HOW HUMAN CYTOMEGALOVIRUS GLYCOPROTEINS MEDIATE ENTRY

By

Paul T. Wille

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

Paul T. Wille

has been approved

David Johnson, Ph.D., Mentor

Klaus Früh, Ph.D., Chair

Jay Nelson, Ph.D.

William Skach, Ph.D.

Scott Wong, Ph.D.

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HCMV: human cytomegalovirus

HSV: herpes simplex virus

MCMV: murine cytomegalovirus

PrV: pseudorabies virus

EBV: Epstein-Barr virus

KSHV: Kaposi's sarcoma associated herpesvirus

RhCMV: rhesus cytomegalovirus

VSV: vesicular stomatitis virus

HHV-6: human herpesvirus 6

HPV: human papillomavirus

HIV: human immunodeficiency virus

AIDS: acquired immunodeficiency syndrome

HAART: highly active anti-retroviral therapy

gB, gC, gD, gE, gH, gI, gL, gM, gN, gO, gQ: glycoprotein B, C, D, E, H, I, L, M, N, O, Q

HSPG: heparan sulfate proteoglycan

GAG: glycosaminoglycan

LTP: large tegument protein

MCP: major capsid protein

mCP: minor capsid protein

mc-BP: minor capsid binding protein

HVEM: herpesvirus entry mediator

HLA: human leukocyte antigen

HA: hemagglutinin

EGFR: epidermal growth factor receptor

DLD: disintegrin-like domain

PDGFRα: platelet-derived growth factor α receptor

GFP: green fluorescent protein

PI(3)K: Phosphoinositide 3-kinase

endo H: endoglycosidase H

DNase: deoxyribonuclease

KLH: keyhole limpet hemocyanin

MAb: monoclonal antibody

BSA: bovine serum albumin

ICP: infected cell protein

pp: phosphoprotein

gp: glycoprotein

kDa: kilodalton

ER: endoplasmic reticulum

RER: rough endoplasmic reticulum

ERC: endosomal recycling compartment

ERGIC: endoplasmic reticulum-Golgi-intermediate compartment

TGN: trans Golgi network

MOC: microtubule organizing center

cp: cytoplasm

pm: plasma membrane

DNA: deoxyribonucleic acid

cDNA: complementary DNA

siRNA: short interfering ribonucleic acid

BAC: bacterial artificial chromosome

PCR: polymerase chain reaction

qPCR: quantitative PCR

FRT: Flp recombination target

IRES: internal ribosomal entry site

wt: wild-type

nt: nucleotide

kan^R: kanamycin resistance

UL: unique long

US: unique short

IE: immediate early

LMP: late myeloid progenitor cell

PBMC: peripheral blood mononuclear cell

CHO: Chinese hamster ovary

NHDF: normal human dermal fibroblasts

AEC: aortic endothelial cell

HPV-AEC: HPV E6/E7 transformed AEC

MEM: minimum essential medium

DMEM: Dulbecco's minimum essential medium

FBS: fetal bovine serum

BGS: bovine growth supplement

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

FFWO: fusion from without

IF: immunofluorescence

EM: electron microscopy

CPE: cytopathic effect

PFU: plaque forming unit

MOI: multiplicity of infection

IU: infectious units

PEG: polyethylene glycol

PBS: phosphate buffered saline

ECL: enhanced chemilluminescence

ATCC: American type culture collection

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ABSTRACT

My studies focused on the role of human cytomegalovirus (HCMV) envelope glycoproteins in viral entry. My first set of studies questioned how a complex of HCMV glycoproteins, gH/gL/gO, functions to promote virus entry. Our laboratory has previously demonstrated that another complex of five HCMV proteins, gH/gL/UL128/UL130/UL131, is necessary for HCMV entry into epithelial and endothelial cells that requires endocytosis and low pH-dependent fusion with endosomal membranes. When I began my studies, it was believed that entry into fibroblasts was facilitated by HCMV gH/gL/gO and occurred at the plasma membrane. However, the exact role of gO in entry had not been thoroughly defined. I studied this genetically by constructing and characterizing a HCMV strain TR gO-null mutant. Together with biochemical characterization of gO, we discovered that in the HCMV strain TR, gO acts as a molecular chaperone to promote gH/gL ER export, then gO dissociates from gH/gL and is not found in the virion envelope. When gO was deleted, gH/gL incorporation into the virion envelope was significantly reduced, and these virus particles were unable to enter both fibroblasts and epithelial and endothelial cells.

My second set of studies focused on HCMV glycoprotein gB. For other herpesviruses, gB is thought to be the fusion protein, causing the virion envelope to fuse with cellular membranes during virus entry. Triggering of gB is thought to involve interactions between gB and gH/gL complexes. However, little is known about how HCMV gB promotes fusion or even if HCMV gB is the fusion protein. I found that HCMV gB could be expressed in cellular membranes and would promote the entry of HCMV virus particles lacking gB. This process, denoted entry *in trans*, was novel in that no one has described an instance in which a viral fusion protein can be expressed in cells and

mediate entry of virus particles lacking that fusion protein. By contrast, cells expressing HCMV gH/gL would not mediate entry of virus particles lacking gH/gL. In the entry *in trans* that I observed, gH/gL complexes are oriented toward the plasma membrane of cells and gB is oriented toward the virion envelope. Thus, these results support two important hypotheses: i) HCMV gH/gL complexes bind cellular ligands or receptors and can interact with gB across the space between the virion envelope and cell membranes and ii) gB is the fusion-inducing protein.

CHAPTER 1: INTRODUCTION

A. HCMV infection and pathogenesis

Human cytomegalovirus (HCMV) is a ubiquitous virus. The distribution of humans with antibodies to HCMV varies between 40% in industrialized nations and 100% in developing nations (115). The differences in seroprevalence have been attributed to crowding rather than geographical location and climate (188). Several routes of transmission lead to infection with HCMV early in life, including congenital (0.2 to 2.2% of live births), perinatal (contracted from the birth canal or after birth from the mother) or between children in group day care facilities, where between 15% and 70% of children acquire an HCMV infection (2, 83, 204-206). While a significant percent of infections occur in childhood, there is a near constant conversion rate from older childhood to middle age, such that there is 70% to 90% seropositivity in the elderly even in populations with lower overall prevalence (210, 235).

A1. Primary Infection. HCMV initiates infection by infecting epithelial cells, either mucosal epithelium or other types of epithelial tissues (144). Congenital infection occurs at the uterine-placenta interface, mediated either by embryonic cells carrying virus back to the embryo, or by hematogenous spread from the maternal blood (reviewed in (162)). In the first scenario, invading embryonic cytotrophoblasts become infected in the uterine wall and then carry virus to the embryo by moving in a retrograde fashion, subsequently infecting the embryonic cells of the anchoring chorionic villi (60). In the second scenario, HCMV present in the maternal blood space that surrounds the anchoring villi is endocytosed by syncytiotrophoblasts and transcytosed to the underlying cytotrophoblasts, which are subsequently infected (60, 133, 164). Perinatal infection

occurs when the neonate acquires virus horizontally, either during birth or soon following birth. The mother transmits virus during birth when her cervix is HCMV positive (179). Following birth, HCMV is transmitted to the neonate by the mother through breast milk (207), or is acquired via blood transfusion (243). Subsequent to the perinatal exposure period, HCMV is acquired in children by exposure to infected saliva and urine leading to infection of mucosal epithelium (159). Adults acquire HCMV through multiple routes, including from the saliva and urine of infected children (158), sexual contact (reviewed in Chapter 9 of (83)), transplanted organs (reviewed in Chapter 13 of (83)), and blood transfusions (reviewed in Chapter 9 of (83)).

A2. HCMV spread in the host. Following primary infection, HCMV spreads widely in its host. Analysis of the dissemination of mouse cytomegalovirus (MCMV) has led to a model where virus is spread from epithelial cells to the vascular endothelium, then subsequently to late myeloid progenitor cells (LMPs) (143, 156). The virus utilizes these leukocytes to spread throughout the body via the peripheral blood as demonstrated by the observation that virus can be recovered from long-term cultures of peripheral blood mononuclear cells (PBMCs) (200), and that monocyte/macrophages spread virus out of the blood and into underlying tissues (67, 132, 196). Following dissemination from the site of infection, virus can be found in many organs, including heart, lungs, spleen, liver, pancreas, adrenal glands, kidney, uterus, fallopian tube, ovary, esophagus, stomach, duodenum, jejunum, cecum, parotid, lymph nodes, breast, lip, skin, colon, pituitary, bone marrow, and brain (15, 153, 236). Cell types infected in these organs include epithelial cells, endothelial cells, macrophages, myocytes, pneumocytes, reticular cells, hepatocytes, acinar cells, mesenchymal cells, fibroblasts, glial cells, and neurons (15, 153, 236). The breadth of infected cells in disseminated HCMV infection

demonstrates that this virus is capable of entering and initiating replication in most cells and tissues.

A3. Epithelial and endothelial cells. Infection of epithelial cells and endothelial cells is critical for both dissemination and pathogenesis. Epithelial cells are infected by CMV at the site of infection and where the virus is transmitted from the body; e.g., salivary glands, mammary glands, the gastrointestinal tract, and the kidney (153). Endothelial cell infection is important both in terms of hematogenous dissemination and pathogenesis. Following the initial epithelial cell infection, HCMV produced by epithelial cells is not likely to be the source of virus that spreads to other cells throughout the body. Invading leukocytes are also unlikely to take virus back out into the blood stream, as these cells do not typically leave tissues they enter. More likely, virus derived from epithelial cells, invading leukocytes and connective tissue cells—e.g., fibroblasts—at the initial site of infection spreads to vascular endothelial cells (143). Then virus replication in endothelial cells leads to dissemination either through infection of leukocytes in the blood that contact infected endothelial cells, or through detachment of the infected endothelial cells, which then infect other cells as they are carried through the blood (143). Although this model predicts that spread of HCMV is mostly dependent upon cell-to-cell transmission, HCMV is also found as cell-free virions in the blood stream (50). Alternatively, monocyte/macrophages may become infected by virus shed from epithelial cells and these cells can move into the blood and infect distant organs either by crossing endothelial barriers or causing infection of endothelial cells.

A4. Latency or persistence. Following primary infection, dissemination, and the initiation of humoral and cellular immune responses, cytomegalovirus likely persists through several mechanisms involving several different cell types. One mechanism is

latency, strictly defined as the maintenance of the viral genome in cells with very limited gene expression and no production of infectious progeny. HCMV genomes are detectable in bone marrow derived cells that are precursors to leukocytes (reviewed in (193)). When these cells are cultured in vitro, they do not replicate the virus, despite containing the HCMV genome (68, 71, 113, 131, 142, 177). However, these cells can be forced to undergo differentiation, and the resulting monocyte-derived macrophages will then replicate virus (200). Although it is unclear the exact population of bone marrow-derived cells that cytomegalovirus initially invades in order to establish latency, it is clear that cytomegalovirus is persistently spread out of the blood compartment for the life of the host by CD34+ cells (193). Persistent infection or latency has also been suggested to occur in endothelial cells (97). In the case of persistence, HCMV might replicate continuously in endothelial cells, monocytes, macrophages or other cells but at a relatively low level, producing cell-free virus or virus that is spread via cell-cell contacts. Low-level replication may not be injurious to persistently infected cells and the virus might defend the cell from attack by immune responses using its many immune evasion proteins (reviewed in (168)). The variety of sites of potential latency and persistence are further evidence that cytomegalovirus relies on several different cell types during each stage of its infection of the host.

