and that the ratio of the two gH/gL complexes can be manipulated through suppression of the UL128-131 proteins. Here, we report further characterizations of strain variability in the abundance of gH/gL complexes in the virion envelope, and relate these differences to the efficiency of entry on different cell types. Comparisons of genetically identical HCMV preparations either enriched in gH/gL/gO, or gH/gL/UL128-131 suggest a model in which gH/gL/gO provides the herpesvirus "core-fusion gH/gL" function of promoting gB-mediated membrane fusion for entry into all cell types, whereas gH/gL/UL128-131 facilitates entry into select cell types, such as epithelial cells, through a distinct, yet necessary mechanism.

MATERIALS AND METHODS

Cell lines. Primary human foreskin fibroblasts (HFF; Life Technologies), MRC-5 fibroblasts (ATCC CCL-171), and HFFtet cells (which express the tetracycline (Tet) repressor protein (177); provided by Richard Stanton (Cardiff University, Cardiff, United Kingdom)) were grown in Dulbecco's modified Eagle's medium

(DMEM; Life Technologies) supplemented with 6% heat-inactivated fetal bovine serum (FBS; Rocky Mountain Biologicals, Inc., Missoula, Montana, USA.) and 6% bovine growth serum (BGS; Rocky Mountain Biologicals, Inc., Missoula, Montana, USA.). Retinal pigment epithelial cell line, ARPE-19 (American Type Culture Collection; Manassas, VA, USA) was grown in a 1:1 dilution mix of DMEM and Ham's F-12 medium (DMEM:F-12; Life Technologies) supplemented with 10% FBS.

HCMV. All HCMV were derived from bacterial artificial chromosome (BAC) clones. BAC clone TR was provided by Jay Nelson (Oregon Health and Sciences University, Portland OR, USA) (41). BAC clone TB40/e (BAC-4) (TB) was provided by Christian Sinzger (University of Ulm, Germany) (184). BAC clone Merlin (ME) (pAL1393), which contains tetracycline operator sequences within the transcriptional promoter of UL130 and UL131, was provided by Richard Stanton (Cardiff University, Cardiff, United Kingdom) (177). Infectious HCMV was recovered by electroporation of BAC DNA into MRC-5 fibroblasts as described by Wille *et al.* (238). Cell-free HCMV stocks were produced by infecting HFF or HFFtet at 2 plaque-forming unit (PFU) per cell. At 8-10 days post infection (when cells were still visually intact), culture supernatants were harvested, and cellular contaminants were removed by centrifugation at 1,000 X g for 10min, and again at 6,000 X g for 10min. Stocks were judged cell-free by the lack of calnexin, and actin in western blot analyses, stored at -80°C. The number of PFU was determined by plaque assay on triplicate HFF or ARPE-19 cultures. Freeze/thaw cycles were avoided.

Antibodies. Monoclonal antibodies (MAbs) specific for HCMV major capsid protein (MCP) 28-4 and gB 27-156 were provided by Bill Britt (University of

Alabama, Birmingham, AL, USA) (45, 267). Anti-UL128 MAb 4B10 was provided by Tom Shenk (Princeton University, Princeton, NJ, USA) (77). Rabbit polyclonal antipeptide antibodies directed against HCMV gH/gL, UL130, and UL131 were provided by David Johnson (Oregon Health and Sciences University, Portland, OR, USA) (80). Rabbit polyclonal antipeptide antibodies directed against MEgO were described in (232).

Western blot. Cell-free virions from culture supernatants (as described above) were concentrated by centrifugation at 50,000 X g for 1 h, and resuspended in 2% SDS in 20mM tris-buffered saline (TBS; pH 6.8). Insoluble material was removed by centrifugation at 16,000 X g for 30min, cleared extracts were heated to 95 °C for 10min. For reducing blots, extracts were adjusted to 2% DTT. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Whatman) in a buffer containing 10mM $NaHCO_3$ and 3mM Na_2CO_3 (PH9.9), plus 10% methanol. Transferred proteins were probed with MAbs or rabbit polyclonal antibodies specific for HCMV proteins, followed by horseradish peroxidase-conjugated secondary antibodies; chemi luminescence was detected using a Bio-Rad ChemiDoc MP imaging system. Band densities were quantified using Bio-Rad Image lab v 5.1 or ImageJ v 1.48 software.

Quantitative PCR (qPCR). 200 µl cell-free HCMV stock was first treated with DNase I to remove DNA not protected within viral capsids. Capsids were disrupted by using the viral lysis buffer, and proteinase K provided in the PureLink Viral RNA/DNA mini kit (Life Technologies). Viral DNA was eluted in 50µl RNAse-free water. A conserved region within UL83 among ME, TR and TB was chosen as

amplicon, and genomes were quantified by real-time qPCR using SYBR Green dye (Bio-Rad): forward primer, TGGTCACCTATCACCTGCAT; reverse primer, GAAAGAGCCCGACGTCTACT. PCR samples were made to a total well volume of 20µl with 1X SYBR Green master mix (Bio-Rad), 1:100 dilution of viral DNA and 500nM forward and reverse primers. PCR products were detected using MyiQ real-time PCR detection system (Bio-Rad). QPCR results were compared to a plasmid pTRUL83 standard and then calculated into genome/ml. Efficiency of DNase I treatment were tested in independent experiments by spiking plasmid DNA. For each experiment, two independent viral DNA extractions and three independent QPCR reactions were performed.

Determination of plaque forming units (PFU). Cell monolayers were infected with cell free HCMV, or HCMV infected cells (infectious centers), and cell culture medium was replaced with a 1:1 mixture of 2XDMEM and 1.2% SeaPlaque agarose to prevent spread of progeny virus by diffusion. Plaques were counted by light microscopy 3 weeks later.

Antibody neutralization of HCMV. 1:100 diluted (vol/vol) anti-UL130 and anti-UL131 rabbit serum were added to culture medium containing cell-free HCMV. The mixture was incubated for at room temperature for 1 h, and then added to HFF or ARPE-19 cultures.

Polyethylene glycol (PEG) treatment. Cell-free HCMV was adsorbed to cells for 2 h at 10°C, under 800 X g centrifugation or at 37°C without centrifugation. Unbound virus was removed by two cold PBS washes, and cells were subsequently treated with 44% PEG (pre-warmed to 37°C) for 30s followed by 10 washes with

warm culture medium to remove PEG. Cultures were incubated 37°C until immunofluorescence analysis was performed. PEG 6000 (Fluka) was prepared as a 60% (wt/wt) solution in PBS, and diluted with PBS to 44%.

Immunofluorescence. HCMV infected cells were fixed with 2% formaldehyde in PBS for 15 min followed by three PBS washes. Fixed cells were incubated, for 1hr in immunofluorescence (IF) buffer (0.5% Triton X-100, 0.5% deoxycholate, 1% bovine serum albumin [BSA], 0.05% sodium azide in PBS). To visualize HCMV infected cells, anti-IE1/IE2 MAb P63-27 was used at 1:50 in IF buffer, and Alexa 488-conjugated goat-anti-mouse antibodies 1:1000 diluted in IF buffer. 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) was used to visualize total cells.

RESULTS

HCMV strains vary in both the ratio of gH/gL/gO to gH/gL/UL128-131, and the total amount of gH/gL complexes. In Chapter 2, gH/gL complexes of different HCMV strains were analyzed by non-reducing western blot. Since the main goal of those experiments was to compare the ratio of gH/gL/gO to gH/gL/UL128-131 among strains, gels loads were adjusted to equalize the total amount of gH/gL. TR and TB40/e (TB) virions were found to contain gH/gL mostly in the form of gH/gL/gO, whereas Merlin (ME) had mostly gH/gL/UL128-131 (232). Those experiments made use of recombinant ME in which the assembly of gH/gL/UL128-131 could be reduced by the tetracycline-repressor protein (TetR), which resulted in ME virions containing mostly gH/gL/gO (denoted Merlin-trimer; ME-T) (177,

232). To further characterize the amounts of gH/gL complexes among HCMV strains, extracellular virions of TB, TR, ME and ME-T (ME virions produced in TetR

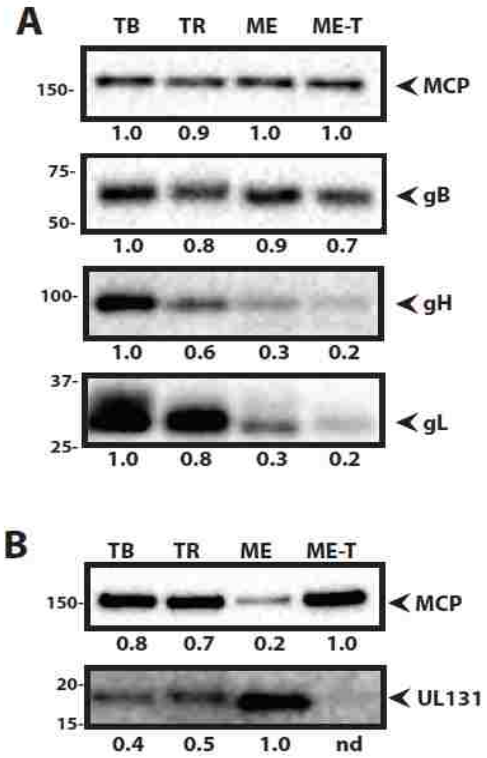

Figure 3-1 Comparison of glycoprotein concentrations in the virion envelope of different HCMV strains.

Cell-free virions of HCMV TB40/e (TB), TR, Merlin (ME) or Merlin-Trimer (ME-T) were separated by reducing SDS-PAGE, and analyzed by western blot with antibodies specific for major capsid protein (MCP), the 55-kDa portion of gB, gH, gL, or UL131. Mass markers (kDa) are shown on the left of each panel. Gel loads were normalized to equal MCP (A), or to 5-fold less MCP for ME (B). Numbers below blots indicate band density analysis, normalized to the most intense band on the same blot. nd: not detected.

expressing cells) were analyzed by western blot under reducing conditions, and gel loads were normalized to major capsid protein to allow for comparison of the amounts of the different glycoproteins per virion (Figure. 3-1). All strains contained comparable amounts of gB, but the amounts of gH/gL varied dramatically. TB contained the most total gH/gL, followed by TR, ME and ME-T virions contained the least total gH/gL (Figure. 3-1A). Despite the low amount of gH/gL, ME virions contained dramatically more UL131 (a marker of the gH/gL/UL128-131 complex) (Figure. 3-1B). Note that in Figure. 3-1B, gel loads were adjusted to allow visualization of the lower amounts of UL131 in TB and TR virions, suggesting that the difference in the level of UL131 between ME and the other viruses was greater then 10-fold. Also note that UL128, UL130 and gO were not directly analyzed in these experiments because amino acid polymorphisms between strains affect cross reactivity of available antibodies (232).

HCMV gH/gL complexes were also analyzed by non-reducing western blot (loads normalized to MCP) (Figure. 3-2). Consistent with analyses reported in Chapter 2, the vast majority of gH/gL in TB, TR and ME-T virions migrated at a size indicative of disulfide-linked gH/gL/gO, whereas most of the gH/gL in ME virions was at the size of disulfide-linked gH/gL/UL128 (Figure. 3-2). Note that UL130 and UL131 are not disulfide-linked to gH/gL/UL128-131, and are thus removed by SDS-PAGE (77, 80). Unbound, or free gH/gL comprised a very small fraction of the total gH/gL in all strains. Whether this represents spontaneous reduction of disulfide bonds in the extract samples, or is indicative of small amounts of free gH/gL in virions is not clear. Band density analyses of the three gH/gL species on non-

reducing blots corresponded well with the quantitation of total gH and gL on reducing blots (compare Figure. 3-1A and 3-2).

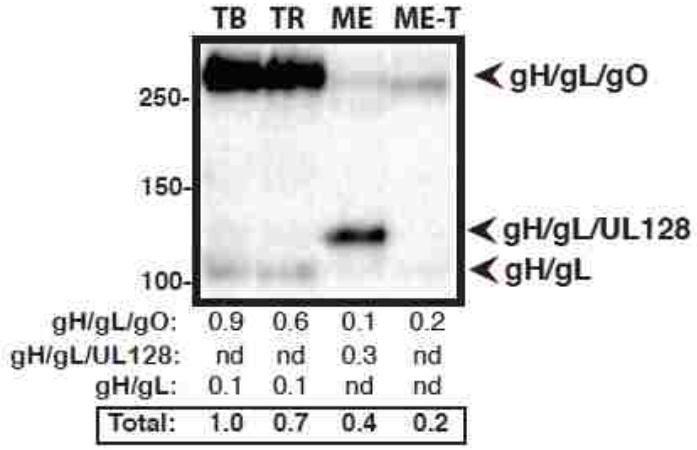

Figure 3-2 Comparison of gH/gL complexes in the virion envelope of different HCMV strains.
Cell-free virions of HCMV TB40/e (TB), TR, Merlin (ME) or Merlin-Trimer (ME-T) were separated by non-reducing SDS-PAGE, and analyzed by western blot with antibodies specific for gL. Mass markers (kDa) are shown on the left. Gel loads were normalized to equal MCP as in Figure. 3-1A (not shown). Numbers below blot indicate analysis of band density for each species of gH/gL. nd: not detected.