A5. HCMV pathology. The pathological consequences of human cytomegalovirus infection are directly tied to the effectiveness of the host immune responses, i.e. whether the host can restrict spread of virus and reduce its replication. Immunocompetent individuals rarely present any symptoms of infection and when they do occur, these symptoms are usually mild and typically range from flulike symptoms to mononucleosis, which is associated with infection of mononuclear leukocytes (3, 173,

180). In extremely rare cases, HCMV is associated with more serious diseases such as pneumonia and encephalitis in otherwise healthy patients (3, 173).

In most cases, HCMV-induced pathologies occur in immunocompromised or immunodeficient patients. The most significant of these populations are AIDS patients, fetuses and newborns, and transplant patients.

AIDS patients are immunocompromised. Thus, when the delicate balance between viral persistence/latency and immunity is upset, HCMV replicates at much higher levels. For AIDS patients, the most clinically relevant result of this increase in HCMV replication involves infection of retinal pigmented epithelial cells and neurons, leading to the destruction of the retina, i.e., retinitis (85, 189). Encephalitis caused by HCMV in AIDS patients is another destructive consequence of uncontrolled HCMV replication (236). Encephalitis in these patients is associated with HCMV infection of capillary endothelial cells, microglial cells and macrophages, and neurons (147). These symptoms can be indirectly ameliorated by highly active anti-retroviral therapy (HAART), which preserves sufficient immunity to prevent uncontrolled HCMV replication and the resulting pathologies (189). HCMV-induced retinitis can also be treated directly with ganciclovir (13).

Fetuses are infected via the placenta and maternal blood space, as described above, and HCMV is believed to cause a wide variety of effects on development, ranging from minor neurological defects and deafness to death (35). Acquisition during the perinatal period has also been associated with disease (5). HCMV causes disease in these circumstances because fetuses and newborns apparently have not developed the immune system functions required to limit HCMV spread (150), and thus several tissues, and therefore cell types, are targeted by the virus.

For transplant patients, intentional immunosuppression necessary to prevent organ rejection is often associated with increased susceptibility to HCMV. Increased virus burden can cause transplant rejection, either as a result of virus invading the organ from the recipient, or virus becoming reactivated in the transplanted organ. For example, in the case of kidney transplant, latently infected kidney capillary endothelial cells or leukocytes give rise to virus that produces disease in the recipient's kidney leading to rejection (82, 86). Treatment with ganciclovir is used frequently to prevent rejection in transplant patients (100).

HCMV has also been linked to several multifactorial inflammatory diseases, including vasculopathies such as atherosclerosis, transplant vascular sclerosis, and restenosis after coronary angioplasty (212). Persistent infections, e.g., in vessel walls, may contribute to inflammation by causing changes in cellular and tissue architecture, increasing immune responses against infected endothelial cells or smooth muscle cells and increasing movement and activation of these cells (212). HCMV infected cells can be detected in these inflammatory lesions. Moreover, *in vitro* infection of several cell types in atherosclerotic plaques (e.g., smooth muscle cells, macrophages, and endothelial cells) leads to phenotypic changes in these cell types including increased migration of smooth muscle cells, properties which may lead to decreased vessel diameter (199, 212-214). Infection of endothelial cells can promote angiogenesis (19).

These and many other studies make it clear that HCMV infects and replicates in a wide spectrum of cell types throughout the body, utilizing epithelial cells, endothelial cells, fibroblasts, leukocytes, monocyte/macrophages, neurons, and microglial cells to initiate infection, disseminate, and cause disease.

B. Overview of the HCMV replication cycle

B1. HCMV entry. To be able to infect all these diverse cell types, HCMV must first gain access into these cells. Simplistically, this involves virus adsorption onto charged structures on the cell surface, binding to more specific receptors, in some cases endocytosis, and membrane fusion between the virion envelope and cellular membranes (plasma membrane or endosomes), so that capsids reach the cytosol (Fig. 1-1, 1-2). Like other herpesviruses, this process begins with adsorption of HCMV onto heparan sulfate proteoglycans (HSPGs) (41). This low affinity adsorption step is dependent on charge attraction between basic residues in GAG consensus binding sites on the surface of the viral glycoproteins (namely glycoproteins B [gB] and M [gM]) and negatively charged HSPGs (41, 80, 108). Attachment is probably followed by more specific protein-protein interactions between HCMV glycoproteins and cell surface proteins that act as receptors. Several different complexes containing the glycoprotein heterodimer gH/gL are likely responsible for receptor binding (183). Based on studies of HSV, receptor binding is believed to trigger the putative fusion protein, glycoprotein B (gB), to promote fusion between the virion envelope and host cell membrane (74). These entry processes are discussed in greater detail below.

Figure 1-1

Overview of HCMV replication cycle.

Illustration by Andrew Townsend.

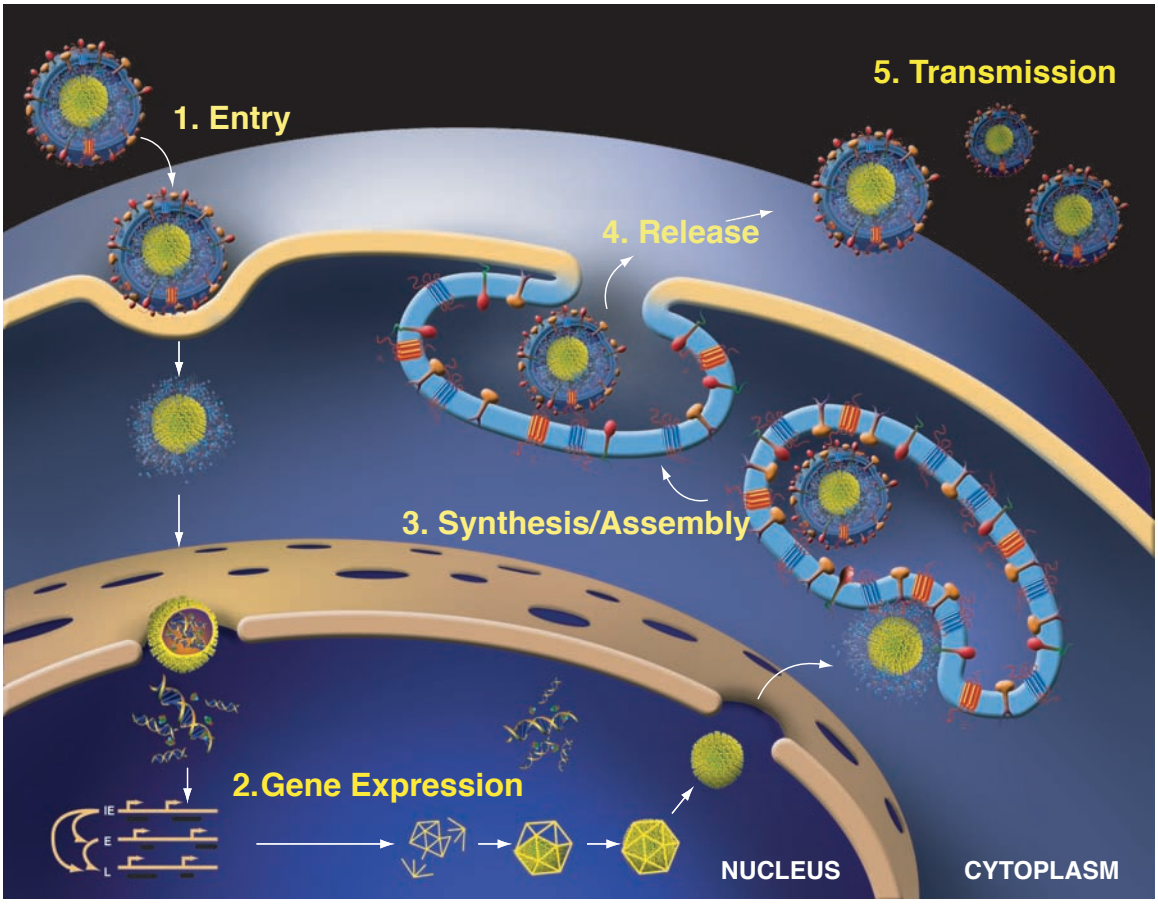
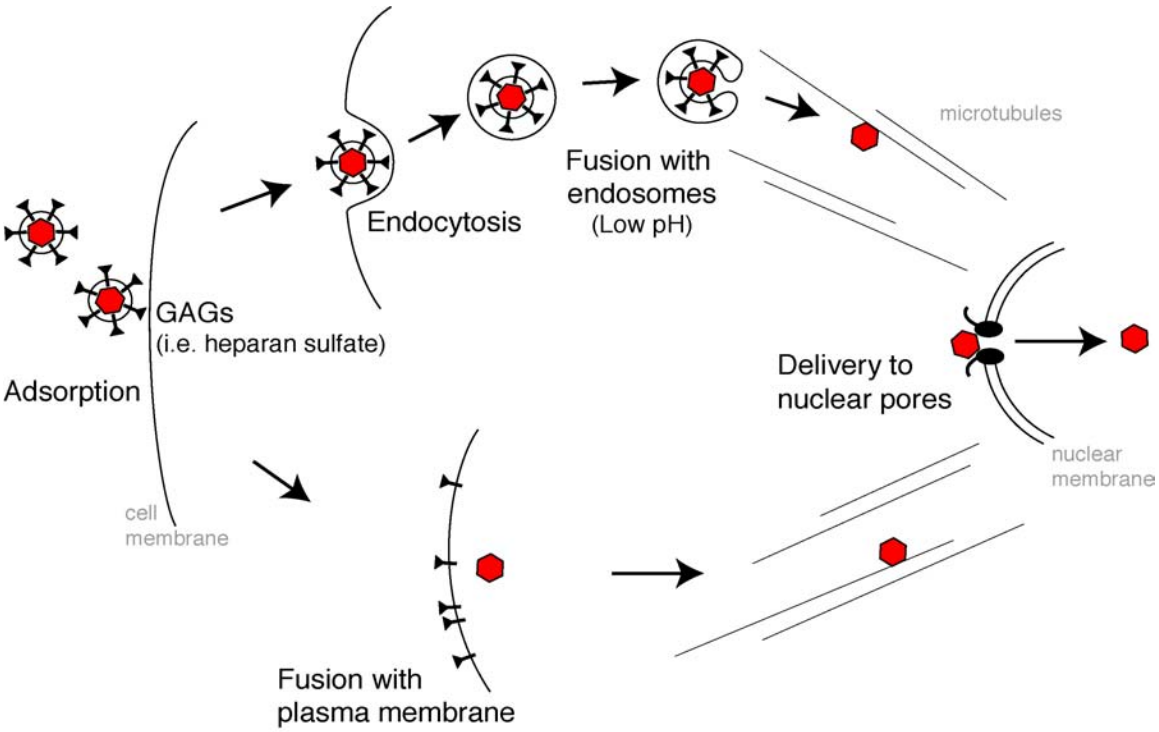


Figure 1-2

Herpesvirus Entry.

Illustration by Tiffani Howard.



B2. HCMV disassembly and access into the nucleus. Following virion envelope fusion with cell membranes, capsid and tegument proteins are released into the cytosol. In order for capsids to reach the nucleus, it is likely capsids must find and attach onto microtubules (157), although there also appears to be an important role for intermediate filaments, e.g., vimentin (139). If HCMV does reach the nucleus via microtubules, it likely does so by using dynein and dynactin motors, similar to HSV (48). Capsids would be pulled towards the microtubule organizing center (MOC) that is proximal to the nucleus. Capsids likely reach nuclear pores via diffusion or other processes and then capsids disassemble and viral genomic DNA is released into the nucleoplasm. This process was described for HSV using a temperature sensitive mutant in UL36 that accumulated capsids at the nuclear pores and did not release viral genomic DNA into the nucleus at the non-permissive temperature (11). The details of this process for HCMV have not been thoroughly characterized, although two HCMV proteins are thought to be important based on their homology to HSV UL36: the large tegument protein (LTP) encoded by UL48 and a LTP binding protein encoded by UL47 (51, 144, 245).

B3. Gene Expression. Once the genome reaches the nucleus, incoming tegument proteins are used to overcome cellular barriers of replication and initiate immediate-early, delayed-early, and late gene expression (reviewed in (211)). Immediate-early gene products have a vast array of functions, most of which alter cellular functions relating to cell cycle and defense mechanisms (reviewed in (144)). HCMV is able to begin robust immediate-early gene transcription in part via the action of the tegument protein pp71, which is brought in by the infecting virion rather than being produced *de novo*. pp71 interacts with several viral and cellular proteins in order to de-

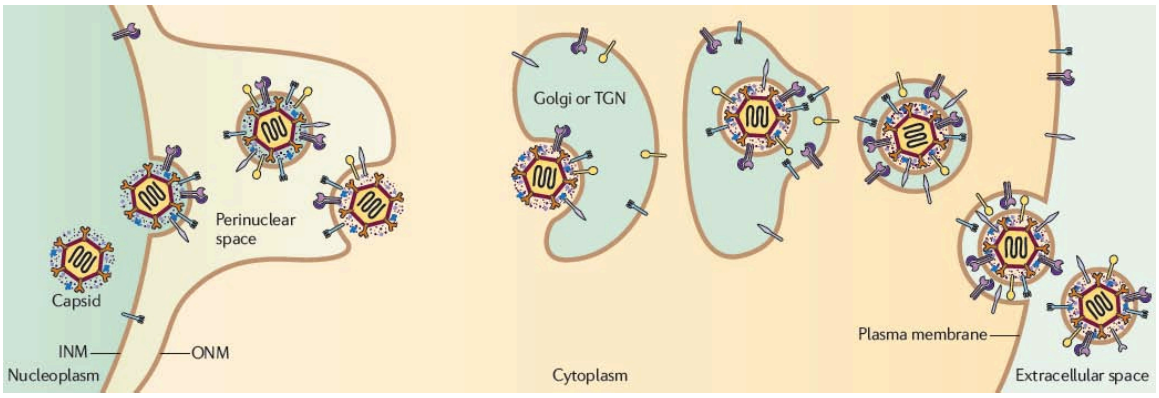
repress viral promoters and initiate transcription. Immediate-early (IE) transcripts encode proteins that are also transcription factors. These IE proteins are thus responsible for initiating delayed-early transcription of genes that encode proteins responsible for DNA replication and for modifying the host cell environment to favor replication (e.g., blocking the immune response). IE and delayed-early proteins are subsequently used to initiate late gene transcription. Late genes mostly encode structural proteins. For HCMV, there appear to be very few true late genes that require genome replication in order to be transcribed and translated (144).

B4. Assembly of capsids. Replicated genomes are packaged into capsids in the nucleus. Capsid proteins initially organize in the cytoplasm after translation, and are transported into the nucleus in multi-protein complexes; the major capsid protein (MCP) is part of the protocapsomers, and the minor capsid protein (mCP) is part of a trimer with its binding protein mc-BP (reviewed in (65)). These two sets of complexes meet in the nucleoplasm to form procapsids. Following proteolysis of scaffolding proteins bound to the MCP, scaffolding remnants are released from the capsid, leaving the empty intermediate “B” capsids. Interactions between the portal protein, terminase complex, and the packaging signal on the genomic DNA initiate packaging, so that DNA is threaded into preformed capsids. Packaging completes when the terminase cleaves the DNA at the next packaging signal, which it encounters because the genome is replicated as a concatamer (65).

Figure 1-3

Herpesvirus egress.

Illustration by Tiffani Howard.



B5. Nuclear egress. Herpesvirus capsids exit the nucleus, gain access to the cytosol, and then gain their final envelope in a strategy denoted envelopment–de-envelopment–re-envelopment (Fig. 1-3, reviewed in (105, 134, 135)). Following genome encapsidation, the final structure of the capsid is set, exposing binding sites for tegument proteins and nonstructural proteins involved in primary egress. Although most tegument proteins are associated with the capsid in the cytosol, some of these proteins, e.g., pp71 (76), are found in the nucleus and bind to the capsid in the nucleus. Primary envelopment occurs at the inner nuclear membrane. The capsid binds to the nuclear egress complex, composed of viral proteins and cellular proteins that are part of the nuclear lamina (30, 138). The nuclear lamina proteins are a blockade to nuclear exit as they are responsible for the structure of the nucleus by forming a dense, highly structured mesh (47). The virus disrupts the lamina in much the same way it is disrupted during cell division, i.e., phosphorylation of the lamins causes their dissociation (47). This process requires a complex of UL31 and UL34 for HSV (178) and M50 and M53 for MCMV (151). Once the capsid has crossed through the lamina, it is able to bud into the inner nuclear membrane, (reviewed in (53)). For HSV and pseudorabies virus (PrV), envelopment at the inner nuclear membrane may also rely on viral envelope glycoproteins, but if so, in a redundant manner since no single envelope glycoprotein is necessary (9, 56, 208, 220, 238). De-envelopment involves enveloped particles in the perinuclear space fusing with the outer nuclear membrane, releasing the capsid and the associated primary tegument layer out into the cytosol. This likely requires envelope glycoproteins, as is the case for HSV (56),

Herpesvirus glycoproteins provide redundant or overlapping functions in de-envelopment. There is evidence that the first egress event, budding into the nuclear

membrane, is maintained in nearly all herpesvirus envelope glycoprotein mutants except for EBV gM/gN mutants, which accumulate capsids in the nucleus (120, 121). Thus, most deletion mutants and combinations thereof maintain the production of primary enveloped particles that bud into the perinuclear space. No other single mutants give rise to defects in nuclear envelopment and de-envelopment. However, for HSV-1 it is clear that there is a role, if redundant, for gB and gH/gL in de-envelopment, as virions from a gB-gH double-null mutant collect in so-called “herniations” in the perinuclear space, and thus cannot undergo de-envelopment (56). Because this is not observed with either single mutant (gB-null or gH-null), this result demonstrates that gB or gH/gL are sufficient to mediate some aspect of primary envelopment/de-envelopment, whether or not that function is membrane fusion, as their role in entry suggests. Double and triple mutants of the related α-herpesvirus pseudorabies virus (PrV) in gB, gH/gL, and/or gD did not show any defects in primary egress (112), nor did a gE/gI/gM triple deletion mutant (21), suggesting PrV uses a different mechanism for de-envelopment than HSV.

B6. Cytoplasmic egress. Following de-envelopment, capsids are delivered into the cytoplasm and additional tegument proteins assemble onto capsids and help properly route them to sites of secondary envelopment. For HCMV, this process occurs in the assembly compartment, a highly organized structure of cytoplasmic membranes involving concentric rings of the TGN and endosomes (innermost), Golgi apparatus and endoplasmic reticulum (outermost) that is rearranged by viral proteins to optimize tegumentation, envelopment, and release (30, 32, 45, 66, 187). Viral glycoproteins traffic to this site of assembly (discussed below) and are incorporated into the infectious virion as the capsid buds into the assembly compartment membranes. At this point, the virion is inside of a vesicle, and must find its way to sites of release from cells. This is either at

cell-cell junctions or apical or basolateral surfaces on cells. Virus released into the space between cells, i.e., at cell-cell junctions, can rapidly spread to the neighboring cell. Whether there is any specific targeting of nascent HCMV virions to sites of cell-cell contact is unclear. HSV exhibits directed transport to epithelial cell-cell junctions (104). Regardless, there is no evidence that the composition of HCMV particles directed to different sites of release are different, such that all infectious virions released from an HCMV infected cell contain the same complement of viral glycoproteins required for entry into any cell type. However, the amounts of certain envelope proteins (e.g., UL128-131) incorporated into virions might be cell-type specific (190).

B7. Secondary envelopment. Like de-envelopment, herpesvirus glycoproteins provide redundant or overlapping functions in secondary envelopment. As was the case with nuclear egress, PrV glycoproteins gB, gH/gL, and gD are not required for secondary envelopment (112). However, a gM/gE/gI triple deletion mutant accumulates capsids in the cytoplasm, in contrast to the single mutants or combinations of double mutants in gE, gI, and gM. This again suggests functional redundancy for glycoprotein complexes during egress (21). For HSV, while single deletion mutants in the envelope glycoproteins do not prevent secondary envelopment, double deletion mutants of gD and gE or gI and a gD/gE/gI triple deletion mutant accumulated unenveloped capsids in the cytoplasm (54). In contrast, a KSHV gB deletion mutant accumulates partially enveloped capsids with uncondensed cores on cytoplasmic membranes, resulting in nearly complete elimination of extracellular virus particles (116). Extracellular virus particle release is also reduced when KSHV glycoprotein K8.1 production is inhibited by siRNA (216). This may also be the case with EBV gB, but unique deletion mutants in EBV gB give conflicting

results, with one mutant defective for release while the other releases the same number of particles as wild-type EBV (79, 155).