The low amounts of gH/gL complexes in ME and ME-T virions made quantitation in these viruses difficult to perform on the same blots as TB and TR. Thus, ME and ME-T virions were also analyzed on separate non-reducing gels with increased gel loads to allow detection in a quantifiable range (Figure. 3-3). Furthermore, since ME and ME-T are genetically identical viruses, these virions could be directly compared by western blots using anti-MEgO and anti-UL128 antibodies (232). As in the previous analyses, ME virions contained more total gH/gL than ME-T virions, although at these increased loads, the difference was less pronounced. This likely reflected the limitations of band density quantitation on the

lower end of the spectrum in Figures. 3-1 and 3-2. Comparison of the amount of gH/gL/gO and gH/gL/UL128-131 in ME and ME-T virions by probing blots for either the common antigen (gL), or the unique antigen, (gO or UL128) confirmed that ME-T virions contained more gH/gL/gO than ME virions, whereas ME virions had more gH/gL/UL128-131 than ME-T virions (compare analogous bands in Figure. 3-3A, B, and C). Together, these analyses indicated that strains of HCMV vary widely in both the total amount of gH/gL complexes in the virion envelope, and also vary in whether that gH/gL is in the form of gH/gL/gO or gH/gL/UL128-131.

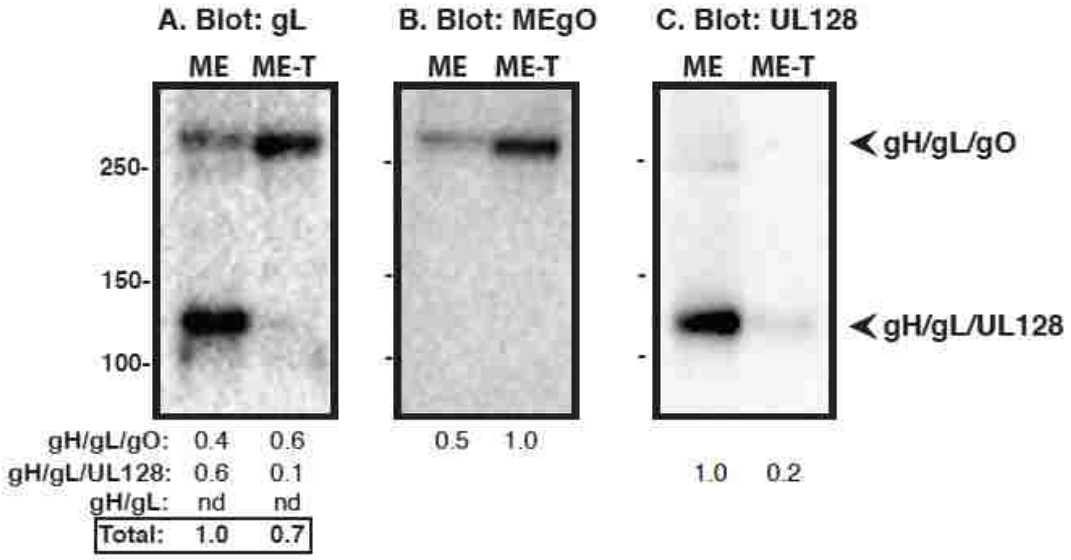

Figure 3-3 Comparison of gH/gL complexes in the virion envelope of Merlin (ME) or Merlin-Trimer (ME-T).

Cell-free virions were separated by non-reducing SDS-PAGE, and analyzed by western blot with antibodies specific for (A) gL, (B), MEgO, or (C) UL128. Mass markers (kDa) are shown on the far left. Gel loads were normalized to equal MCP (not shown). Numbers below blots indicate analysis of band density analysis, normalized to the more intense band within the same blot. nd: not detected.

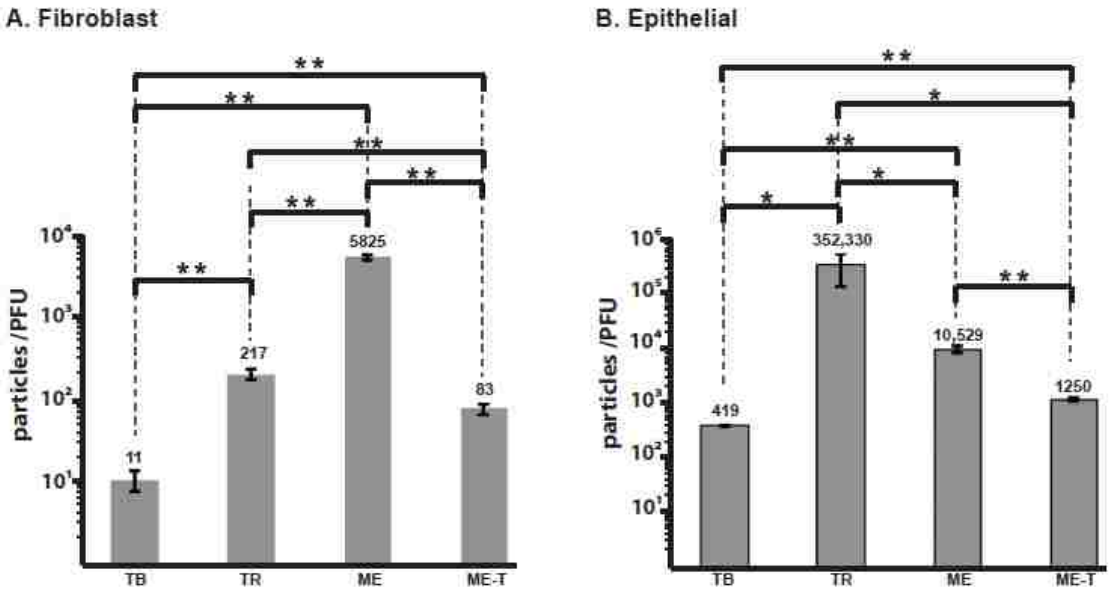

Figure 3-4 Particle-to-PFU analysis of HCMV.
Cell free stocks of TB40/e (TB), TR, Merlin (ME), or Merlin-trimer (ME-T) virions were analyzed by qPCR to quantitate genome-containing virions, and plaque-forming units (PFU) were determined on fibroblast (A) or epithelial (B) cell cultures. Specific infectivity was calculated as the ratio of average genomes per mL to average PFU per mL. Averages and standard deviations of three experiments are plotted. Brackets above columns indicate results of 2-tailed, unpaired Student's T-test. P values: * = 0.04 - 0.05; ** < 0.04.

Particle-to-PFU analysis of strains of HCMV that contain different amounts of gH/gL complexes. One model of HCMV tropism suggests that gH/gL/gO and gH/gL/UL128-131 serve mechanistically analogous roles for entry into fibroblasts and epithelial cells, respectively. However, that model is inconsistent with observations that a gO-null mutant TR was impaired for entry into fibroblasts and epithelial cells (238). To help resolve this issue, we reasoned that specific infectivity (particle-to-PFU ratio) of HCMV on both fibroblasts and epithelial cells should be influenced by the amounts of the gH/gL complexes in the virion envelope. Thus, cell free virions of TB, TR, ME, and ME-T were quantitated by real-time quantitative PCR (qPCR) for viral genomes, titered by plaque assay on both fibroblasts and epithelial cells and the specific infectivity was calculated (Figure. 3-4).

Particle-to-PFU measurements on fibroblasts varied widely between strains, and were statistically significant in all comparisons (Figure. 3-4A). The extremes were TB and ME at 11, and 5825 particles-per-PFU respectively, which correlated with the respectively high and low amounts of gH/gL/gO detected in these strains (Figure. 3-2). Values for TR and ME-T were intermediate (217 and 83 respectively), which generally correlated with their intermediate amounts of gH/gL/gO relative to TB and ME. Notably however, TR contained considerably more gH/gL/gO than ME-T, but was 2.6-fold less infectious than ME-T. It is possible that this relatively modest difference might indicate that the level of gH/gL/gO in ME-T represents a minimum threshold. Additionally, this might reflect other genetic differences between the strains. Thus, the comparison of ME with ME-T is highly informative

since these are genetically identical viruses that likely differ mostly, if not exclusively, in the amounts of gH/gL/gO and gH/gL/UL128-131.

Particle-to-PFU ratios were generally higher on epithelial cells, consistent with previous observations that these cells are more difficult to infect with HCMV than are fibroblasts, perhaps reflecting the more complicated route of entry which involves internalization followed by fusion from within endosomes (79). TB was the most infectious on epithelial cells (419 particles/PFU), followed by ME-T (1250 particles/PFU), ME (10,529 particles/PFU), and TR (325,330 particles/PFU) (Figure. 3-4B). All comparisons were statistically significant. Given that ME virions contained dramatically more gH/gL/UL128-131 than the other viruses, the observation that ME was nearly 10-fold less infectious than ME-T, and 25-fold less infectious than TB on epithelial cells was inconsistent with the model that gH/gL/UL128-131 performs the conserved herpesvirus gH/gL function of promoting gB mediated fusion on these cells. Rather, the comparatively poor infectivity of ME on both fibroblast and epithelial cells is more consistent with the hypothesis that gH/gL/gO provides the core fusion function for entry into all cell types, and that gH/gL/UL128-131 provides a mechanistically distinct, additional function necessary for entry into epithelial cells. However, the particle-to-PFU ratio of TR on epithelial cells was log-folds higher than any of the other viruses, despite being relatively rich in gH/gL/gO. This discrepancy was likely due in part to the fact that plaque formation is not only a function of initial entry of cell free virions, but also of replication and spread.

To address post-entry differences in plaque formation between these viruses (i.e., replication and spread), an infectious center experiment was performed. Fibroblast or epithelial cells were infected with TB, TR, or ME, and at 2 dpi. cells were detached, and re-plated onto monolayers of the homologous cell type, and resultant plaques were counted (Figure. 3-5). Note that since ME-T virions were produced by propagating ME in cells expressing TetR to repress the transcription from the UL128 locus (177, 232), they are genetically identical to ME virions. Thus, there was no logical distinction to be made between ME and ME-T in these infectious center experiments. On fibroblasts, TB, TR, and ME were all comparably efficient at forming plaques from infectious centers (40-60 plaques per 100 infectious centers seeded) (Figure. 3-5A). On epithelial cells, TB and ME formed plaques from infectious centers as efficiently as they did on fibroblasts, (approx. 50%), but TR was notably less efficient, yielding only 10 plaques per 100 infectious centers (Figure. 3-5B). In interpreting these results, it is important to note that gO-null TR was severely impaired for entry, but more efficient at cell-cell spread in these epithelial cells compared to the parental wild type TR (238), suggesting a mechanistic distinction between entry and spread on these cells. Thus, the poor ability of TR to form plaques on epithelial cells from infectious centers helps to explain the apparently high particle-to-PFU ratio of TR on these cells, and is also consistent with reports indicating that HCMV tropism is not solely determined at the level of entry (268, 269).

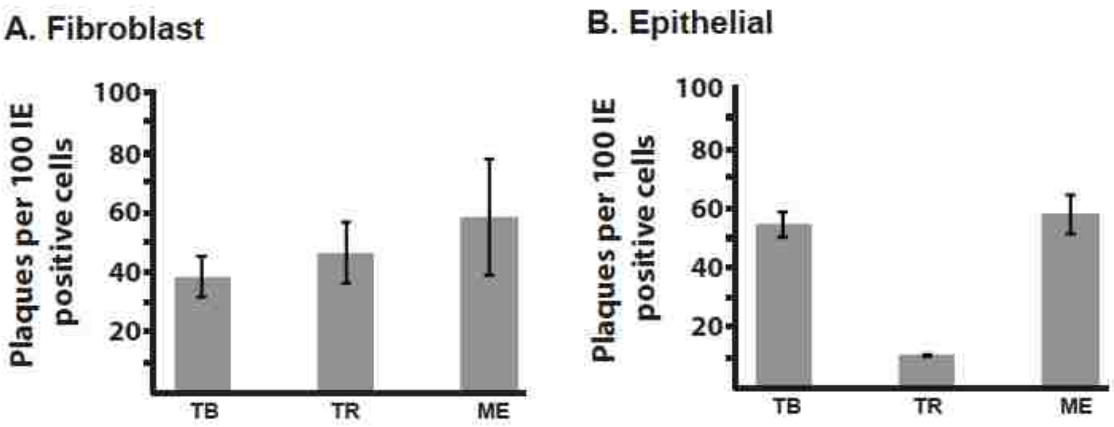

Figure 3-5 HCMV plaque formation from infectious centers.
Replicate cultures of fibroblasts (A), or epithelial cells (B) were infected were infected with TB, TR or ME. At 2 dpi., one set of cultures was fixed and the fraction of infected cells was quantified for each infection by immunofluorescence detection of immediate early gene expression. Cells in replicate cultures were detached, counted and an amount of cells corresponding to 100 IE-positive cells were plated onto cultures of the homologous cell type. Plaques were allowed to develop and then counted. Averages and standard deviations from three experiments are plotted.