C. Roles of HCMV and herpesvirus glycoproteins in virus assembly and egress

C1. HCMV glycoprotein intracellular transport. Herpesvirus envelope glycoproteins are involved in numerous steps during the replication cycle. For HCMV, these proteins include, but are not limited to, gB, gH/gL, gM/gN, gO, UL128, UL130, and UL131. Following translation and translocation into the endoplasmic reticulum membrane, the glycoproteins are modified by oligosaccharyltransferase to yield N-linked high-mannose oligosaccharides (reviewed in (114)). During and after translation, these HCMV envelope proteins associate with binding partners, e.g. gH/gL and gM/gN, and this assembly is required for their further progression through the secretory pathway (129, 202). Other proteins, gO and UL128, UL130, and UL131, can also associate with gH/gL in two independent complexes: gH/gL/gO and gH/gL/UL128/UL130/UL131 (87, 124, 186, 232). As the glycoproteins move from the RER to the trans-Golgi apparatus, N-linked oligosaccharides on all of the glycoproteins are modified by removal and addition of sugars leaving the proteins with complex, N-linked oligosaccharides. Completion of this processing and transport to the Golgi apparatus can be assessed by treating glycoproteins with the enzyme endoglycosidase H (endo H), which cleaves immature, mannose-rich N-linked oligosaccharides found on glycoproteins in the ER, but not complex oligosaccharides found on glycoproteins in the trans-Golgi apparatus (114). The removal of immature, high-mannose oligosaccharides from glycoproteins by endo H can be assayed by changes in the electrophoretic mobility of the protein in SDS-PAGE (see for example (184)). It is important to understand that without this proper assembly of multi-protein complexes and intracellular transport from the ER to the Golgi apparatus,

HCMV glycoproteins will not be incorporated into the virion envelope and cannot function properly. Thus, when HCMV mutants are made or when HCMV glycoproteins are expressed outside the context of virus infection, these effects must be considered.

C2. Roles of HCMV glycoproteins in virus egress. The immature, high-mannose form of gB is found in the inner nuclear membrane (52), and gB has specific nuclear trafficking and retention signals (136, 137). Any role gB plays during assembly, though, is likely redundant with other HCMV glycoproteins, because a gB mutant lacking the cytoplasmic domain of the protein produces enveloped virions (215), and a HCMV gB-null mutant releases normal levels of virions (94). Newly described HCMV and rhesus CMV (RhCMV) gL deletion mutants also had no defects in assembly (20). Thus, although functional redundancy is possible, it appears that the two major HCMV glycoproteins gB and gH/gL are not necessary for any aspect of virus egress. However, like HSV (56), it is possible that deletion of both gB and gH/gL might compromise virus egress.

HCMV glycoproteins gM and gN form a heterodimer, gM/gN, that plays a role in virus assembly and egress (118, 130). gM- and gN-null mutants do not release normal levels of virions, and exhibit increased concentrations of unenveloped capsids in the cytoplasm (118, 130). This suggests that gM/gN is necessary for secondary envelopment. The role of gM/gN during secondary envelopment is not entirely clear; one model suggests that gM/gN helps deliver other HCMV membrane proteins to the sites of secondary envelopment, either through organizing the envelope glycoproteins in the assembly complex or binding tegument proteins so that they are brought into the virion (118). There is precedent for HSV gM causing relocalization of several viral glycoproteins from cell surfaces to the TGN (44). The HCMV gM cytoplasmic tail was

recently shown to bind to Rab11 effector protein FIP4, a protein involved in vesicular trafficking in the endosomal recycling compartment (ERC). This interaction may be critical for assembly compartment formation by concentrating gM/gN in the ERC and assembly compartments (119).

The role of the gB in secondary envelopment is unclear. gB moves through the exocytic pathway to the cell surface (163), where it is subsequently retrieved (171), sorted to endocytic compartments (59, 221) and finally to TGN-derived compartment by PACS-1 (43, 98). It is unclear whether this final step is necessary for gB to be re-localized to the site of secondary envelopment, as membrane acquisition colocalizes with both TGN and early endosome markers at the center of the assembly compartment (45, 187). The large-scale membrane reorganization initiated by HCMV, however, may make these markers irrelevant, as virus membranes contain both TGN markers and early endosome markers (32), and thus the cellular proteins that gB utilizes to traffic to the site of assembly may be associated with either compartment. After reaching the assembly compartment, gB is incorporated into enveloped virions. However, as described above, an HCMV gB-null mutant produces enveloped virions, and thus gB is not required for secondary envelopment, or is used in a redundant fashion as with HSV gB (106).

The role of gH/gL complexes in secondary envelopment is also unclear. Like gB, gH/gL traffics through the exocytic pathway and can be found on the cell surface (109). Unlike gB, PACS-1 is not required for gH localization to TGN-derived membranes (43). How gH/gL complexes are sorted to sites of secondary envelopment and incorporated into the virion envelope is unknown. Given that HCMV and rhesus CMV (RhCMV) gL-

null mutants do not have defects in assembly (20), it seems unlikely that gH/gL is required for secondary envelopment.

D. HSV and EBV glycoprotein function during virus entry

It is useful at this point to describe the pathways used by HSV and EBV to enter cells because more is known about these viruses compared with HCMV. This sets the scene for my studies of HCMV entry into cells. All herpesviruses apparently require gB and gH/gL for entry and for the related process of cell-cell fusion, which is often used to study herpesvirus entry machinery.

D1. Herpes simplex virus. HSV requires three envelope glycoproteins for receptor binding and fusion: gD, gH/gL, and gB. Entry is initiated by attachment, which involves either gB or gC that bind to heparan sulfate glycosaminoglycans (GAGs) (77, 78, 241). Entry requires an additional binding to cellular ligands, involving gD which interacts with receptors: e.g., nectin-1 or HVEM (117, 146). Subsequent to receptor binding, gD triggers conformational changes in gH/gL and/or gB (7). In virus particles lacking gD, entry does not progress past attachment onto heparan GAGs (127). The precise function of gH/gL during HSV entry is unclear. Virus particles lacking gH/gL do not progress beyond receptor binding to fusion of the virion envelope with cellular membranes (61). There are two models for the role of gH/gL in entry. First, gH/gL might act as a fusogen, providing the necessary energy to fuse the viral envelope and cellular membrane. Supporting this model, Subramanian and Geraghty suggested that HSV particles containing gH/gL and gD promoted hemifusion, while gB, gH/gL and gD promoted full fusion of both leaflets of the membrane bilayers (217). This observation was contradicted by observations that gD, gH/gL and gB are required for both hemifusion and fusion (96). Second, gH/gL could relay the signal from gD (that binds gD

receptors) to gB (that acts as the fusion protein) by changing conformation in response to gD receptor binding and transmitting these changes to gB. The Cohen and Eisenberg laboratories demonstrated that gB directly associates with membranes and is required for the interaction of gH/gL with membranes (7, 31). This suggested that gB is the primary fusion protein and is triggered by gH/gL, which associated with membranes only through binding to gB. The structure of HSV gB also argues very strongly that gB is a fusion protein. gB has the structure of a class III fusion protein similar to VSV-G and baculovirus gp64 (75, 107, 181). It appears that gB associates with membranes and mediates fusion involving so called "fusion loops," which are hydrophobic loops embedded in the center of the molecule that enter cellular membranes during fusion (72). Together, these two observations suggest that gH/gL does not act on its own as a membrane fusion protein. It is more likely that gB is the main fusion protein and gH/gL triggers gB. The gB-mediated fusion reaction likely involves insertion of gB fusion loops into the apposing cellular membranes followed by rearrangement of the structure of gB, drawing viral and cellular membranes together and promoting their fusion (reviewed in (74)).

D2. Epstein-Barr virus. EBV entry contrasts with HSV in that EBV enters cells in a cell-type specific manner, using different viral and cellular molecules to enter B cells versus epithelial cells (reviewed in (91)). For B cells, EBV attaches via high-affinity binding interactions between gp350/220 in the virion envelope and CD21 (CR2) on the cell surface (154, 218). Similar to HSV gB and gC interactions with heparan GAGs, the interaction between gp350/220 and CD21 is not sufficient for entry. Instead, EBV assembles a gH/gL complex containing another glycoprotein, gp42, which binds directly to HLA class II molecules present on B cells and this binding of gH/gL/gp42 to class II

likely triggers gB for entry fusion (125, 126). This entry into B cells also requires endocytosis (140). EBV entry into epithelial cells involves gH/gL (without gp42) which bind integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ (37) and does not require endocytosis (140). Again, gH/gL bound to integrins likely triggers conformational changes in gB which cause fusion. EBV virus particles assembled in epithelial cells contain primarily gH/gL/gp42 that facilitates entry into B cells. When virus is assembled in B cells, however, gp42 is sequestered by HLA class II molecules, preventing its association with gH/gL (18). Thus, EBV particles derived from B cells contain less gp42 (approximately 100-fold decreased), and instead primarily enter epithelial cells. This provides a switch in virus tropism, such that virus derived from epithelial cells infects B cells well and virus from B cells infects epithelial cells well.

E. Roles of HCMV membrane glycoproteins in virus entry

E1. HCMV Attachment. As with HSV and EBV, HCMV attaches to cells via envelope glycoproteins; specifically gB and gM appear to play a redundant role in this process. These proteins bind to heparan GAGs (41, 108). The attachment step is not cell-type specific, and does not initiate the fusion process at the plasma membrane (41). More specific receptor-ligand interactions are necessary for the remaining entry steps. Once attached, the virion is close enough to the cell to find and bind to other receptors that trigger fusion with the plasma membrane or endocytosis, depending upon the cell type.

E2. Membrane penetration. HCMV penetrates cellular membranes either at the plasma membrane or internal membranes following endocytosis (40, 185). This correlates with pH-dependence: pH-independent membrane fusion occurs at the cell

surface, and low pH-dependent fusion occurs following endocytosis. The particular route of entry appears to be cell-type specific: HCMV enters fibroblasts by fusing at the plasma membrane (40), but enters endothelial and epithelial cells by fusing with internal membranes in a process that depends upon endosomal acidification (185). This specificity is likely mediated by cell-specific receptor binding.

E3. The role of HCMV gH/gL complexes in virus entry. Analogous to other herpesviruses, HCMV gH/gL is required for entry and likely participates in fusion. Neutralizing antibodies to HCMV gH initially established its role in entry (42, 175, 176). gH antibodies were also shown to block fusion of U373 cells initiated by high-titer HCMV infection (i.e., fusion from without [FFWO], the ability of a high concentration of virus particles to fuse neighboring cells during infection) (141). Entry can also be blocked by heptad-repeat peptides derived from gH. These peptides overlap putative coiled-coil domains, motifs which have been implicated as important for the function of other fusion proteins (128). Similar to other herpesviruses, HCMV gH was found to form a heterodimer with gL (109), and that gH/gL complex formation was necessary for both gH and gL to exit the ER and get to the cell surface (202). gH/gL was most rigorously implicated in fusion using cell-cell fusion assays: epithelial cells fuse when gH/gL and gB are expressed in isolation from other HCMV proteins (227). Recently, the role of gH/gL in entry was shown genetically, as a gL-null HCMV is defective in entry and cell-cell spread (20). In accordance with a putative role in entry, several receptors have been proposed for gH/gL, including a 92.5 kDa protein found in fibroblasts that was identified using gH-derived anti-idiotype antibodies (110), and $\alpha_v\beta_3$ integrin (233). However, there is no evidence to date that either of these molecules is a trigger for entry fusion. Overall,

it has become clear that HCMV gH/gL has a function in entry similar to its herpesvirus homologues.

Similar to EBV gH/gL and gH/gL/gp42, HCMV gH/gL forms multiple complexes with other viral proteins. The discovery of the two independent HCMV gH/gL complexes provided important clues into how gH/gL functions to mediate entry into different cell types.

gH/gL/gO was originally described as the predominant HCMV gH/gL complex in experiments using laboratory strain AD169 (87, 88, 124). While the HCMV homologues of HSV gH and gL had been previously identified, when both were expressed by vaccinia virus vectors they did not reconstitute the gH/gL complexes found in AD169 virion envelopes (124). A third glycoprotein found covalently bound to gH was subsequently recognized as the final component of glycoprotein complex III (gcIII) of the HCMV envelope (87, 124), identified as the gene product of UL74, and named gO (88). In cells infected with the laboratory-adapted HCMV strain AD169, gO binds to gH/gL as a glycosylated precursor, and gH/gL/gO subsequently reaches the cell surface decorated with endo H resistant oligosaccharides (89). gO has a single hydrophobic domain that is used as a signal peptide for translocation into the ER, and thus gO is a peripheral membrane protein via its interactions with gH/gL (219).

Studies similar to those that determined the role of gH/gL in entry and fusion have been used to better understand the role of gO in these processes. Antibodies to gO prevent fusion from without in a manner similar to gH antibodies (160). Prior to the gO-null mutant described herein, two other gO mutants have been characterized. An AD169 gO-null mutant was able to replicate in fibroblasts and spread, but has a small plaque phenotype and a 100-1000 fold reduction in titer as compared to a repaired virus (84).

These results suggested that AD169 gO-null was defective in cell-cell spread and/or virus release. A gO-null mutant in strain TB40/E has a similar phenotype, and was also demonstrated to spread normally in endothelial cells (101). Defects in replication in fibroblasts were attributed to a reduction in virus assembly (as determined by electron microscopy). Aside from the reduction in plaque size and titer, no assay performed with these two mutants demonstrated a direct role for gO in entry; in particular, there is no evidence that gO participates in receptor binding or fusion. The role of gO in HCMV replication is investigated in depth in Chapter 2.

gH/gL/UL128-131. It was observed that AD169, Towne and other HCMV laboratory strains that have been serially passaged long-term on fibroblasts are greatly reduced in their ability to infect cell types such as endothelial and epithelial cells (29, 81, 172, 197, 229, 230). Later studies revealed that AD169 cannot enter these cells because it cannot construct a second gH/gL-containing complex with three additional proteins: UL128, UL130 and UL131 (4, 64, 70, 231). It was deduced that serial passage in fibroblasts led to mutations in the UL128, UL130, or UL131 genes which are part of the ULb' region of HCMV that is frequently mutated in laboratory-adapted HCMV strains (169). The absence of one or more of the UL128-131 genes prevents assembly of the gH/gL/UL128-131 complex in laboratory strains passaged long-term on fibroblasts (70). Biochemical studies of the gH/gL/UL128-131 complex revealed that all five members are required to form a complex, demonstrating why the loss of only one of UL128, UL130, or UL131 leads to the loss of the entire gH/gL/UL128-131 complex in laboratory strains. Complexes of gH/gL missing any of UL128, UL130, or UL131 cannot exit the ER and thus cannot be incorporated into the virion envelope (186). Work from the laboratory of Thomas Shenk showed that when UL131 expression was restored in strain AD169, that

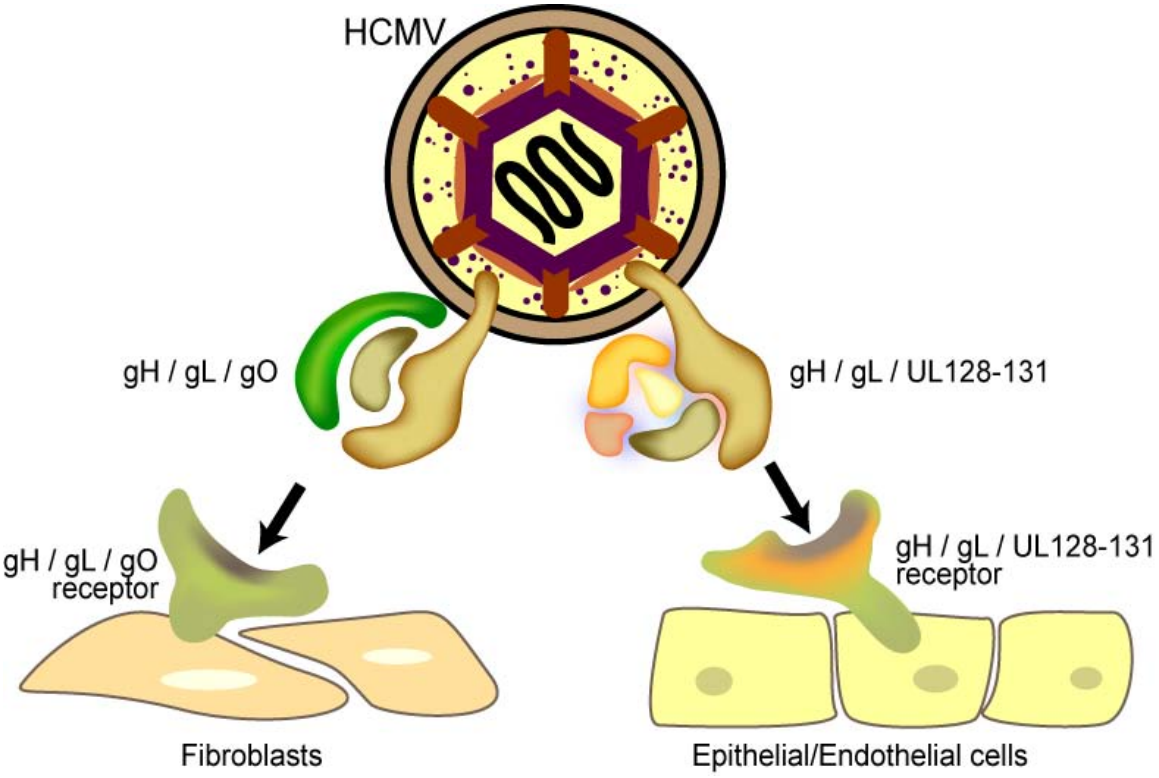
two gH/gL complexes, gH/gL/UL128/UL130/UL131 and gH/gL/gO, were produced and appeared to be separate from one another (232). Restoration of gH/gL/UL128-131 in AD169 significantly increased its ability to infect endothelial and epithelial cells without compromising fibroblast infection (232). In agreement with this observation, the UL128, UL130, and UL131 genes are highly conserved in all clinically derived HCMV (10). Null mutants of any or all of UL128, UL130, UL131 in clinical strains are likewise unable to infect epithelial and endothelial cells (185, 186). The defect in replication was narrowed to entry, as virions lacking gH/gL/UL128-131 complexes can be forced to enter epithelial and endothelial cells by the chemical fusogen polyethylene glycol (PEG) and will initiate the replication cycle but not spread cell-to-cell (185, 186). The role of gH/gL/UL128-131 was subsequently demonstrated to be at the stage of receptor binding by using an interference assay (183). Interference occurs when viral proteins (in this case, gH/gL/UL128-131) are expressed in a susceptible cell, and these bind to putative receptors, preventing the receptor from being utilized by incoming virus. Brent Ryckman also demonstrated that gH/gL/UL128-131 interfered with HCMV infection of epithelial cells when all five proteins were expressed using non-replicating adenovirus vectors before the cells were challenged with HCMV. Interference only occurred in cell types in which gH/gL/UL128-131 was required for entry; i.e., endothelial and epithelial cells, and not fibroblasts. There was no interference in epithelial cells transduced with gH/gL or gB (183). This reduction in infectivity is strong evidence that the viral proteins bind to a saturable receptor required by HCMV to enter epithelial cells. These data further refined the model of entry to include gH/gL/UL128-131 as a receptor binding protein complex that is required for the endocytic route of entry into epithelial and endothelial cells.

Thus, it was clear from these studies that gH/gL complexes for HCMV were a key tropism determinant, i.e., gH/gL/gO was associated with fibroblast infection and gH/gL/UL128-131 was critical for endothelial and epithelial cell infection (Fig 1-4). However, there was less information on how gH/gL/gO functions, whether in entry or in some other process (e.g., assembly) before my studies.

Figure 1-4

gH/gL complexes are receptor binding proteins.

Illustration by Tiffani Howard.



E4. The role of HCMV gB in virus entry. HCMV gB has been demonstrated to be critical for viral membrane fusion during entry. Initial biochemical studies involved mapping monoclonal neutralizing antibodies to the exposed surfaces of gB (27). Neutralizing antibodies are made to several HCMV envelope glycoproteins (e.g., gH), thus neutralization via gB-specific antibodies did not show that gB was critical for entry or how. HCMV gB was found to be proteolytically cleaved similar to the influenza virus hemagglutinin (HA) and HIV gp120, both of which are responsible for receptor binding and entry fusion for their respective viruses (22, 203). During transit through the TGN, gB undergoes endoproteolytic cleavage by furin from a 160 kDa precursor into 100 kDa and 58 kDa fragments which are disulfide linked (24, 27, 228). Also like other fusion proteins, gB oligomerizes, forming a dimer in the ER (14, 25). gB causes syncytium formation when constitutively expressed independent of infection in stably-transfected U373 cell clones (222). Mutations in the transmembrane domain and cytoplasmic tail of gB reduced the number of synctia formed in these cells, although how this relates to gB function is unclear, as syncytia formation was partially retained when half of the transmembrane domain was deleted (223). It is difficult to justify a model in which gB could induce fusion as a soluble protein, as all known cellular and viral fusogens require a membrane anchor in order to function. Background fusion by the parental U373 cells also confounds these results, and this assay was not repeated with any other HCMV proteins, including gH/gL, as controls. Thus, although these data suggested a role for gB in fusion, because of its limitations, a U373 cell-cell fusion assay does not provide conclusive evidence of the role of gB acting as the fusion protein. Stronger evidence has come from experiments in which entry of HCMV was blocked by peptides of putative coiled-coil domains in both gB and gH (128). Coiled-coil domains have been implicated

in formation of hairpins found in the post-fusion structures of fusion proteins. However, this result did not distinguish between gH and gB as being the fusion proteins. Kinzler and Compton demonstrated low levels of fusion between cells expressing only gH/gL, and addition of gB or gO did not increase the number of syncytia (111). However, this result was again confounded by background fusion between CHO cells when no glycoprotein was added. Moreover, these results do not fit with many observations that gB is required for cell-cell fusion in other herpesviruses. These results were also contradicted by experiments by Adam Vanarsdall in our laboratory who found that both gB and gH/gL were required for cell-cell fusion of ARPE-19 epithelial cells (227). Neither gB or gH/gL alone were able to mediate any detectable fusion and these cells do not fuse without glycoprotein present. These results were extended to several other cell lines that also did not display background fusion.

The strongest evidence supporting an essential role for HCMV gB in entry came from studies of a gB-null mutant (94). The gB-null mutant failed to enter fibroblasts at a post-attachment step. This defect can be overcome with PEG, demonstrating that the defect involves receptor binding or membrane fusion. Again, these results did not directly determine whether gB or gH/gL is the fusion protein for HCMV. However, based on the preponderance of evidence listed above involving HCMV gH/gL complexes and other herpesviruses (HSV and EBV), it is very likely that HCMV gB is the fusion protein, while gH/gL and perhaps other glycoproteins bind receptors and trigger gB.