Perhaps the most surprising result of the particle-to-PFU analysis presented in Figure. 3-4 was that ME-T was approximately 10-fold more efficient than ME on epithelial cells (Figure. 3-4B). Western blot analysis clearly demonstrated that ME-T virions contain mostly gH/gL/gO, and very little gH/gL/UL128-131, whereas ME contains abundant gH/gL/UL128-131, and smaller amounts of gH/gL/gO (Figure. 3-1~3-3). It was possible that these differences in gH/gL complexes affected the fundamental mechanism, or route of entry for ME and ME-T into these cells. To test this, antibody neutralization experiments were performed using anti-UL130 and anti-UL131, which have been shown previously to block HCMV infection of epithelial cells, but not fibroblasts (270) (Figure. 3-6). Anti-UL130/131 antibodies blocked infection of both ME and ME-T on epithelial but not on fibroblasts, indicating that for both viruses, infection of epithelial cells but not fibroblasts was gH/gL/UL128-131-dependent. These results indicate that 1) ME-T virions were not completely devoid of gH/gL/UL128-131, and 2) the enhanced infectivity of ME-T on epithelial cells was not due to an aberrant, gH/gL/UL128-131-independent process. Taken together, these analyses strongly contradict the model that gH/gL/gO and gH/gL/UL128-131 serve mechanistically analogous roles for entry of HCMV into fibroblasts and epithelial cells, respectively. Instead, it seems more likely that the two complexes act through distinct mechanisms to facilitate entry into these cell types.

The specific infectivity difference between ME and ME-T virions can be overcome with polyethylene glycol. Since ME and ME-T are genetically identical viruses, their post entry replication and spread characteristics are identical in cells

that do not express TetR (177, 232). Thus, the observed differences in particle-to-PFU ratios (Figure. 3-4) were likely indicative of differences in the entry process as a direct result of the amounts of gH/gL/gO and gH/gL/UL128-131 in the virion envelope, likely acting on the ability of fusion. To test this hypothesis, equivalent amounts of ME or ME-T were adsorbed onto fibroblasts, or epithelial cells, and then both viruses were provided with an equivalent, surrogate fusion mechanism, polyethylene glycol (PEG) treatment (Figure. 3-7) (79).

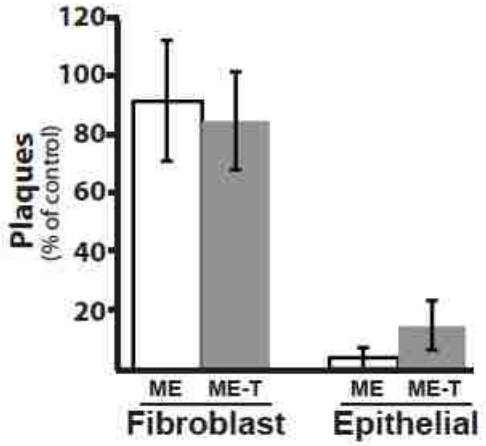

Figure 3-6 Neutralization of HCMV by UL130, and UL131 specific antibodies.
Cell free Merlin (ME) or Merlin-trimer (ME-T) were plated on fibroblast or epithelial cell cultures in the presence of anti-UL130 and anti-UL131 antibodies, and the number of resultant plaques was determined. The average number of plaques as percent of control (no antibody) and standard deviations from three experiments are plotted. For each experiment, the number of plaque under control conditions ranged from 80-120.

ME-T efficiently infected fibroblast cells, but ME did not (66% and 2.5%, respectively; Figure. 3-7A). PEG treatment had no significant effect on the number of cells infected with ME-T suggesting that most ME-T virions were capable of

efficient entry without PEG. This was consistent with the relatively low particle-to-PFU ratio of ME-T on these cells (Figure. 3-4A). In contrast, PEG resulted in an approximately 20-fold increase in the numbers of cells infected with ME, bringing the percent of cells infected with ME very near the values for ME-T. These results confirmed that ME virions were poorly infectious on fibroblasts largely due to an inefficient fusion mechanism.

At the multiplicity used in these experiments (150 particles per cell), neither ME nor ME-T infected measurable numbers of epithelial cells (Figure. 3-7B). This was consistent with the much higher particle-to-PFU ratio measured on these cells (Figure. 3-4B). PEG treatment resulted in a similar number of infected cells for both ME and ME-T, consistent with the notion that the high particle-to-PFU ratio of ME compared to ME-T (Figure. 3-4B) reflected a reduced ability of ME virus to accomplish the fusion step of entry, not later stages of the replication cycle. However, because of the relatively high particle-to-PFU ratios for both ME and ME-T on these cells (1250 and 10,529, respectively; Figure. 3-4B), it was possible that the 150 particles per cell used in these experiments did not reflect the replication-competent virions in each population. To address this caveat, another set of experiments were performed in which 10, or 30 PFU were plated on epithelial cells, virus entry was induced with PEG, and the number of resultant plaques was determined (Figure. 3-8). For ME, PEG treatment resulted in plaque numbers 4-5 fold greater than the inputs, but PEG had no significant effect on the numbers of ME-T plaques. The lack of effect on ME-T plaque formation suggests that a large portion

of the 1250 particle-to-PFU ratio may represent virions with post-entry defects. Indeed, infectious center experiments suggested that approximately 50% of virus

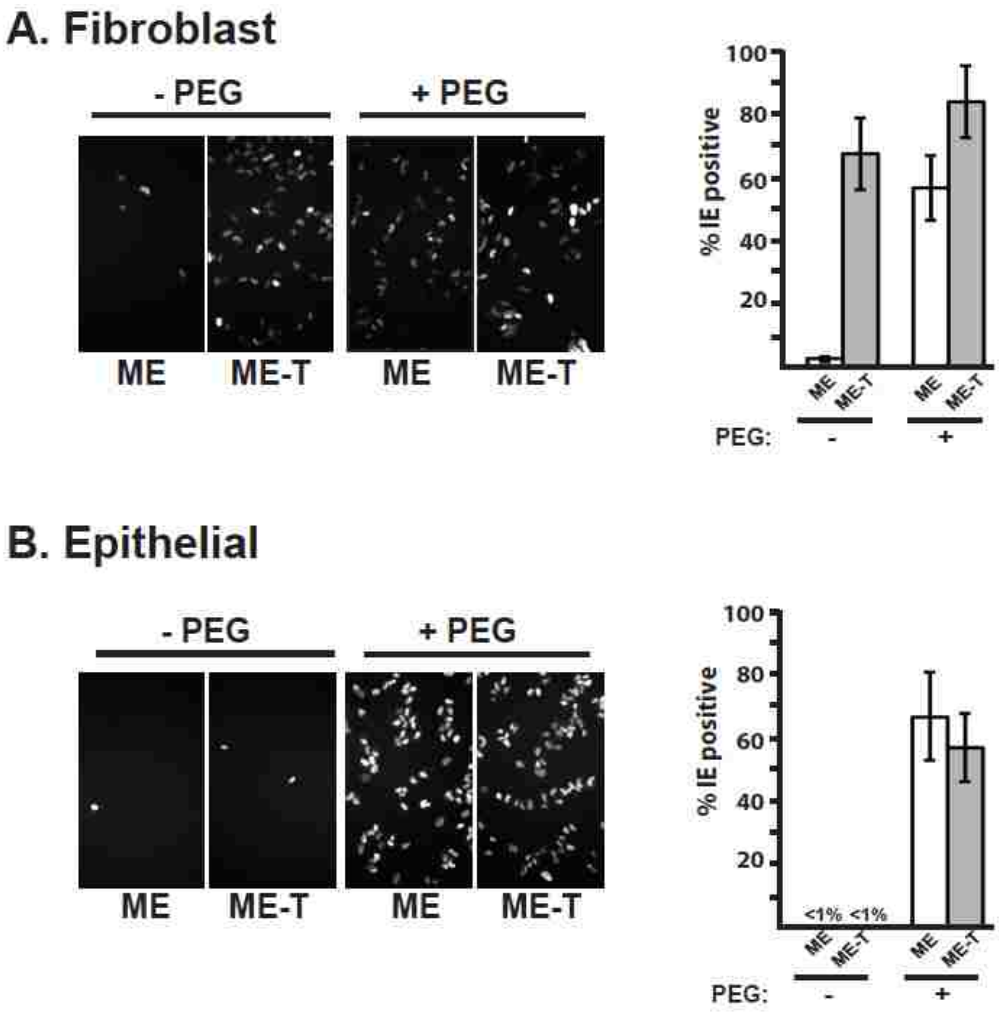

Figure 3-7 Effects of polyethylene glycol (PEG) on infection by gH/gL/UL128-131-rich, and gH/gL/gO-rich HCMV.
Cell free Merlin (ME) or Merlin-trimer (ME-T) virions were plated on fibroblast (A) or epithelial cell (B) cultures at 150 particles per cell, and then treated with either PBS (-) or PEG (+). Cells were fixed 48h later, and analyzed by immunofluorescence to detect HCMV IE gene expression. Representative microscopic fields are shown (left), and the average percent of cells infected among 6 microscope fields (> 80 cells each) are plotted (right). Error bars represent standard deviations.

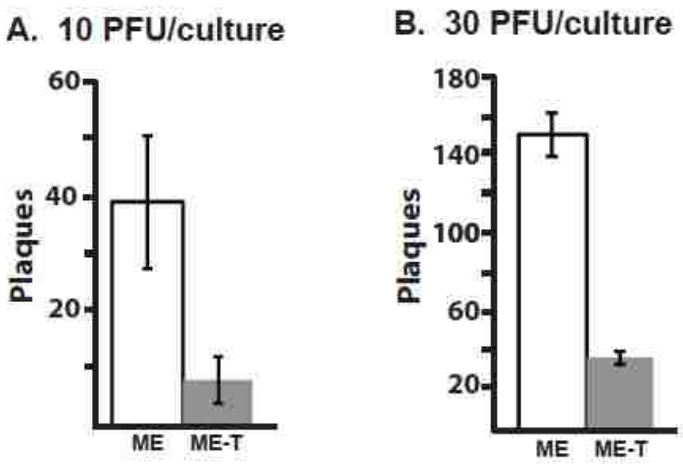

Figure 3-8 Effects of polyethylene glycol (PEG) on HCMV plaque formation on epithelial cells.
10 PFU (A), or 30 PFU (B) of Merlin (ME) or Merlin-trimer (ME-T) were plated on epithelial cell cultures, treated with PEG, and the number of resultant plaques was determined. The average number of plaques and standard deviations from three experiments are plotted.

entry events that progress to IE expression fail to result in a plaque on these cells (Figure. 3-5B.) However, it may also reflect limitations in PEG-induction of virus entry. The observed enhancement of ME plaque formation by PEG strongly suggests that ME virions were less infectious on epithelial cells due to an inability to accomplish the fusion stage of entry, despite their elevated levels of gH/gL/UL128-131.

DISCUSSION

Specific infectivity (also referred to as "plating efficiency") was among the earliest established parameters of virus characterization, and has been generally expressed as some unit of physical quantitation of virus (e.g., grams of protein or nucleic acid, number of capsids, number of genomes, etc.) per unit of virus infectivity (e.g., plaque forming units (PFU), lethal/infectious dose (LD/ID) 50, tissue culture infectious dose (TCID) 50, etc.) (271–273). High particle-to-PFU ratios are common, and reflect the fact that some viral particles in a given preparation are defective, and incapable of initiating or completing a replication cycle. However, irrespective of bona fide defective virions, high specific infectivity ratios also reflect the generally low probability that any given replication-competent virion within a population will successfully accomplish a measurable replication event in an inherently time-limited assay. Indeed, Thomas *et al.* reported that high particle-to-PFU ratios of HIV in part reflect the poor odds that any given virion will even attach to a host cell (274). It has been suggested that the envelope composition of HCMV virions within a population is heterogeneous (259, 260), and it is plausible that this phenomenon contributes to high and variable particle-to-PFU ratios, through stochastic probability effects.

Early analyses of the specific infectivity of HCMV involved counting particles by electron microscopy, and particle-to-PFU ratios ranging from approximately 10^2 to 10^8 were reported (275–277). The wide ranges, and high values were likely due in part to variable numbers of dense bodies (DB) and non-infectious enveloped particles (NIEPs), which lack genomes, and therefore cannot be PFUs (49, 126, 278).

Our use of quantitative PCR avoided particles that lack genomes, and consequently, values obtained were log-orders lower, more consistent between independent samples of the same virus, and consistent with previous uses of this approach (279).

While the specific infectivity measurements reported herein were highly reproducible for any given strain of HCMV, there were large differences between strains, and between cell types. Values on epithelial cells were considerably higher than on fibroblasts for all strains analyzed. This indicates that the susceptibility and permissiveness of different cell types to HCMV is not a simple "yes-no" binary, but rather a continuum that likely reflects complex differences including receptor availabilities, and the mechanism of entry and spread on different cell types. Indeed, entry of HCMV into fibroblasts likely occurs through fusion at the cell surface, whereas entry into other cell types, such as epithelial cells, likely involves internalization of the virus by endocytosis-like processes, and subsequent fusion from within endosomes (79, 102, 280). Additionally, the large differences between strains of HCMV observed likely have multi-locus genetic underpinnings, since plaque formation is a function of every stage of the replication cycle.