E5. Binding of cellular proteins by HCMV gB and gH/gL and involvement in virus entry. There has been some evidence that HCMV gB can bind cell surface proteins, but it is frequently unclear whether these interactions mediate entry or, alternatively, activate signaling cascades that are important for downstream replication

events. Several of these gB receptors have been proposed for HCMV only to be subsequently ruled out as bona fide entry-fusion receptors, and more evidence is needed to confirm the role of several other putative receptors (reviewed in (16, 93)). gB binds to annexin II (240), but evidence was subsequently presented that annexin II is not an important receptor for HCMV (166). Similarly, gB was found to bind to epidermal growth factor receptor (EGFR) and there was evidence that this receptor can mediate entry (234). However, HCMV entered EGFR-null fibroblasts and cells treated with EGFR-neutralizing antibodies and thus it was argued that EGFR is not an important entry receptor for HCMV (38, 92). Studies by Adam Vanarsdall in our laboratory have recently shown that EGFR plays no important role in HCMV entry into any cell tested. Integrins have also been implicated in post-attachment steps of HCMV entry; a peptide derived from the disintegrin-like domain (DLD) of gB—a recombinant soluble version of the gB-DLD—and β_1-integrin antibodies partially block HCMV entry at a step between attachment and delivery of the tegument protein pp65 into the cytosol (57, 58). Again there is no direct evidence that this interaction is a trigger for entry fusion, or whether integrin signaling is necessary for a post-fusion entry event like EGFR. A second report suggested that gH/gL interacts with integrins (233), but the evidence for this involved coprecipitation of integrins and gH/gL and focused on signaling rather than virus entry.

Another cell surface signaling molecule, platelet-derived growth factor-α receptor (PDGFRα) was found to be bound by HCMV gB and activated by either HCMV infection or soluble gB (201). In this case, however, blocking downstream signaling events by suppressing the PI(3)K pathway only delayed gene expression and did not prevent entry. Thus, it is possible that PDGFRα acts as an entry mediator in a manner distinct from signal transduction, or that HCMV replication requires PDGFRα signaling either for early

replication or entry. Recent studies by Adam Vanarsdall have shown that PDGFRα can mediate HCMV entry into a variety of cell types, but that PDGFRα does not explain HCMV entry into some relevant cell types including epithelial and endothelial cells.

E6. Other HCMV receptors. All the receptors described above were identified and characterized by using HCMV laboratory strain AD169. AD169 does not express gH/gL/UL128-131 and does not enter epithelial and endothelial cells or monocyte-macrophages. Therefore, there must be other important receptors used by wild-type HCMV. Moreover, interference experiments in which gH/gL/UL128-131 was expressed in epithelial cells suggested that there are molecules expressed in these cells and bound by gH/gL/UL128-131 that act as receptors. By definition, these receptors cannot be EGFR, PDGFRα or integrins, because those molecules were discovered using AD169 that does not express gH/gL/UL128-131. Moreover, gB has been expressed in fibroblasts, epithelial and endothelial cells and in no case was interference observed. Adam Vanarsdall also has evidence that expression of gH/gL/gO in fibroblasts can mediate interference. Therefore, none of our results support HCMV gB acting as a receptor binding protein and so there must be important receptors beyond integrins, PDGFRα and EGFR.

F. Summary

Despite significant advances in our understanding of HCMV entry, two aspects of this process remain poorly defined. First, it is not clear how the two gH/gL complexes function during entry. Several lines of data suggest that gH/gL complexes are receptor-binding proteins; the interference assay suggested that gH/gL/UL128-131 bind epithelial and endothelial cell receptors while gH/gL/gO binds fibroblast receptors. This suggests

that different gH/gL complexes are used depending on the route of entry and/or receptor availability, and that these interactions then trigger entry fusion, likely promoted by gB. However, before my studies we knew little of how gH/gL/gO was assembled and how this complex functions in entry into different cell types. The second aspect of HCMV entry that is poorly understood involves how gB participates in HCMV entry and specifically whether gB acts as the viral fusion protein. gB is clearly necessary for HCMV entry and is probably the fusion protein for all herpesviruses. However, for HCMV there is little information pointing to gB acting in membrane fusion and yet extensive information that gB binds receptors. Experiments in the results below attempt to separate these functions and more clearly define whether gB is the fusion protein.

CHAPTER 2: AN HCMV gO-NULL MUTANT FAILS TO INCORPORATE gH/gL INTO THE VIRION ENVELOPE AND IS UNABLE TO ENTER FIBROBLASTS, EPITHELIAL, AND ENDOTHELIAL CELLS

Paul T. Wille, Amber J. Knoche, Jay A. Nelson, Michael A. Jarvis, and David C. Johnson

A. Statement of Author Contributions and Copyright

P.T.W. and D.C.J. designed research; P.T.W. performed research; A.J.K., J.A.N., and M.A.J. contributed reagents; P.T.W. and D.C.J. analyzed data; and P.T.W., J.A.N., M.A.J., and D.C.J. wrote and reviewed manuscript.

B. Abstract

Human cytomegalovirus (HCMV) depends upon a five protein complex, gH/gL/UL128-UL131, to enter epithelial and endothelial cells. A separate HCMV gH/gL-containing complex, gH/gL/gO, has been described. Our prevailing model is that gH/gL/UL128-131 is required for entry into biologically important epithelial and endothelial cells and gH/gL/gO is required for infection of fibroblasts. Genes encoding UL128-131 are rapidly mutated during laboratory propagation of HCMV on fibroblasts, apparently related to selective pressure for the fibroblast entry pathway. Arguing against this model in Ryckman et al. (184), we describe evidence that clinical HCMV strain TR expresses a gO molecule that acts to promote ER export of gH/gL and gO is not stably incorporated into the virus envelope. This was different from results involving fibroblast-adapted HCMV strain AD169 that incorporates gO into the virion envelope. Here, we

constructed a TR gO-null mutant, TRΔgO, that replicated to low titers, spread poorly between fibroblasts, but produced normal quantities of extracellular virus particles. TRΔgO particles released from fibroblasts failed to infect fibroblasts, epithelial and endothelial cells, but the chemical fusogen polyethylene glycol (PEG) could partially overcome defects in infection. Therefore, TRΔgO is defective for entry into all three cell types. Defects in entry were explained by observations that TRΔgO incorporated about 5% the quantities of gH/gL in extracellular virus particles compared with wild type virions. Although TRΔgO particles could not enter cells, cell-to-cell spread involving epithelial and endothelial cells was increased relative to TR, apparently resulting from increased quantities of gH/gL/UL128-131 in virions. Together, our data suggest that TR gO acts as a chaperone to promote ER export and incorporation of gH/gL complexes into the HCMV envelope. Moreover, the data suggest that it is gH/gL, and not gH/gL/gO, that is present in virions and is required for infection of fibroblasts, epithelial and endothelial cells. Our observations that both gH/gL and gH/gL/UL128-131 are required for entry into epithelial/endothelial cells differ from models for other β- and γ-herpesviruses that use one of two different gH/gL complexes to enter different cells.

C. Introduction

Human cytomegalovirus (HCMV) infects a broad spectrum of cell types in vivo including epithelial and endothelial cells, fibroblasts, monocyte-macrophages, dendritic cells, hepatocytes, neurons, glial cells and leukocytes (26, 122, 167). Infection of this diverse spectrum of cell types contributes to the multiplicity of CMV-associated disease. HCMV infection of hepatocytes and epithelial cells in the gut and lungs following transplant immunosuppression is directly associated with CMV disease (15, 195). HCMV

can be transported in the blood by monocyte-macrophages and virus produced in these cells can infect endothelial cells leading to virus spread into solid tissues such as the brain, liver, lung, etc. (63). Despite the broad spectrum of cells infected *in vivo*, propagation of HCMV in the laboratory is largely limited to normal human fibroblasts because other cells produce little virus. HCMV rapidly adapts to laboratory propagation in fibroblasts, however, losing the capacity to infect other cell types, i.e., epithelial and endothelial cells and monocyte-macrophages (33, 63, 70, 194). This adaptation to fibroblasts involves mutations in the ULb' region of the HCMV genome which includes 22 genes (33). Targeted mutation of three of the ULb' genes: UL128, UL130 and UL131 abolished HCMV infection of endothelial cells, transmission to leukocytes and infection of dendritic cells (62, 70). Restoration of UL128-131 genes in HCMV laboratory strain AD169 (which cannot infect epithelial and endothelial cells) produced viruses capable of infecting these cells (70, 231). There is also evidence that the UL128-UL131 proteins are deleterious to HCMV replication in fibroblasts, resulting in rapid loss or mutation of one or more of the UL128-131 genes during passage in fibroblasts (4).

A major step forward in understanding how the UL128-131 genes promote HCMV infection of epithelial and endothelial cells involved observations that the UL128-131 proteins assemble onto the extracellular domain of the membrane-anchored HCMV glycoprotein heterodimer gH/gL (1, 232). Antibodies to each of UL128, UL130 and UL131 neutralized HCMV for infection of endothelial or epithelial cells (1, 232). All herpesviruses express gH/gL homologues and, where this has been tested, all depend upon gH/gL for replication and, more specifically, for entry into cells (51, 61, 145, 182). Indeed, we showed the gH/gL/UL128-131 complex mediated entry into epithelial and endothelial cells (185). All five members of the gH/gL/UL128-131 complex were required

for proper assembly and export from the endoplasmic reticulum (ER) and for function (183, 186). In addition, the expression of gH/gL/UL128-131, but not gH/gL or gB, in epithelial cells interfered with HCMV entry into these cells (183). This interference suggested that there are saturable gH/gL/UL128-131 receptors present on epithelial cells, molecules that HCMV uses for entry. There was no interference in fibroblasts expressing gH/gL/UL128-131, although some interference was observed with gH/gL (183). As noted above, gH/gL/UL128-131 plays no obvious role in entry into fibroblasts and, in fact, appears to be deleterious in this respect (4, 70, 185).

HCMV also expresses a second gH/gL complex: gH/gL/gO (87-89, 124, 231). Fibroblast-adapted HCMV strain AD169 expresses a gO protein that is a 110-125 kDa glycoprotein (88). Pulse-chase studies suggest that gH/gL assembles first in the ER before binding and forming disulfide links with gO (88, 89). The 220 kDa immature gH/gL/gO complex is transported from the ER to the Golgi apparatus and increases in size to ≈280-300 kDa before incorporation into the virion envelope (88). gH/gL/gO complexes are apparently distinct from gH/gL/UL128-131 complexes because gO-specific antibodies do not detect complexes containing either UL128 or UL130 and UL128-specific antibodies do not precipitate gO (232). Laboratory strains Towne and AD169 gO-null mutants can produce small plaques on fibroblasts, leading to the conclusion that gO is not essential. However, the AD169 and Towne mutants produced ≈ 1000 fold less infectious virus compared with wild type HCMV (51, 84), which might also be interpreted to mean that gO is very important or even essential for replication. Thus, the prevailing model has been that wild type HCMV particles contain two gH/gL complexes: gH/gL/gO that promotes infection of fibroblasts and gH/gL/UL128-131 that promotes entry into epithelial and endothelial cells. Supporting this model, there are two

different entry mechanisms: HCMV enters fibroblasts by fusion at the plasma membrane at neutral pH (40), whereas entry into epithelial and endothelial cells involves endocytosis and a low pH-dependent fusion with endosomes (185). This model of HCMV entry parallels models for Epstein-Barr virus (EBV) entry that uses gH/gL to enter epithelial cells and gH/gL/gp42 to enter B cells (91). Similarly, HHV-6 uses gH/gL/gO and gH/gL/gQ that bind to different receptors (149).