Comparative western blot analyses indicated considerable variation in the amounts of gH/gL complexes in the virion envelope between strains of HCMV. TB and TR were found to contain gH/gL mostly in the form of gH/gL/gO, and comparatively less gH/gL/UL128-131, whereas ME virions contained much less total gH/gL, and that was mostly in the form of gH/gL/UL128-131. These results were consistent with the previous data reported in Chapter 2 (232). Importantly, despite the overall low amount of the amount of gH/gL in ME, the amount of

gH/gL/UL128-131 was dramatically more than was detected in TB or TR virions. While the HCMV virions analyzed here were produced in fibroblasts, Murrell *et al.* produced virions in epithelial cells and found that TR contained amounts of gH/gL/UL128-131 comparable to ME, and TB had noticeably less (281). These discrepancies suggest that factors of the host cell type can influence the assembly of HCMV gH/gL complexes. Furthermore, our analyses suggest that the gH/gL deficit in ME represents a deficit of gH/gL/gO. This was reversed by Tet-repression of gH/gL/UL128-131, resulting in ME virions contained similar amounts of total gH/gL, but it was mostly in the form of gH/gL/gO ((232) and Figure. 3-3). This virus was denoted ME-trimer (ME-T). These characterizations of gH/gL complexes among HCMV strains allowed for studies into the mechanisms by which gH/gL/gO and gH/gL/UL128-131 facilitate entry into different cell types.

Characterization of gH/gL/gO and gH/gL/UL128-131 mechanisms has relied largely on the analysis of UL128-131, and gO deletion mutants. (77–79, 227) (42, 234, 235, 238). UL128-131 mutants replicate well on fibroblasts, allowing them to be easily propagated, and clearly characterized for their defective(s) on select cells such as epithelial, and endothelial cells. In contrast, gO mutants are severely impaired on all cell types. This suggests a fundamental role for gH/gL/gO in entry, but without a complementation system, further characterization of mechanism has been difficult since the rare infection events observed likely represent non-physiologic mechanisms. Thus, there has been no clear data to distinguish two mutually exclusive models for how HCMV gH/gL complexes facilitate entry into different cell types. A commonly assumed model is one in which gH/gL/gO and

gH/gL/UL128-131 each performs a mechanistically analogous function for entry into different cell types. Under this model, that analogous function is likely the "core" herpesvirus gH/gL function of promoting gB-mediated membrane fusion during entry, and the basis of cell type selectivity is alternate receptor binding. An alternative model however, is that gH/gL/gO promotes gB-mediated fusion for entry into all cell types, where as gH/gL/UL128-131 facilitates infection of select cell type, including epithelial cells, though a distinct mechanism that does not functionally replace the need for gH/gL/gO.

Our use of ME virions enriched for either gH/gL/UL128-131 or gH/gL/gO (ME and ME-T, respectively) avoided many of the caveats associated with null mutants. Since the viruses are not devoid of either gH/gL/gO, or gH/gL/UL128-131, they are theoretically capable of physiologically relevant viral entry mechanisms on all cell types, albeit with different efficiencies. Furthermore, since they are genetically identical viruses, after the initial entry event, replication and plaque spread should be identical. Indeed, plaque size and morphologies were indistinguishable on all cells lacking TetR expression (177). The higher particle-to-PFU ratio of ME compared to ME-T on both fibroblasts and epithelial cells indicated that a greater fraction of virions in ME stocks failed to form plaques. Experiments involving PEG-induced fusion indicated that these ME virions failed largely because they could not accomplish the fusion event of entry. The fate of non-fusing virions is unclear but may include endocytosis, and destruction within the lysosome. Regardless, these results are inconsistent with the model that gH/gL/gO and gH/gL/UL128-131 are each sufficient as a "herpesvirus core-fusion gH/gL" for entry

into fibroblasts and epithelial cells, respectively. Rather, it is more likely that only gH/gL/gO performs the conserved "herpesvirus gH/gL function" of promoting gB-mediated membrane fusion during entry into all cell types, and gH/gL/UL128-131 provides a distinct, yet necessary function for entry into select cell types, such as epithelial cells.

The exceedingly high particle-to-PFU ratio of TR on epithelial cells was seemingly incongruent with the model of distinct functions for gH/gL complexes since TR virions contained comparable amounts of gH/gL/gO and gH/gL/gUL128-131 as TB, and contained more of both gH/gL complexes than ME-T virions, yet both TB and ME-T were vastly more efficient at plaque formation than TR. However, as noted above, plaque formation reflects the entire entry-replication-spread cycle. Analysis of the efficiency of post-entry replication and spread indicated that TR was much less likely to progress from entry to form an observable plaque on epithelial cells than were TB or ME (Figure. 3-5). Furthermore, plaques on epithelial cells that were observed for TR were notably smaller than those for TB and ME. Thus, it seems likely that the poor plaque formation of TR on epithelial cells reflects genetic differences between strains loci other than gO and UL128-131, and underscores observations that HCMV tropism is not only determined by entry mechanisms (268, 269).

The model of distinct mechanisms for HCMV gH/gL complexes fits well with other recent observations of CMV entry and spread. First, HCMV entry into epithelial cells likely involves membrane fusion from within low-pH endosomes (79, 190, 280). Nogalski *et al.* characterized the interaction of HCMV with monocytes, a cell

type for which infection is also gH/gL/UL128-131 dependent (230, 266). They found positive correlation between the level of gH/gL/UL128-131 in the virion envelope, and the intensity of Src kinase activation following virion attachment (230). Thus, one possibility is that signaling through gH/gL/UL128-131 receptors might affect the nature of the endosome in a way that fosters subsequent membrane fusion mediated by gB and gH/gL/gO. Second, while data presented here indicate that gH/gL/gO is important for infection of all cell types by cell-free HCMV, analyses of gO mutants suggested that cell-to-cell spread on epithelial or endothelial cells and monolayers does not require gH/gL/gO (235, 238). Furthermore, a recent *in vivo* study involving murine CMV suggesting that gH/gL/gO is important for initial infection of mice, but less important for subsequence spread to distal organs and tissues (282).

The proposed mechanistic distinction between gH/gL/gO and gH/gL/UL128-131 may provide a unique opportunity to study the general principles of how the herpesvirus gH/gL promotes fusion by gB (i.e., the "core fusion" function of gH/gL). Herpesvirus fusion has been most extensively studied for HSV, which has only one form of gH/gL, and EBV, which has two, gH/gL, and gH/gL/gp42 (reviewed in (189)). In the case of EBV, both complexes appear to perform the conserved function of promoting gB-mediated membrane fusion for entry into either epithelial, or B-cells, indicating that gp42 does not cover, or otherwise alter the profusion surfaces of gH/gL. Fusion by HCMV also likely involves interactions between gB and surfaces of gH/gL (122, 216). However, data presented here suggest that gH/gL cannot promote gB fusion when the UL128-131

proteins are bound. This could be due to differential allosteric effects on gH/gL conformation as the result of gO versus UL128-131 binding. Alternatively, it could be that gO and UL128-131 physically cover different surfaces of gH/gL. Recent cryo-EM analyses reported by Ciferri *et al.* indicated that gO and UL128-131 bind the same face of gH/gL, but the data provided neither support nor contradiction to either aforementioned possibility (215). It is also noteworthy that Ciferri *et al.* suggested that gH/gL/UL128-131 was capable of facilitating gB-mediated membrane fusion in cell-cell fusion experiments. However, these data were difficult to interpret because in the same experiments, unbound gH/gL (lacking UL128-131 or gO) failed to promote gB-mediated fusion. This was an important discrepancy with the results of Vanarsdall *et al.* showing that gH/gL alone was capable of promoting gB fusion in the same type of cell-cell fusion experiment, though Ciferri *et al.* did not comment on this (215, 216).

Several recent studies have characterized the antibody response to the pentameric gH/gL/UL128-131 with an aim towards vaccine development (283–285). Pentamer vaccines clearly elicit antibodies that are specific to epitopes that are contained on the UL128-131 proteins surfaces themselves, or present on the gH/gL surfaces that are allosterically dependent on UL128-131 (i.e., pentamer-specific antibodies), as well as antibodies that react to surfaces of gH/gL that are independent of the UL128-131 proteins (i.e., gH/gL-specific antibodies). Pentamer-specific antibodies powerfully neutralize HCMV infection of epithelial cells, but show little to no neutralizing potency on fibroblasts. In contrast, the gH/gL-specific antibodies have significantly lower neutralizing potency, but block infection of both

fibroblasts and epithelial cells with comparable potency. In the context of the model of distinct mechanisms proposed here, it seems likely that the pentamer-specific antibodies interfere with the binding of the pentamer to cell-type specific receptors, and thus block the subsequent signaling cascades that enhance infection of select cell types, whereas the gH/gL-specific antibodies block the role of gH/gL/gO in promoting gB fusion on all cell types. The disparity in potency between pentamer-specific and gH/gL-specific antibodies may be due to any of several, non-mutually exclusive factors including; 1) the differential abundance of gH/gL/gO and gH/gL/UL128-131 in the virion envelope, 2) the apparent fact that infection of epithelial cells can likely be inhibited at either of two independent mechanisms, internalization or fusion, compared to only fusion on fibroblasts, 3) the relevant gH/gL-specific epitopes may be differentially displayed due to different allosteric effects of UL128-131 binding compared to gO binding, or by the presence of the extensive gO glycan network. The latter possibility seems especially plausible given that gH/gL-specific antibodies described have been raised either against gH/gL alone, or gH/gL/UL128-131 (i.e., in the absence of gO).

In summary, the observations reported here and elsewhere strongly argue that the fusion event of entry of HCMV into all cell types is mediated by gB and gH/gL/gO. On some cell types, such as epithelial cells, efficient fusion must be preceded by a process mediated by the pentameric gH/gL/UL128-131 complex. This model of entry and tropism may have important implications for the development of HCMV vaccines as well as broader implications for understanding the principles of that the herpesvirus gH/gL does to promote gB-fusion.

CHAPTER 4. CHARACTERIZATION OF THE VIRAL FACTORS THAT INFLUENCE HUMAN CYTOMEGALOVIRUS gH/gL COMPLEXES ASSEMBLY

INTRODUCTION

Human cytomegalovirus (HCMV) is a member of beta-herpesvirus family, which is widespread and can infect up to 95% of the population in the United States (9). The primary infection of HCMV in healthy host is normally subclinical, followed by life-long persistent and latent infection. However, with immunocompromised (e.g., HIV/AIDS patients) or immunosuppressed (e.g., transplant recipients) individuals, HCMV primary infection, reinfection by another strain, or reactivation from latency can cause life-threatening diseases (reviewed in (240)). The virus is also a leading cause for congenital birth defects. Each year about 0.2% of all live births are infected by HCMV, resulting in approximately 1% death and 20% with permanent neurological impairments (18). HCMV can infect a wide spectrum of cell types in the human body, including epithelial/endothelial cells, fibroblasts, monocytes, placental trophoblasts, smooth muscle cells, hepatocyte and neuronal cells (147, 149–157). The broad cell tropism reflects that this virus is capable of entering numerous cell types. No matter which cell type the virus encounters, as an enveloped virus, the key event is to fuse viral envelope with cellular membranes, which is mediated by the viral envelope glycoproteins.

All herpesviruses share conserved fusion machinery comprised of gB and heterodimeric complex gH/gL. gB is the fusion protein, while gH/gL provides essential regulatory role, probably involving in direct interaction with gB (67, 189, 198, 209, 210, 213, 214, 219, 286). Besides gB and gH/gL, each herpesvirus also encodes accessory proteins that can regulate the action of the core fusion machinery, for instance, herpes simplex virus (HSV) gD, and Epstein-Barr virus

(EBV) gp42 (214, 218, 221–223, 247, 262). The accessory proteins could either transiently or stably interact with gH/gL, and are often involved in receptor binding and can act as viral tropism determinants. For both HSV and EBV, their unbound gH/gL or gH/gL complex could provide essential regulation for gB's fusion activity, which is referred to as the "core fusion function".

HCMV gH/gL has two sets of binding partners, gO and UL128-131, forming gH/gL/gO and gH/gL/UL128-131 complexes on the virion envelope (73, 74, 77, 80, 227). Both complexes could influence HCMV cell tropism, but the mechanisms are poorly understood. *In vitro* cell-cell fusion assay demonstrated that gB and gH/gL formed the minimal set of HCMV glycoproteins that are required for membrane fusion, while adding UL128-131 or gO didn't inhibit or enhance cell-cell fusion efficiency (216, 239). However, there is little, if any, unbound gH/gL dimer on the virion envelope (data reported in Chapter 2), thus it is possible that either one of HCMV gH/gL complexes or both of them could provide the "core fusion function". Data reported in Chapter 3 supported a model where gH/gL/gO provides the conserved herpesvirus "core fusion function" of promoting gB fusion for entry into all cell types, whereas gH/gL/UL128-131 provides a non-fusion, yet necessary function on select cell types (i.e. epithelial/endothelial cells). This model is consistent with the phenotype of UL128-131, and gO deletion mutants: the UL128-131 mutants fail to infect epithelial/endothelial cells but can still infect fibroblast cells as efficiently as wild type viruses while the gO mutants fail to enter any cell type (77–79, 227, 238).

The other important finding in both Chapter 2 and Chapter 3 was that strain Merlin (ME) contained less total amount of gH/gL than strains TB40/e (TB) or TR, and the majority of ME gH/gL was in the form of gH/gL/UL128-131 while TB and TR contained mostly gH/gL/gO. The aim of the research in this chapter was to characterize the mechanism(s) that regulates the differential assembly of gH/gL complexes among strains of HCMV. Strain TR and ME were chosen to represent gH/gL/gO-rich and gH/gL/UL128-131-rich viruses respectively for studies carried out in this Chapter.