Many of the studies of gH/gL/gO have involved the fibroblast-adapted HCMV strain AD169 that fails to express UL131 and assemble gH/gL/UL128-131 or AD169 recombinants in which UL131 expression was restored (87-89, 231, 232). It seemed possible that the adaptation of AD169 to long-term passage in fibroblasts might also involve alterations in gO. HCMV gO is unusually variable (15-25% amino acid differences) among different HCMV strains, compared with other viral genes (49, 152, 160, 174, 209). In recent studies, Jiang et al. (101) described a gO-null mutant derived from the HCMV strain TB40/E, a strain that can infect endothelial cells following extensive passage on these cells. The TB40/E gO-null mutant spread poorly on fibroblasts compared with wild type TB40/E and there was little infectious virus detected in fibroblast culture supernatants. However, the few TB40/E gO-null mutant particles produced by fibroblasts that could initiate infection of endothelial cells were able to spread to form normal sized plaques on endothelial cells. These results further supported the model in which gH/gL/gO is required for infection of fibroblasts, but not for epithelial/endothelial cells. These authors also concluded that gO is important for assembly of enveloped particles in fibroblasts, based on observations of few infectious virus particles in supernatants and cytoplasmic accumulation of unenveloped capsids (101).

Our studies of gH/gL/UL128-131 have involved the clinical HCMV strain TR (183, 185, 186, 227). HCMV TR was originally an ocular isolate from an AIDS patient (198) and was passaged only a few times on fibroblasts before being genetically frozen in the form of a bacterial artificial chromosome (BAC) (152, 185). HCMV TR infects epithelial and endothelial cells (185) and monocyte-macrophages well (D. Streblow and J. Nelson, unpublished results). In the accompanying paper (184), we characterized the biochemistry and intracellular trafficking of TR gO. TR gO expressed either in TR-infected cells or by using adenovirus vectors (expressed without other HCMV proteins) was largely retained in the ER. Co-expression of gO with gH/gL promoted transport of gH/gL beyond the ER. Importantly, TR gO was not found in extracellular virions. By contrast, AD169 gO was present in extracellular virus particles, as described previously (87, 88). We concluded that TR gO is a chaperone that promotes ER export of the gH/gL complex but gO dissociates prior to incorporation into the virus envelope. Moreover, these differences highlight major differences between gO molecules expressed by fibroblast-adapted strain AD169 and low-passage TR.

To extend these results and characterize how TR gO functions, whether in virus entry or virus assembly/egress, we constructed a TR gO-null mutant. TRΔgO exhibited major defects in entering fibroblasts, as evidenced by increased virus infection following treatment with the chemical fusogen PEG. Unexpectedly, the mutant also failed to enter epithelial and endothelial cells and, again, PEG partially restored entry. Relatively normal numbers of TRΔgO particles were produced and released into cell culture supernatants, although even with PEG treatment most of these virus particles remained defective in initiating immediate early HCMV protein synthesis. Western blot analyses of TRΔgO extracellular particles demonstrated very low levels of gH/gL incorporated into

virions, which likely explains reduced entry of TRΔgO. However, the low amounts of gH/gL complexes that were present in TRΔgO virions were associated with increased quantities of UL130 and these TRΔgO particles spread better compared with wild type HCMV on epithelial cell monolayers. Together with the results in the accompanying paper (184), we concluded that HCMV TR gO functions as a chaperone to promote ER export of gH/gL to HCMV assembly compartments and incorporation of gH/gL into the virion envelope. The highly reduced quantities of gH/gL in virions are apparently responsible for the inability of TRΔgO to enter fibroblasts, epithelial and endothelial cells. These results suggest a modified version of our model in which gH/gL, not gH/gL/gO, mediates entry into fibroblasts and both gH/gL and gH/gL/UL128-131 are required for entry into epithelial and endothelial cells.

D. Materials and Methods

D1. Cells and viruses. MRC-5 fibroblasts were obtained from the American Type Culture Collection (ATCC-CCL-171) and neonatal normal human dermal fibroblasts (NHDF) were obtained from Cascade Biologics (Portland, OR) and were grown in Minimum Essential Medium (MEM, Invitrogen) and Dulbecco's Minimum Essential Medium (DMEM, Invitrogen), respectively, that was supplemented with 5% fetal bovine serum (FBS, Hyclone) and 5% bovine growth supplement (BGS, Hyclone). The retinal pigment epithelial cell line ARPE-19 was obtained from the ATCC and was grown in a 1:1 mix of DMEM and Ham’s F-12 medium (Invitrogen) supplemented with 10% FBS. HPV E6/E7 transformed aortic endothelial cells (HPV-AEC) were provided by Ashlee Moses (Oregon Health & Science University) and were grown in Medium 200 (Cascade M-200-500) supplemented with low serum growth supplement (Cascade).

HCMV strain TR was derived from the ocular vitreous fluid of an AIDS patient (198) and was stabilized in the form of a bacterial artificial chromosome (BAC) (152). Wild-type TR was propagated on NHDF cells by infection at a multiplicity of less than 0.1 in DMEM plus 5% FBS for 10 to 16 days. Virus particles were concentrated from infected cell supernatants by centrifugation at 50,000 X g for 1 h through a 20% sorbitol cushion.

D2. Construction of recombinant HCMV TRΔgO. The UL74 gene of BAC-TR was mutated by using lambda phage linear recombination as previously described (23, 123, 244). Briefly, a 1.5-kb PCR product containing a kanamycin resistance (kan^R) gene cassette flanked by Flp recombination target (FRT) sites and with HCMV sequences near the 5' end of the UL74 gene (60 nt, corresponding to nt 144593 to 14534 of the published TR sequence, accession no. AC146906) and sequences near the 3' end of the UL74 gene (61 nt, corresponding to nt. 143393 to 143333) was generated with primers:
GTACACGCAGGCAAGCCAAACCACAAGGCAGACGGACGGTGCGGGGTCTCCTCCT CTGTCGTAAAACGACGGCCAGT and
TAGGTGTAGTTTCGGAAGCCGTTTTGTTTTCCACGAACATGGTTTCGTTGTAATATA AGGATTACAGGAAACAGCTATGAC using a PCR template from pCP015 (36). Following transformation of EL250 bacteria containing TR-BAC with the kan^R PCR product, bacteria were selected for growth on kanamycin. Kan^R clones were analyzed by Southern blot analyses using probes specific for the UL74 gene and for the kan^R sequences following EcoRI restriction. Clones were also confirmed by direct DNA sequence analysis. The kan^R cassette was removed from BAC-TRΔgO kan^R clones by inducing Flp recombinase expression in EL250 host bacteria (123). Colonies were replica plated on chloramphenicol and kanamycin-containing plates to screen for BAC

lacking kan^R sequences and clones were confirmed by Southern blotting. Infectious HCMV TRΔgO was derived by electroporation of BAC DNA (2 μg) into MRC-5 cells (5 X 10^6 cells). Following electroporation, the MRC-5 cells were washed, plated in 100-mm dishes in DMEM containing 10% serum, allowed to spread on the plastic, and fresh medium was added after 24 h. When these cells became confluent, the cells were trypsinized and replated at a lower cell density, until TRΔgO produced cytopathic effect in ≈20% of the cells after 42 days.

D3. Production of TRΔgO virus stocks and PEG treatment to enhance entry. Initially, TRΔgO was propagated using BAC-TRΔgO transfected MRC-5 cells by trypsinizing the cells and plating infected cells with other, uninfected MRC-5 cells. Cell culture supernatants were harvested, and viruses were concentrated either by centrifugation at 50,000 X g for 1 h or through 20% sorbitol at 50,000 X g for 1 h. Later, TRΔgO virus stocks were produced using NHDFs infected with extracellular TRΔgO virus particles by enhancing virus entry using a combination of low speed centrifugation and PEG treatment. Briefly, cells and virus were mixed and immediately centrifuged at 800 x g for 1 h at 15˚C. Cells were washed once with warm PBS then treated with 44% (wt/wt) PEG (Fluka) in PBS at 37°C for 30 sec, and then washed immediately four times with warm PBS.

D4. Quantitative PCR (qPCR) of HCMV DNA. Cell culture supernatants from HCMV-infected cells were treated with DNase-I (Roche) to remove any DNA not protected within viral capsids. Capsids were disrupted by using the detergents and proteinase K in the QIAamp MinElute Virus Spin Kit (Qiagen). DNA was resuspended in 50 μL nuclease-free water and genomes were quantified by real-time qPCR using UL115 primers and TaqMan probes (Applied Biosystems): forward

GTAGCCATAACCTGTCAATCATCCTGTA, reverse GTATTGTAGCGTGTAATTTAGGTTGCACT, probe 6FAM-TTGCGGTGGATGAAGAAGAGCCAGAACTG. PCR samples were made to a total volume of 25 μL with 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 10 μL of 1:100, 1:1,000, or 1:10,000 dilutions of cDNA, 600 nM forward and reverse primers, 150 nM probe, and nuclease-free water. Sequences were detected using an ABI Prism 7700 Sequence Detection System. Samples were compared to a plasmid pcDNA3.1(+)gLSP standard.

D5. Antibodies. Mouse monoclonal antibodies (MAb) specific for the HCMV major capsid protein (28-4), tegument protein p65 (28-19), and glycoproteins gB (27-156) and gN (14-16A) were kindly provided by Bill Britt (University of Alabama, Birmingham) (27, 28, 34). Rabbit polyclonal anti-IE86 antibody (R683) has been described previously (99). Rabbit polyclonal antisera directed against HCMV gH, gL, and UL130 were previously described (186). Polyclonal antisera directed against a peptide (KRKQAPVKEQSEKKSKKSC) derived from HCMV TR gO was produced by coupling the peptide to keyhole limpet hemocyanin using m-maleimidobenzoyl-N-hydroxysuccinimide ester (Sigma, St. Louis, MO) and vaccinating rabbits as described (186).

D6. Immunofluorescence. HCMV-infected cells were fixed with 50:50 methanol:acetone for 10 min, washed 3 times with phosphate buffered saline (PBS), and then permeabilized using IF buffer (0.5% Triton X-100, 0.5% deoxycholate, 1% BSA, 0.05% sodium azide in PBS). The cells were stained with anti-IE86 rabbit sera diluted in IF buffer, washed with IF buffer, and then incubated with Alexa-594-conjugated goat anti-rabbit secondary antibody obtained from Molecular Probes (Eugene, OR). In some

experiments, cells were also stained with SYTO 13 green fluorescent nucleic acid stain (Invitrogen) in PBS for 10 min. The cells were analyzed using a Nikon Eclipse TS100 fluorescent microscope.