Herpesvirus glycoproteins are synthesized in the endoplasmic reticulum (ER), and have to be transported to trans-Golgi-derived viral assembly compartment in order to be incorporated into the virion envelope. So either, or both of the glycoprotein expression level and trafficking efficiency from ER to Golgi compartment could affect the assembly of glycoprotein complexes. Moreover, UL148, an ER-chaperone protein, was recently reported to favor the formation of gH/gL/gO (287). Comparison of the glycoprotein levels in the infected cells showed that ME expressed much more of UL128-131 proteins, less of UL148, and comparable amount of gH/gL as compared to TR. However while TR gH/gL were efficiently transported to the trans-Golgi derived virus assembly compartment, ME gH/gL remained mostly in the ER. Moreover, the transport of ME gH/gL out of ER was even worse in fibroblast cells where UL128-131 expression was repressed, suggesting that ME gH/gL/gO assembly was inherently inefficient. It is likely that both high expression of UL128-131 and low expression of UL148 contributed to the poor assembly of ME gH/gL/gO in ER, which dampened the transport of gH/gL to

trans-Golgi derived assembly compartment, and thus resulted in the less total gH/gL, and dearth of gH/gL/gO in ME virion envelope. Further characterization showed that neither gO sequence diversity nor gH N-term polymorphisms had great affect in regulating the gH/gL complexes assembly. Taken together, report here demonstrated that multiple viral factors are involved in the regulation of gH/gL complexes assembly, and helped us better understand how HCMV adjusts tropism and navigates through the host.

MATERIALS AND METHODS

Cell lines. Primary human foreskin fibroblasts (HFF) (Life Technologies), HFF tet cells (which express the tetracycline repressor protein (177)), and MRC-5 fibroblasts (ATCC CCL-171) were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 6% heat-inactivated fetal bovine serum (FBS) and 6% bovine growth serum (Rocky Mountain Biologicals, Inc.). The retinal pigment epithelial cell line ARPE-19 (American Type Culture Collection, Manassas, VA, USA) was grown in a 1:1 dilution mixture of DMEM and Ham's F-12 medium (DMEM: F12) (Life Technologies) supplemented with 10% FBS.

HCMV. The HCMV strains were derived from bacterial artificial chromosome (BAC) clones. BAC clone TR was provided by Jay Nelson (Oregon Health and Science University, Portland, OR, USA) (41). BAC clone Merlin (ME) (pAL1393), which contains tetracycline operator sequences inserted in front of the promoter of UL130 and UL131, was provided by Richard Stanton (Cardiff University, Cardiff, United Kingdom) (177). Infectious HCMV was recovered by electroporation of BAC DNA

into MRC-5 fibroblasts, as described by Wille *et al.* (238). Cell-free TR and ME stocks were produced by infecting HFF and HFFtet at 0.1 PFU per cell respectively. At 8 to 14 days postinfection (when 90% of the cells were still visually intact), culture supernatants were harvested, and cellular contaminants were removed by centrifugation at 1,000X g for 10 min and again at 6,000Xg for 10 min. Stocks were judged cell free by the lack of calnexin in western blotting analyses, and were aliquoted in adequate amounts and stored at -80°C. The number of PFU was determined by plaque assay on triplicate HFF.

Recombineering. The gO inter-strain swap mutants were constructed by Richard Stanton (Cardiff University, Cardiff, United Kingdom) using two-step galactokinase (*GalK)* selection as described previously (281). The gH N-term inter-strain swap mutants were generated in our lab by *en passant* mutagenesis (288, 289). A selectable kanamycin (*Kan*)-resistance cassette with endonuclease I-SceI restriction site, duplications of the target gene sequences and short homologous sequences flanking at either end was PCR amplified. Insertion of the cassette into the region to be modified on the BAC DNA that contained chloamphenicol (*Cm*) resistance gene was allowed by recombination at the short homologous sequences, followed by positive selection for *Cm* and *Kan* resistance. Next, expression of I-SceI was induced and the BAC DNA was cleaved into linear dsDNA. The resulting dsDNA ends recombined at the duplicated sequences of target gene, resulting in the completely removal of all foreign sequences including the selection marker. The recombined BAC was selected by *Cm* resistance.

Antibodies. Monoclonal antibodies (MAbs) specific for HCMV major capsid protein (MCP) 28-4 and gB 27-156 were provided by Bill Britt (University of Alabama, Birmingham, AL, USA) (45, 267). Anti-UL128 MAb 4B10 was provided by Tom Shenk (Princeton University, Princeton, NJ, USA) (77). Rabbit polyclonal antipeptide antibodies directed against HCMV gH, gL, UL130, and Ul131 were provided by David Johnson (Oregon Health and Sciences University, Portland, OR, USA) (80). Rabbit polyclonal antipeptide antibody directed against HCMV UL148 was provided by Jeremy Kamil (Louisiana State University, Health Sciences Center, Shreveport, LA, USA) (287). Anti-IE1/2 MAb was commercially purchased from abcam.

Western blotting analysis. Cell-free virions from culture supernatants were concentrated by centrifugation at 50,000Xg for 1 h and resuspended in 2% SDS, 20mM Tris-buffered saline (TBS) (pH 6.8), followed by heating at 95°C for 10 min. Insoluble material was removed by centrifugation at 16,000Xg for 30 min, and the cleared supernatants were saved as virion extract and stored at -80°C. For cell extract, HCMV infected cells were scraped into medium at either 2 dpi or 5 dpi, and cell fractions were separated by centrifugation at 1,000Xg for 10 min. Cell pellets were resuspended in 2% SDS, 20 mM TBS, followed by heating at 95°C for 10 min. Insoluble material was removed by centrifugation at 16,000Xg for 30 min, and the cleared supernatants were saved as cell extract and stored at -80°C. For endoglycosidase H (endo H) and peptide N-glycosidase F (PNGase F) treatment, cell extract were incubated with endo H or PNGase F for 3~5 h at 37°C in the buffer specified by the enzyme manufacturer (New England Biolabs). For reducing blots,

extracts were then adjusted to 25mM dithiothreitol (DTT), and proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Whatman) in a buffer containing 10 mM $NaHCO_3$ and 3 mM Na_2CO_3 (pH 9.9) plus 10% Methanol. The transferred proteins were probed with MAbs or rabbit polyclonal antibodies specific for HCMV proteins, followed by horseradish peroxidase-conjugated secondary antibodies; chemiluminescence was detected using a Bio-Rad ChemiDoc MP imaging system.

qPCR. Cell-free HCMV stock (20 µl) was first treated with DNase I to remove DNA not protected within viral capsids. Capsids were disrupted by using the viral lysis buffer and proteinase K provided in the PureLink Viral RNA/DNA minikit (Life Technologies). Viral DNA was eluted in 50 µl RNAase-free water. A region within UL83 conserved among TR and ME was chosen as the amplicon (as described in Chapter 3), and genomes were quantified by real-time quantitative PCR (qPCR) using SYBR green dye (Bio-Rad) and the forward primer TGGTCACCTATCACCTGCAT and the reverse primer GAAAGAGCCCGACGTCTACT. PCR samples were made to a total well volume of 20 µl with 1X SYBR green master mix (Bio-Rad), a 1:100 dilution of viral DNA, and 500 nM forward and reverse primers. PCR products were detected using MyiQ real-time PCR detection system (Bio-Rad). The qPCR results were compared to a plasmid pTRUL83 standard and then calculated as genomes per ml. For each experiment, two independent viral DNA extractions and three independent qPCRs were performed.

Plaque assay. Cell-free HCMV were serially 10 fold diluted in DMEM medium and subsequently used to infect monolayers of HFF or ARPE-19 cells. After 2 h

incubation, cells were overlaid by 1:1 mixture of 2X DMEM, and 1.2% Sea-Plaque agarose to prevent the spread of progeny virus by diffusion. Plaques were counted by light microscopy three weeks later and titer was calculated as plaque forming unit (PFU) per ml. For each experiment, three independent plaque assays were performed.

Multi-step growth curve. HFF cells were infected by HCMV at 0.1 PFU per cell, and cell-free supernatant viruses were harvested at various time points (4, 8, 14, 21 dpi) as described above. Quantitation of cell-free virus infectivity was performed by plaque assay on HFF cells.

RESULTS

Analysis of viral glycoprotein expression levels and trafficking efficiency in fibroblast cells infected by different HCMV strains. To compare the glycoprotein expression level, fibroblast HFF cells were infected with either TR or ME at MOI=1. Cell lysate were prepared at different time points post infection and analyzed by western blotting under reducing conditions. Gel loads were normalized to viral immediate-early (IE) gene products (IE1 and IE2) at 2 days post infection (2 dpi) to normalize the input of viral gene expression (Figure. 4-1A). Note that only IE1 was detected at 2 dpi while all the other viral glycoproteins of interest were not (data not shown), which was consistent with the kinetic profiles of those proteins.

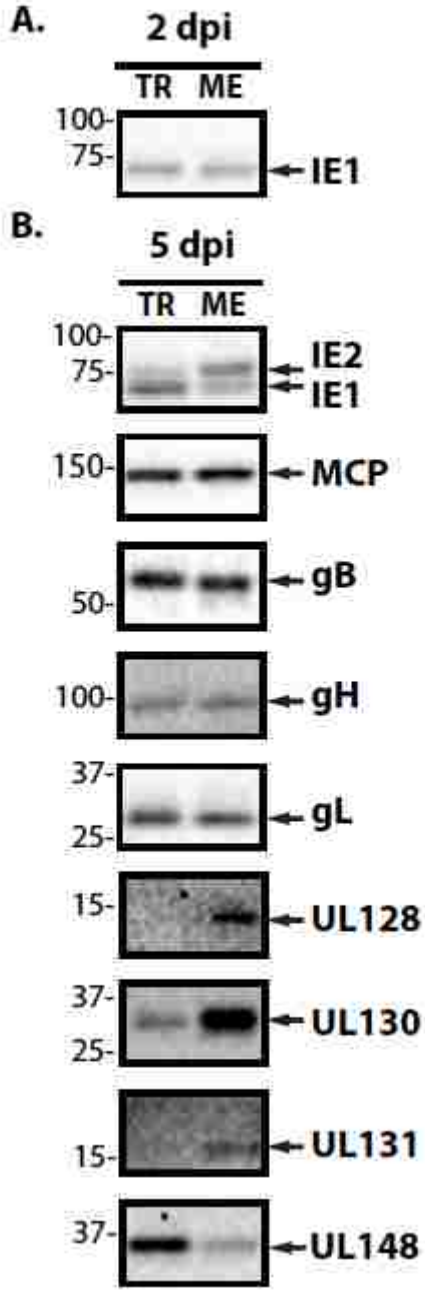

Figure 4-1 Comparison of glycoproteins expression level in the fibroblasts cells infected with strains of HCMV TR and ME.

Cell lysate harvested from 2 dpi (A) or 5 dpi (B) were separated by reducing SDS-PAGE and analyzed by western blotting with antibodies specific for IE1/2, MCP, the 55-kDa fragment of gB, gH, gL, UL128-131 respectively, and UL148. The gel loads were normalized to equal amount IE1 at 2 dpi. Mass markers (kilodaltons) are shown on the left.

The steady-state levels of glycoproteins were then compared at 5 dpi., by the time of which virus assembly occurred. One interesting observation was that IE gene products appeared to show different patterns between TR and ME, with IE1 being the predominant species in TR infected cells whereas IE2 in ME infected cells (Figure. 4-1B). The significance of this difference is beyond the scope of study in this dissertation, but since IE1 and IE2 are known as crucial transcriptional activators of multiple viral and cellular genes (reviewed in (290)), this data might have implication in interpreting potential strain-specific viral replication or virus-host interaction characteristics. Nevertheless, the combined amounts of IE were comparable at 5 dpi. The blotting of major capsid protein (MCP), gB, gH and gL showed comparable amount of those proteins as well (Figure. 4-1B). Conversely, ME infected cells contained dramatically more UL128, UL130 and UL131 proteins than TR infected cells (Figure. 4-1B). This result correlated well with the amount of UL128-131 detected in ME, and TR virions respectively, which may help explain why ME is a gH/gL/UL128-131-rich virus. Note that gO could not be compared here because the anti-gO antibodies lacked cross-reactivity as shown in Figure. 2-4.

However, the comparable amount of gH and gL expressed in the infected cells was not consistent with the amount of those proteins detected in the virus envelope, where TR virions contained greatly more total amount of gH/gL than ME. This discrepancy suggests that ME gH/gL might not be efficiently transported to trans-Golgi-derived assembly compartment. To test this hypothesis, cell lysate were treated with endoglycosidase H (endo H), which removes the high-mannose N-linked glycans that are added to proteins in the ER, or peptide N-glycosidase F

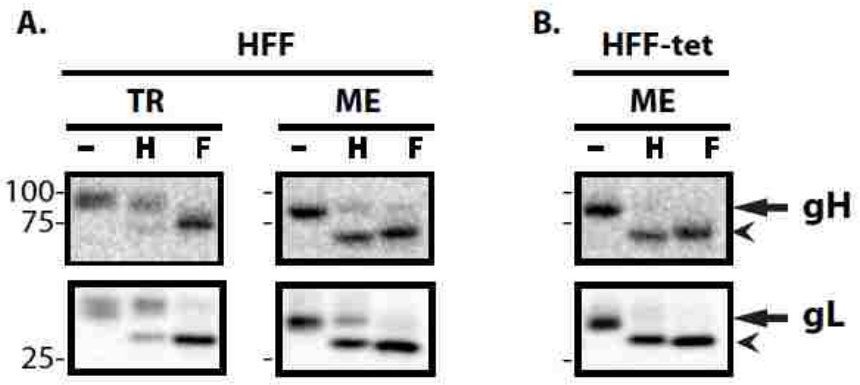

Figure 4-2 Western blotting analysis of the maturation of HCMV gH/gL in the infected fibroblast cells.

TR- and ME-infected HFF cell lysate (A), or ME-infected HFFtet cell lysate (B) were either left untreated (-), or treated with endo H (H) or PNGase F (F), and then were separated by reducing SDS-PAGE and analyzed by western blotting with antibodies specific to gH or gL. Bands corresponding to glycosylated form, or endo H resistant form of the protein were pointed by arrow; and the deglycosylated forms of the protein either by endo H or PNGase F was pointed by arrowhead. Mass markers (kilodaltons) are shown on the left.

(PNGase F), which removes all types of N-linked glycans, including those ones that have been modified by enzymes in the Golgi compartments. Thus, glycoproteins retained in the ER should stay endo H sensitive, resulting in a faster migrating band on SDS-PAGE gels, while the ones present in Golgi should be endo H resistant. And glycoproteins in either ER or Golgi compartments should be sensitive to PNGase F. The treated samples were analyzed by western blotting under reducing conditions and assessed for protein mobility. As expected, gH and gL in both TR and ME infected cells were fully sensitive to PNGase F. However, the majority of gH and gL were in the faster-migrating, endo H sensitive form for ME, while the reverse pattern was true for TR, with larger proportion of gH and gL remaining endo H resistant (Figure. 4-2A). This result confirmed the hypothesis that most of ME gH/gL

are not efficiently transported out of ER, which correlated with the significant lower amount of total gH/gL present in ME virion envelope.

ER to trans-Golgi transport of gH/gL is largely dependent on binding of gO or the UL128-131 proteins (80). Analysis of the cell lysate from ME infected fibroblasts where the expression of UL128-131was repressed (HFF-tet) showed that there was even a smaller proportion of endo H resistant gH and gL detected (Figure. 4-2B), as compared to that in regular fibroblast cells as shown in Figure. 4-2A. When the assembly of gH/gL/UL128-131was suppressed, the transport of gH/gL should be mainly dependent on the assembly of gH/gL/gO, thus this result indicated that ME gH/gL/gO assembly was inherently not efficient even without the competitor UL128-131. This finding was also consistent with previous reports that ME virions produced from HFF-tet cells contained elevated level of gH/gL/gO, but that amount of gH/gL/gO didn't compensate the amount of gH/gL that was used to be in the form of gH/gL/UL128-131. In summary, these results suggested that for ME, the endo H resistant gH/gL observed in Figure. 4-2A was largely due to the formation of gH/gL/UL128-131, and that the deficit of total gH/gL in the virion represents an inefficient assembly of gH/gL/gO.

Recently, Li *et al.* reported that a viral protein, UL148, likely acted as an ER-chaperone that favors the formation of gH/gL/gO (287). Analysis of TR and ME infected fibroblast cell lysate showed that ME expressed quite less of UL148 protein (Figure. 4-1B). This result was consistent with the model that UL148 promotes the assembly of gH/gL/gO, and suggested that the dearth of gH/gL/gO in ME virions was likely due to both high expression of UL128-131 and low expression of UL148.

Characterizations of HCMV gO inter-strain swap mutants. Compared to the more conserved gH, gL and Ul128-131 proteins, gO is one of the most diverse proteins encoded by the virus, with 10 to 30% diversity at amino acid sequence level among the eight families that are categorized based on phylogenetic analysis of more than 40 clinical isolates (reported in (89) and Chapter 2). Because gH/gL/gO and gH/gL/UL128-131 are formed through competitive interactions, one compelling hypothesis was that gO sequence diversity might influence the assembly of the two gH/gL complexes through differential binding affinity to gH/gL. To test this hypothesis, gO inter-strain mutants were constructed by swapping the UL74 ORFs between strain ME and TR. ME virus with TRgO was designated as MEdeltaTRgO, and TR virus with MEgO was designated as TRdeltaMEgO. Both mutant viruses were viable, and further characterizations were performed. HFF cells were infected with MEdeltaTRgO, ME, TR and TRdeltaMEgO at MOI=1, and extracellular virions were analyzed by western blotting under non-reducing conditions. Gel loads were normalized to total amount of gH/gL for better comparison of the ratio. Probing with common antigen gL showed that MEdeltaTRgO was a gH/gL/UL128-131-rich virus as wild type ME, and TRdeltaMEgO was a gH/gL/gO-rich virus as wild type TR. There might be slightly more gH/gL/gO in MEdeltaTRgO virus than ME, but this clearly did not fully explain why ME virion is deficient in gH/gL/gO (Figure. 4-3A). Therefore, swapping gO did not drastically affect the assembly of gH/gL complexes, suggesting that the sequence diversity of gO did not greatly influence the binding affinity of gO to gH/gL.

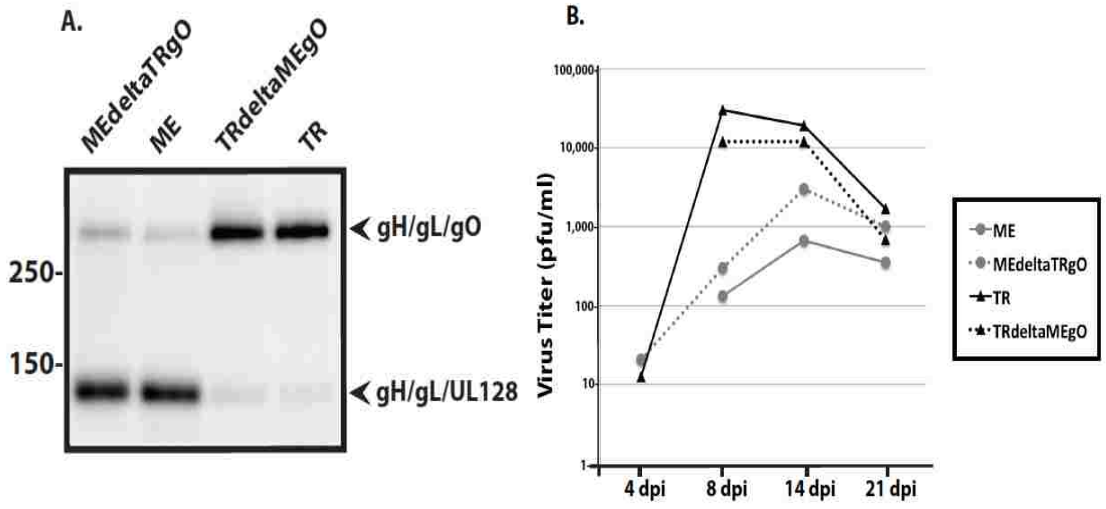

Figure 4-3 Characterizations of HCMV gO inter-strain swap mutants.
(A) Cell-free virions of MEdeltaTRgO, ME, TR and TRdeltaMEgO were analyzed by western blotting under non-reducing condition. Gel loads were normalized to equal amount of total gL and blot was probed with antibody specific to gL. Mass markers (kilodaltons) are shown on the left. (B) HFF cells were infected with the indicated viruses at MOI=0.1, and cell-free supernatants were retained at different time points after infection and then titrated on HFF to measure the infectivity of cell-free progeny viruses. (Note: titer of ME and TRdeltaMEgO were zero at 4 dpi, which cannot be plotted on a log scale axis).

To test whether the virus infectivity is altered in the presence of different gO isoform, multi-step growth curve analysis was performed. HFF cells were infected by the indicated viruses at MOI=0.1. Extracellular viruses were harvested at various time points post infection, and the infectivity was measured by plaque assay on HFF cells. TR produced higher peak titers of cell-free virus than those of ME by more than 40-fold, which is consistent with previous report in Chapter 3 that TR had a better specific infectivity on fibroblasts than ME (291). Mutant viruses exhibited roughly similar pattern as their corresponding wild type viruses. However, in both genetic backgrounds, TRgO seemed to correlate with a more robust replication of

the virus (Figure. 4-3B). Since this experiment was only performed once, no definitive conclusion can be drawn so far.

Characterizations of HCMV gH N-term inter-strain swap mutants. There are small amino acids polymorphisms residing on the N-term of gH. It seems possible that these polymorphisms might influence the assembly of gH/gL complexes. To test this hypothesis, gH N-term inter-strain mutants were constructed by swapping between the N-term of 10~36 amino acids sequence of ME gH and 10~37 amino acid sequence of TR gH. Designations of the mutant viruses mimic gO swap mutants for the purpose of consistency, with MEdeltaTRgH and TRdeltaMEgH respectively.

To characterize the level of gH/gL/gO and gH/gL/UL128-131 on virion envelope, fibroblast HFF cells were infected by TR, TRdeltaMEgH, ME and MEdeltaTRgH at MOI=1. Extracellular virions were harvested and analyzed by western blotting under reducing conditions (Figure. 4-4A). When gel loads were normalized to equal amount of MCP, all viruses contained comparable amount of gB, while TR contained more total amount of gH/gL than ME (consistent with the report in Chapter 3). Swapping the N-term of gH didn't dramatically change this pattern. Analysis of gH/gL complexes ratio by non-reducing western blotting showed that the vast majority of gH/gL was in the form of gH/gL/gO in TR while most of gH/gL in ME was in the form of gH/gL/UL128-131 (consistent with report in Chapter 3). The corresponding mutant viruses didn't change the ratio pattern either (Figure. 4-4B). Thus, N-term polymorphisms in gH didn't dramatically influence the assembly of gH/gL complexes.

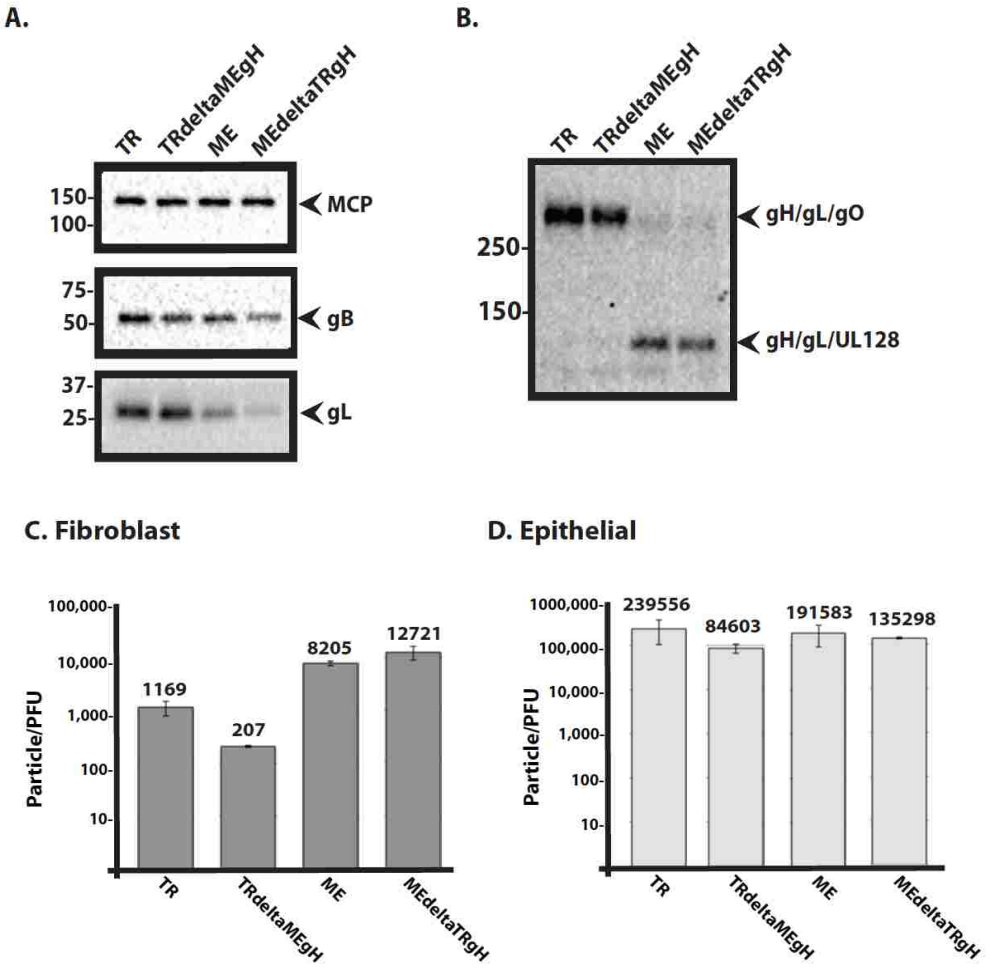

Figure 4-4 Characterizations of HCMV gH N-term inter-strain swap mutants.
Cell-free virions of HCMV TR, TRdeltaMEgH, ME, and MEdeltaTRgH were analyzed by western blotting under reducing (A) and non-reducing (B) conditions. Gel loads were normalized to equal amount of MCP. Reducing blots were probed with antibodies specific for the 55-kDa portion of gB, and gL. Non-reducing blot was probed with antibody to gL. Mass markers (kilodaltons) are shown on the left. Cell-free stocks of indicated viruses were analyzed by qPCR to quantitate genome-containing virions, and PFU were determined on fibroblasts (C) or epithelial cells (D). The particle-to-PFU ratio was calculated as the number of genomes per milliliter to the number of PFU per milliliter. The averages and standard deviations of three experiments are plotted.

To test whether gH N-term sequence polymorphisms affect gH/gL complexes functions, particle-to-PFU ratio on either fibroblasts or epithelial cells were characterized. Cell-free virions of indicated viruses were analyzed by qPCR to

measure genome-containing virions and plaque assay on both cells type to determine titers, and particle-to-PFU ratio was calculated. Consistent with the report in Chapter 3, on fibroblasts TR was more infectious than ME. Interestingly, TRdeltaMEgH was about 5-fold more infectious than TR while MEdeltaTRgH was reciprocally less infectious than ME, although with less than 2-fold difference (Figure. 4-4C). Since the amount of gH/gL/gO was not greatly changed, it is possible that the gained replication efficiency of TRdeltaMEgH on fibroblast cells is due to the qualitative differences in gH/gL/gO complex exerted by ME N-term of gH. Particle-to-PFU measurements were generally higher on epithelial cells. Extremes were TR and TRdeltaMEgH, again with TRdeltaMEgH slightly more infectious than TR; MEdeltaTRgH and ME were comparable to each other at intermediate values (Figure. 4-4D). Therefore, the specific infectivity on epithelial cells was roughly unaffected by swapping the N-term of gH.

DISCUSSION

During initial infection, dissemination, establishment of latent/persistent infection, and reactivation/shedding, HCMV requires a "entry machinery" that enables itself to enter a wide array of distinct cell types in the host. HCMV employs two gH/gL complexes that could influence cell tropism. Our current model suggests that gH/gL/gO provides the "core-fusion function" for entry into all cell types, probably through interaction with the fusion protein gB. Meanwhile, gH/gL/UL128-131 acts through a distinct function for virus entry into select cell types, like epithelial/endothelial cells, which broadens viral tropism. The fact that different

HCMV strains vary in the relative amount of gH/gL/gO and gH/gL/UL128-131 on the virion envelope indicates that the amount of the two gH/gL complexes could be adjusted by the virus differentially. However, the mechanisms that regulate the assembly of the two gH/gL complexes have remained mostly elusive.

Comparison of glycoproteins steady-state levels in the infected cells showed that strain ME expressed considerably more UL128-131 proteins, which correlated well with the high level of gH/gL/Ul128-131 in its virion. And this result is consistent with the observation reported in Murrell *et al.,* where polymorphisms in the UL128-131 locus of strains TB40/E and FIX, both of which contained little gH/gL/UL128-131 in the virion, when introduced into ME, were found to reduce the incorporation of gH/gL/UL128-131 (281). However, ME is not only gH/gL/UL128-131-rich, but also contains less total amount of gH/gL, and apparently the UL128-131 locus cannot be the sole reason to explain why ME behaves the way it does. Intracellular trafficking studies showed that ME gH/gL was poorly transported from ER to trans-Golgi apparatus, albeit the similar expression levels of gH/gL compared to strain TR, which contains a lot more total amount of gH/gL in the virion. The finding that even less efficient transport of ME gH/gL from ER to trans-Golgi apparatus in fibroblast cells where the expression of UL128-131 were repressed suggested that the inefficient transport of ME gH/gL was likely due to the poor assembly of gH/gL/gO.

Recently, Li *et al.* reported that another viral glycoprotein UL148 could increase the virion incorporation of gH/gL/gO, and this effect may or may not comes at the expense of gH/gL/UL128-131 (287). Given the positive role of UL148 in favoring

the formation of gH/gL/gO, it is possible that the expression level of UL148 could affect the assembly of the gH/gL complexes. Data here showed that ME expressed considerably less UL148, which correlated with its poor assembly efficiency of gH/gL/gO. In the future, it would be important to characterize whether boost of ME UL148 level by trans-expression would influence the abundance of gH/gL/gO in ME virion, and ask why different strains of HCMV differ in the expression level of UL148.

Disruption of UL148 in TB decreased the level of gH/gL/gO in virion envelope without largely increasing the amount of gH/gL/UL128-131. The resulting UL148-null virus had similar infectivity on fibroblast cells, but improved replication efficiency on epithelial cells as compared to wild type TB (287). On the other hand, disruption of UL148 in ME resulted in a decreased infectivity on epithelial cells (Brent's communication with Richard Stanton), although the influence on the level of gH/gL complexes hasn't been looked carefully to date. Thus, the effect of UL148 on HCMV infectivity seems to vary depending on the genetic background of the virus. And this discrepancy draws attention to the unaddressed question, what is the minimum amount of either gH/gL/gO or gH/gL/UL128-131 required for their function respectively. It is likely that the amount of gH/gL/gO in TB virus is in large excess above the "threshold" of gH/gL/gO required to promote gB-fusion, such that even though the amount of gH/gL/gO was reduced in UL148-null virus, that amount is still ample to keep gH/gL/gO at comparable efficiency. The little amount of gH/gL/UL128-131 in TB is probably close to the "threshold" of gH/gL/UL128-131 required for its function on select cell types, and the increased infectivity of TB UL148-null virus on epithelial cells could be due to the possibility of slightly

increased gH/gL/UL128-131, which was not detected in Li *et al.* (287). However, for ME, a gH/gL/gO-deficit virus, the amount of gH/gL/gO it contains might be closer to, or even below the "threshold", such that decreasing the amount of gH/gL/gO might result in a "sharp" drop in infectivity. Disruption of UL148 in ME might result in reduced amount of gH/gL/gO, which decreases the virus infectivity, like on epithelial cells, as mentioned above. Therefore, the different amount of gH/gL complexes that strains of HCMV contain indicates where they are relative to the minimum amount of either gH/gL/gO or gH/gL/UL128-131 required, and that would be important reference to the interpretation of mutant phenotypes, when the abundances of gH/gL complexes are changed.

The mechanisms by which UL148 works remain largely uncharacterized at this time. Interestingly, what Li *et al.* found is that UL148, an ER retained protein, exclusively interacted with gH/gL complexes containing UL130 and UL131, but not with UL128 or gO (287), suggesting that UL148 might compete with either gO or UL128 for binding onto gH/gL, but the binding of UL148 apparently doesn't hurt the loading of UL130/UL131. Therefore, UL148 seems to temporally "reserve" a reservoir of gH/gL in ER from forming either gH/gL/gO or gH/gL/UL128-131 complexes. But how does UL148 eventually favor the assembly of gH/gL/gO? One possibility is that UL148 passively promote gH/gL/gO formation by occluding gH/gL/UL128-131. Because gO and UL128 each covalently binds to gH/gL through the disulfide-bond with the same cysteine residue on gL, whereas UL130 and UL131 do not (76, 77, 80, 215), binding of UL148 with gH/gL may prevent UL128 from forming a disulfide-bond with gL, which could be an irreversible process required

for gH/gL/UL128-131 assembly. On the other hand, the resulting intermediate complex "gH/gL/UL148/U130/UL131" might be less stable given the noncovalent linkage between UL130/UL131 and gH/gL. Thus, at some point this complex would dissociate, providing the opportunity for gO to load to gH/gL, which then allows the assembly of gH/gL/gO. Alternatively, another non-mutually exclusive possibility is that binding of UL148 to gH/gL may actively help gH/gL fold into a favorable conformation for gO to bind. Since whether UL148 specifically binds to the immature form of gH/gL/UL130/UL131 or also binds to gH/gL dimer in the absence of UL128-131 is currently not known, it is hard to distinguish the abovementioned "passive" versus "active" mechanisms. But if UL148 could bind to gH/gL dimer in the absence of UL128-131, and still promote the formation of gH/gL/gO, it would suggest UL148 could act actively for gH/gL/gO assembly. Nevertheless, in either scenario, the loading of gO would likely displace UL148 in some manner in order to form gH/gL/gO. Characterization of the working mechanism of UL148 would be an interesting spot of research in the future.

Although neither gO or gH N-term inter-strain swap mutants showed obvious changes on the viral composition of gH/gL complexes, there were slightly better replication efficiency associated with TRgO, and the N-term of MEgH on fibroblast cells respectively. It is not clear yet if the increased infectivity is the result of slight changes in the amount of gH/gL/gO, or is indicative of functional differences of TRgO and N-term of MEgH. Future work is needed to clarify those possibilities in detail, and if the growth difference is not due to the alteration in the abundance of gH/gL/gO, these results would be the first piece of evidence suggesting that the

sequence diversity of gO or the polymorphisms of gH N-term could influence the function of gH/gL/gO.

In summary, studies in this chapter helped to sort out some viral factors that underpin the differential regulation of the gH/gL complexes assembly, that was expression levels of UL128-131 and UL148 likely contribute to the regulation through the influence on the competitive assembly between gH/gL/gO and gH/gL/UL128-131.

CHAPTER 5. GENERAL DISCUSSION AND FUTURE DIRECTIONS

HCMV is a significant public health concern due to the severe diseases that it can cause in immunocompromised and/or immunoimmature individuals. The toxicity and drug-resistance associated with antiviral drugs and the lack of licensed vaccine have highlighted the need for a better understanding of the molecular biology of the virus, which will, in turn, aid the development of effective vaccines against this virus, as well as the design of antiviral therapeutic treatment in the future.

The overall focus of this dissertation is on the mechanisms by which HCMV manages to enter different cell types. The current consensus is that the interaction between gH/gL and the fusion protein gB serves as the conserved entry mechanism among all herpesviruses (reviewed in (189)). Additionally, each herpesvirus encodes accessory proteins that bind to gH/gL, and can regulate the action of the core fusion machinery. HCMV gH/gL can be bound by gO, or by the set of proteins UL128, UL130, and UL131, forming gH/gL/gO and gH/gL/UL128-131 complexes on the virion envelope (73, 74, 76, 77, 80, 227). There has been a lot of interest in these complexes as targets for vaccine development. However, the mechanisms by which these complexes facilitate virus entry are still poorly understood. Moreover, the specific function of gO in gH/gL/gO complex has not been addressed in detail yet.

gO is among the most diverse proteins encoded by the virus. There are at least 8 isoforms of gO in nature, varying by 10~30% at amino acid sequence level (89). It is reasonable to hypothesize that the amino acid differences of gO might affect the properties of gO isoforms. Thus, the research work in this dissertation was started by addressing the function of gO through comparative analysis of different gO

isoforms. The results of these studies, as detailed in **Chapter 2**, indicated that gO and UL128-131 competed for binding to gH/gL, and that the ratio of the two complexes on the virion envelope can vary dramatically among strains. The finding that some strains contain mostly gH/gL/gO, whereas others contain mostly gH/gL/UL128-131 was quite surprising. While these studies did not shed light on the function of gO, they did provide an opportunity to address the important roles that the two gH/gL complexes play in virus entry into different cell types.

gH/gL and gB comprised the minimum set of requirement for HCMV fusion in cell-cell fusion experiment, suggesting HCMV gH/gL is able to promote gB-mediated fusion (216). This function of gH/gL is referred to as the "core fusion function". However, in virus scenario, there is little, if any, unbound gH/gL. Most of the gH/gL is in the form of gH/gL/gO or gH/gL/UL128-131 complexes, thus it is possible that either one of those two complexes or both of them can perform the core fusion function.

The functions of HCMV gH/gL complexes have been mostly studied with deletion mutants. UL128-131 deletion mutants replicate well on fibroblasts but fail to enter epithelial/endothelial cells, suggesting that gH/gL/UL128-131 is required for entry into epithelial/endothelial cells but is dispensable on fibroblasts. This phenotype is often interpreted to mean that entry into epithelial/endothelial cells is facilitated by gH/gL/UL128-131, whereas entry into fibroblasts is facilitated by gH/gL/gO. The resulting model suggests that both gH/gL/UL128-131, and gH/gL/gO provide essentially the same function for entry into different cell types, i.e., promoting gB-mediated membrane fusion. This was an attractive model due to

the analogy with the well-studied Epstein-Barr virus (EBV) tropism model. EBV's gH/gL exists in two forms as well, naked gH/gL or gH/gL/gp42 complex. Entry into epithelial cells is facilitated by gH/gL, through interactions with specific integrin on the cell surface. Binding with gp42 blocks the interaction of gH/gL with integrin, instead, gH/gL/gp42 interacts with MHCII molecule on B cell surface, thus facilitates virus entry into B cells. Therefore, EBV gH/gL and gH/gL/gp42 can both promote gB-mediated fusion into different cell types, with gp42 acting like a tropism switch adapter. While UL128-131 deletion mutants replicate well on fibroblasts, gO deletion mutants fail to enter any cell type, suggesting that gH/gL/UL128-131 is not sufficient to promote gB-mediated fusion into epithelial/endothelial cells, and entry into that cell type requires both complexes. However, further analysis is largely limited due to the severe growth defect of the gO mutants and the lack of a complementation system.

The work in **Chapter 3** avoided the caveats of gO-null mutants by employing a panel of HCMV strains that contained different amounts of gH/gL/gO and gH/gL/UL128-131 complexes. Since none of the viruses were absolutely lacking either complex, they did not exhibit severe growth defect as observed in gO-null mutants, thus the infectivity measured were more biologically relevant. Cell-type specific particle-to-PFU ratios were determined and showed that infection of both fibroblasts and epithelial cells was positively correlated with the abundance of gH/gL/gO, but not with gH/gL/UL128-131. And the low infectivity of the gH/gL/gO-deficit virus on both cell types could be recovered by treatment with chemical fusogen PEG, indicating that the deficiency of infection was due to less efficient

fusion. These results confirmed that infection of fibroblasts requires gH/gL/gO, and further showed that infection of epithelial cells requires both gH/gL/gO and gH/gL/UL128-131. The sufficient role of gH/gL/gO in promoting gB-mediated fusion on fibroblasts demonstrated that gH/gL/gO is clearly fusion competent. The necessary role of gH/gL/gO on epithelial cells suggested that gH/gL/gO likely acts as the fusion competent complex on that cell type as well. This speculation supports the data described above better than either of the other two explanations: 1) gH/gL/gO is fusion competent on fibroblasts but not on epithelial cells, instead it performs an alternate function. 2) The two different gH/gL complexes perform the same (redundant) core fusion function on epithelial cells. Therefore, we proposed a new model where gH/gL/gO provides the conserved herpesvirus gH/gL "core fusion function" of promoting gB-mediated fusion for entry into all cell types, whereas gH/gL/UL128-131 provides a non-fusion, yet necessary mechanism to allow infection of select cell types (i.e. epithelial/endothelial cells). This is a lot different from the EBV tropism model, and to our knowledge, is also the first report of a fusion-incompetent gH/gL complexes in the herpesvirus field, which might provide an important opportunity to study the fundamental mechanism by which gH/gL regulates gB fusion. These results also help explained the cell type-dependent virus neutralization exhibited by anti-gH/gL/UL128-131 antibodies, and underscore the importance of gH/gL/gO as a potential HCMV vaccine or therapeutic target.

Several important questions regarding to the model are still remaining to be addressed: 1) what underpins the distinct functions exhibited in gH/gL/gO and gH/gL/UL128-131? , 2) what is the non-fusion function of gH/gL/UL128-131? , and

3) What is gO's role in the fusion competent complex gH/gL/gO? Some speculations regarding those questions will be discussed below.

What underpins the distinct functions exhibited in gH/gL/gO and gH/gL/UL128-131? Studies of HSV and EBV suggest that the surfaces of gH/gL could directly contact with gB to promote membrane fusion (reviewed in (189)). For HCMV, cell-cell fusion assay demonstrated that gH/gL and gB comprised the minimum requirement for membrane fusion, indicating that HCMV gH/gL is capable of triggering gB-fusion as well (216). The distinct function model raises the possibility that the "profusion" surfaces of HCMV gH/gL may be differentially formed or exposed on gH/gL/gO versus gH/gL/UL128-UL131. This discrepancy might be due to the direct binding with gO versus UL128-131, or allosteric conformation changes of gH/gL as a result of the binding with gO versus UL128-131. Although Cryo EM revealed overall similar architecture between the two complexes (215, 215), detailed structure differences were not resolved in those studies. Our lab have created a library of gH and gL charge-cluster-to-alanine mutants, which might allow more detailed structure differences between gH/gL/gO and gH/gL/UL128-131 to be elucidated in the future.

The other possibility is that gH/gL/gO and gH/gL/UL128-131 might not be uniformly distributed on the virion envelope; in other words, they might cluster in different "landscape". One speculation could be that the fusion competent gH/gL/gO complex might either be spatially closer to gB or be more flexible in mobility on the viral membrane, such that upon triggering, it could interact with gB with proximity or "swim" around and have higher probability to interact with gB. In contrast, fusion

incompetent gH/gL/UL128-131 complex might be more spatially sequestered or restricted in mobility. Precedents have been reported in herpesvirus that glycoproteins could be associated with the less fluid lipid raft on membranes (292–294). It could be possible that lipid raft might play a role in the distinct functions model of gH/gL/gO and gH/gL/UL128-131.

What is the non-fusion function of gH/gL/UL128-131? gH/gL/UL128-131 null virus cannot replicate on epithelial/endothelial cells, but with the aid of chemical fusogen PEG, the mutant virus could establish infection as well as wild type virus (79), suggesting that the deficiency is at pre-fusion but not post-fusion steps. Infection of HCMV on epithelial/endothelial cells has been showed to happen through endocytosis in a pH-dependent manner (79). Trans-expressed gH/gL/UL128-131 was showed to exert interference on epithelial cells, indicating that the complex is involved in receptor interaction (229). Moreover, recent study showed that gH/gL/UL128-131 triggered specific signaling transduction pathway for virus internalization into monocytes, the cell type where entry requires gH/gL/UL128-131 as epithelial/endothelial cells does (230). Taken the above evidences together, one compelling model would be that gH/gL/UL128-131 interacts with its receptors on epithelial/endothelial cell surface and triggers specific signaling/endocytosis pathway, the process of which is required for subsequent gH/gL/gO-promoted core fusion event to occur.

However, it is worth to note that gH/gL/UL128-131-null viruses are still efficiently internalized into epithelial/endothelial cells (79, 231, 258, 295), suggesting gH/gL/UL128-131 is dispensable for the internalization of the virus.

Perhaps, it is not surprising that non-specific endocytosis could be triggered even without gH/gL/UL128-131 given that the virus contains numerous different glycoproteins on the virion envelop. However, the non-specific endocytosis would not lead to productive infection, indicating that efficient fusion can only occur in specific endosome compartments triggered by gH/gL/UL128-131-dependent endocytosis.

What is gO's role in the fusion-competent complex gH/gL/gO? It seems quite clear now that at least one conserved function of gO is to facilitate the incorporation of the fusion-competent gH/gL/gO into the virion envelope. But does gO perform more function other than that?

gH/gL/gO expressed in trans system exerted interference on fibroblasts but not on epithelial cells (239), suggesting that the complex engages some currently uncharacterized receptor on the surface of fibroblasts. This receptor-gH/gL/gO interaction likely triggers gB-mediated fusion, resulting in virus entry into fibroblasts through either direct fusion with plasma membrane or from within endosomes followed by macropinocytosis at neutral pH (185, 186). The negative data of interference on epithelial cells could be explained at least by the following two possibilities: 1) in a trans-expressed system, gH/gL/gO cannot reach to a compartment where its receptor resides. In fact, trans-expressed gH/gL/gO was showed to be mostly retained in Golgi-like compartments (239). It is possible that receptor of gH/gL/gO in epithelial cells is sequestered in different compartments, such as early endosomes or multivesicular bodies, thus making gH/gL/gO-receptor interaction spatially impossible. 2) Or simply the receptor for gH/gL/gO is not

available on epithelial cells, suggesting that the conformational change of the complex on epithelial cells does not require a receptor; instead, the ligand could be a proton, which is acquired in low-pH endosomes after endocytosis. Recently, there is evidence suggesting that gH/gL/gO can bind to PDGFRα, which is selectively expressed on fibroblasts but not on epithelial cells (Manuscript reviewed by Brent). This seems to favor the latter possibility, and also help to explain that the requirement for gH/gL/UL128-131 on epithelial cells is probably to overcome the lack of gH/gL/gO receptor, and that gH/gL/UL128-131-dependent endocytosis somehow provides an alternative way for gH/gL/gO-mediated gB fusion to occur.

gO might directly involve in gH/gL/gO's function (i.e. receptor interaction), and it is conceivable that gO sequence diversity might affect the "efficiency" of gH/gL/gO in performing its job. In line with this hypothesis, ME-TR inter-strain gO swap mutants displayed slight growth difference as compared to wild type virus (**data in Chapter 4**). However, more detailed studies in the future are warranted to verify whether the growth difference is due to entry, and also whether this observation applies to other gO isoforms. Moreover, since gO is heavily glycosylated, it is possible that it could also act like a glycan shield, protecting gH/gL from neutralizing antibodies. No research has been carried out in testing this hypothesis in the context of gO sequence diversity. Our lab has created a library of anti-peptide antibodies raised against gH and gL and there are also anti-gH mAbs library created by Brent's colleague Bill Britt. So future research could include the screening of the strain-specific neutralizing antibodies from the above antibody resources and testing the correlation of neutralization specificity with gO isoforms.

In summary, the work in Chapter 2 and Chapter 3 showed that different strains of HCMV varied dramatically in the relative amount of gH/gL/gO and gH/gL/UL128-131, and this could influence virus infectivity on different cell types due to the distinct functions of the two gH/gL complexes during entry and tropism. The next question was that why strains differ in the abundance of the gH/gL /gO and gH/gL/UL128-131. Recently, Li *et al.* reported a viral protein, UL148, which might act as an ER-chaperone to promote the formation of gH/gL/gO over gH/gL/UL128-131 (287). The research work in **Chapter 4** found that strains of HCMV varied in the protein expression level in the infected cells. ME expressed much more of UL128-131 proteins, but less of UL148 protein as compared to TR. gH/gL levels were more comparable, however while TR gH/gL were efficiently transported to the trans-Golgi derived virus assembly compartment, ME gH/gL remained mostly in the ER. These results suggested that both high expression of UL128-131 and the low expression of UL148 probably contributed to the poor assembly of ME gH/gL/gO in ER, which likely dampened the transport of gH/gL to trans-Golgi derived assembly compartment, and thus resulted in the less total gH/gL, and dearth of gH/gL/gO in ME virion envelope. Therefore, the assembly of gH/gL/gO and gH/gL/UL128-131 is probably regulated through interstrain variability at multiple loci. In the future, a further characterization of how the virus adjusts the level of those viral factors (i.e., Ul128-131 and UL148) would help us better understand how HCMV determines tropism and navigates through the host.

The abovementioned distinct function model is focused on the contribution of gH/gL complexes to cell-free entry of HCMV. Another way of virus infection is via

cell-to-cell spread. While cell-free entry is apparently important for HCMV to establish infection in a new host, cell-to-cell spread is probably essential for virus dissemination within a host and can be one of the strategies that the virus utilize to evade immune surveillance. Cell-free infection and cell-to-cell spread could be mechanistically distinguished by using neutralizing antibodies: cell-free entry is sensitive to neutralizing antibodies whereas cell-to-cell spread is largely resistant. How HCMV spreads cell-to-cell is currently unknown. The fact that double deletions of gO and UL128-131 proteins are lethal to the virus indicates that viral spread absolutely depends on either of, or both of gH/gL/gO and gH/gL/UL128-131, but not on gH/gL dimer (180). Single deletion of either gO or UL128-131 proteins results in a phenotype with similar spreading efficiency on fibroblast cells, suggesting that gH/gL/gO and gH/gL/UL128-131 probably provide redundant function for viral spread on fibroblast cells (79, 180). On epithelial cells, single deletion mutants of UL128-131 proteins cannot spread, while single deletion mutants of gO spread slightly better, suggesting that gH/gL/UL128-131 is necessary and sufficient for cell-to-cell spread on epithelial cells, but gH/gL/gO is dispensable (79, 238). Note how gH/gL/gO and gH/gL/UL128-131 are differentially required in cell-free entry versus cell-to-cell spread, and this discrepancy can be important consideration to take when it comes to studies on the molecular biology of HCMV transmission and dissemination. An intriguing difference between virus cell-to-cell spread and transiently cell-cell fusion is that gH/gL dimer is sufficient to trigger gB-mediated cell-cell fusion but it cannot do it in the context of virus cell-to-cell spread. This fits so well with the observation that HCMV infection usually would not result

in the formation syncytium (massive cell-cell fusion), while gH/gL and gB expressed in trans exclusively generates syncytium, indicating that the cell-to-cell spread during virus infection is somehow less "disruptive" and mechanistically different from gH/gL and gB promoted cell-cell fusion in trans. One speculation could be that during HCMV infection, gH/gL dimer is prevented to reach to the cell surface or cell-cell junction where it can interact with gB to trigger cell-cell fusion. If so, UL148 might be a potential candidate since it is an ER-chaperone that can bind to gH/gL (Kamil's new abstract), and probably holds off the immature gH/gL in ER, thus negatively controls the extent of cell-cell fusion during virus infection.

HCMV is a virus that has been successfully adapted to the human host, which exhibits significant levels of genetic diversity between individuals (interhost diversity) and also within a single individual (intrahost diversity) (reviewed in (142)). The intrahost diversity of HCMV has been largely underappreciated previously in the field, and it might have important influence on virus pathogenesis. This diversity may provide a mechanism by which the virus could rapidly adapt to the changing host environments through influences on virus ability of dissemination. The possible role in dissemination is likely based on viral glycoproteins, which are involved in virus entry and tropism. Even with the glycoproteins that this dissertation studies, there was a dramatic variation that different HCMV strains exhibit in the relative amount of gH/gL/gO and gH/gL/UL128-131 complexes on the virion envelope and this variation significantly influenced virus infectivity on different cell types. It is conceivable that there are genetic bases that attribute to this trait. One possibility is that during infection in human host, HCMV exists as a

diverse population that may harbor different genetic variations affecting the relative abundance of gH/gL/gO and gH/gL/UL128-131, such that upon encountering distinct host tissue/cell, subset of the population could replicate better than the others, resulting in overall efficient dissemination. Although no clear conclusion can be drawn to date on the correlation between the variation of HCMV genotypes and the risk of virus transmission, or the outcome and severity of diseases, HCMV intrahost diversity is an important aspect that needs more appreciation and might help with future vaccine or antiviral drug design.

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