D7. HCMV cell-to-cell spread assay. NHDF, MRC-5, and ARPE-19 cells were grown to near confluence and infected with 100-200 PFU of wild type TR or TRΔgO using centrifugation (800 x g for 1 h at 15˚C) and PEG treatment. Cells were maintained in media containing 0.3% pooled human immunoglobulin (a source of HCMV neutralizing antibodies) for 10 and 20 days then fixed and analyzed by immunofluorescence for IE86.

D8. HCMV release assay. NHDF cultured in 12 well dishes (Falcon) were infected with wild type TR and TRΔgO using doses of virus that resulted in 20% infection by using PEG treatment. Cell culture medium was changed 1 day post-infection with 1 mL fresh DMEM containing 10% serum. Cell culture supernatants were collected after 2, 4, 6, and 8 days. Cell debris was removed from supernatants by centrifugation at 20,000 x g for 10 min. 200 μL of each sample was DNase I treated and then viral DNA was subsequently harvested with QIAamp MinElute Virus Spin Kit (Qiagen). Genomes were measured by qPCR (as described above). At these same times, the cells were fixed and analyzed by immunofluorescence for IE86 expression.

D9. Immunoblotting. Cell culture supernatants derived from HCMV-infected cells were clarified by centrifugation at 6,000 x g for 15 minutes then virus particles pelleted at 50,000 x g for 1 h through 20% sorbitol cushions (185). Virus pellets or HCMV-infected cells were lysed in 50 mM Tris-HCl, pH 6.8 buffer containing 2% SDS and 2% ß-mercaptoethanol, then proteins were separated using SDS-polyacrylamide gels. Proteins were electrophoretically transferred to Immobilon membranes (Millipore) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. HCMV proteins

were detected with rabbit polyclonal antibodies or MAb, followed by horseradish peroxidase-conjugated secondary antibodies, and chemiluminescence imaging as described (90, 239).

D10. Electron microscopy. NHDF were infected with wild type TR or TRΔgO by using centrifugation and PEG enhancement. After 7 days the cells were washed with 0.1 M sodium cacodylate buffer, pH 7.2 and fixed in Ito and Karnovsky's fixative (1.6% paraformaldehyde, 2.5% glutaraldehyde, and 0.5% picric acid in 0.1 M sodium cacodylate, pH 7.2) for 30 min at 23°C. The samples were post-fixed in 1.5% osmium tetroxide, rinsed, post-fixed in 4% paraformaldehyde, then dehydrated in a graded acetone series and embedded in epoxy resin, and ultrathin sections were double stained in uranyl acetate and lead citrate and viewed with a Philips EM 300 electron microscope.

E. Results

E1. Construction of a HCMV TR mutant unable to express gO. The previous evidence that HCMV gH/gL/UL128-131 promotes virus entry (185), suggested the possibility that gH/gL/gO might also participate in entry. This point has not been studied for any HCMV strain, either AD169 or the endotheliotropic TB40/E strain (101). We routinely use HCMV strain TR that can infect fibroblasts, epithelial and endothelial cells well (152, 185, 198). To construct a gO-null mutant, a BAC containing TR sequences was used in conjunction with homologous recombination targeting the UL74 (gO) gene, as described (23, 185). First, the N-terminal 1,141 nucleotides of the UL74 (gO) gene beginning with the start codon were replaced with a kanamycin resistance (kan^R) cassette flanked by FRT sites (Fig. 1A). The gO sequences targeted were carefully designed to avoid disruption of the promoters and polyadenylation sites for flanking genes (gH and gN). Bacterial clones were selected for resistance to kanamycin then the

kan^R cassette was removed by induction of flp recombinase. Southern blot analysis revealed that the kan^R cassette replaced the UL74 gene in BAC-TRΔgO kan^R clones #2 and #3 and the kan^R cassette was removed in BAC-TRΔgO clones #2.1 and #3.1 (Fig. 1B).

BAC-TRΔgO clones #2.1 and #3.1 were separately introduced into MRC-5 fibroblasts using electroporation. After 5 days, there was visible cytopathic effect (CPE) in a small number of cells and there was limited virus spread of both TRΔgO clones over the subsequent 5-7 days. This spread of CPE was substantially (2-10%) less when compared with other dishes of MRC-5 cells that had been transfected with BAC TR (wild-type). Since the majority of MRC-5 cells transfected with both clones of BAC-TRΔgO did not display CPE, we replated these cells with other, uninfected MRC-5 cells for several rounds to amplify virus. Over the course of 6 weeks, infections with both clones spread to ≈20% of the cells. Other attempts to transfect BAC-TRΔgO using normal human dermal fibroblasts (NHDF) produced virus but CPE was observed even later. Virus derived from one of these BAC clones: BAC-TRΔgO #3.1, was denoted HCMV TRΔgO and studied further by western blot analysis and most other analyses. However, a second virus derived from BAC-TRΔgO #2.1 was also characterized in a more limited number of experiments described below. TRΔgO expressed gB, gH, gL, and gN, but not gO (Fig. 1C). There were reduced quantities of all HCMV proteins in TRΔgO-infected fibroblasts compared with wild type TR but these reductions in HCMV proteins were in line with reduced numbers of infected cells (not shown). Importantly, the reduced expression of gH and gN could be explained by these reduced numbers of cells

and was similar to expression of gB, demonstrating the adjacent (UL73 and UL75) genes were not negatively affected by mutation of the gO (UL74) gene.

E2. TRΔgO particles are defective for entry into human fibroblasts. We attempted to characterize entry of TRΔgO virus particles into fibroblasts by using particles derived from cell culture supernatants. Because infection and spread of TRΔgO in fibroblast monolayers was markedly reduced, compared with wild type TR, many fewer cells were infected and fewer virus particles were produced into cell culture supernatants. However, virus particles in supernatants could be concentrated by centrifugation and quantified by measuring HCMV DNA by quantitative PCR. Thus, by using the content of viral DNA we could apply similar quantities of virus particles to cells. Wild type TR and TRΔ131, a mutant unable to assemble gH/gL/UL128-131 complexes (186) were applied to NHDFs, representing 1 PFU/cell of each virus. A similar quantity of TRΔgO particles (normalized using genome copies) was also incubated with fibroblasts. Infection of these fibroblasts was measured by staining cells using IE86-specific antibodies. Wild type TR and TRΔ131 infected 78.4% and 52.6% of fibroblasts, respectively (Fig. 2). By contrast, we did not detect any IE86+ fibroblasts following inoculation with a comparable amount of TRΔgO particles (Fig. 2, TRΔgO 1X). When the TRΔgO inoculum was increased 10-fold, we observed infection of 5-10 cells/well representing 0.025-0.04% of the total cells in the dish (Fig. 2, TRΔgO 10X). Thus, extracellular TRΔgO virus particles cannot initiate infection of fibroblasts well. It is important to note that these infections involved extracellular TRΔgO particles and are different from our propagation of TRΔgO which involved plating TRΔgO-infected fibroblasts with uninfected fibroblasts.

We previously showed that HCMV UL128-131 mutants failed to enter epithelial and endothelial cells, but this defect was reversed by treating cells and virus with the chemical fusogen PEG (185). PEG promotes entry of mutant viruses that are bound onto cell surfaces, but unable to enter because the virus particles are blocked in the capacity to fuse with cellular membranes. Fibroblasts were inoculated with TRΔgO using quantities of virus particles corresponding to 1 PFU/cell of wild type TR, then treated with PEG and ≈ 7% of the cells were IE86+ (Fig. 2, TRΔgO 1X). Using 10-fold more TRΔgO and PEG, 26% of the fibroblasts expressed IE86 (Fig. 2, TRΔgO 10X). In separate experiments using concentrated stocks of TRΔgO, we could obtain 5-40% infection of fibroblasts using PEG treatment (not shown). Therefore, the markedly enhanced expression of IE86 following PEG treatment demonstrated that TRΔgO was severely defective for entry into human fibroblasts. However, the low levels of infection observed even with 10 fold more TRΔgO virions and PEG treatment, suggested that many of the TRΔgO particles were defective in processes that preceded or followed fusion. This phenotype was different from UL128-131 mutants (186).

A very similar phenotype was observed with HCMV derived from the second BAC clone, BAC-TRΔgO #2.1, described above, i.e., very few or no IE86+ fibroblasts were detected without PEG and 5-40% IE86+ cells with PEG (not shown). This provided evidence that this phenotype was not due to secondary mutations.

E3. TRΔgO particles are defective for entry into epithelial and endothelial cells. Our model suggested that gH/gL/gO might mediate infection of fibroblasts (40, 185). To test this model, we investigated whether TRΔgO could enter ARPE-19 retinal epithelial cells. Infection of ARPE-19 cells requires higher inputs of HCMV (40, 185) and

consequently, cells were infected using 10 PFU/cell of wild type TR and an equal quantity of TRΔgO particles (based on quantities of viral DNA). All the epithelial cells were infected with wild type TR (Fig. 3). By contrast, very few of the ARPE-19 cells were infected with TRΔgO. In this particular experiment, no IE86+ cells were detected in several wells infected with TRΔgO, although in other experiments 1-10 IE86+ cells per well (0.005-0.04% of the cells) were detected. PEG treatment increased the number of IE86+ epithelial cells infected by TRΔgO to 7% (Fig. 3).

To determine whether TRΔgO could infect endothelial cells, HPV E6/E7 transformed aortic endothelial cells (HPV-AEC) were also infected with wild type TR, TRΔ131 or TRΔgO. Again, similar quantities of TRΔgO particles were applied to cells using PCR to quantify viral genomes in preparations of supernatant virus particles. Wild type TR infected 54.7% of the endothelial cells using 1 PFU/cell, whereas no cells were infected with either TRΔgO or TRΔ131 at this dose of virus (Fig. 4). Using 10 fold more TRΔgO (Fig. 4, TRΔgO 10X) less than 0.1% of the endothelial cells were infected in this experiment (Fig. 4) and no cells in other experiments (not shown). PEG treatment increased TRΔ131 infection at the lower dose of virus (1 PFU/cell) to 43.3%. TRΔgO at the lower dose (TRΔgO 1X) infected ≈1% of endothelial cells following PEG treatment and 10 fold more TRΔgO particles infected 4.8% of the cells (Fig. 4, TRΔgO 10X). These observations suggested that TR gO is important for HCMV entry into both epithelial and endothelial cells. This conclusion was surprising given our model that gH/gL/gO functions primarily in fibroblasts and not in epithelial and endothelial cells. However, in contrast to TRΔ131, there were defects beyond entry fusion, because PEG enhancement did not restore infection to wild type levels.

E4. TRΔgO cell-to-cell spread. Cell-to-cell spread of TRΔgO between fibroblasts and between epithelial cells was measured by using PEG to enhance virus entry into cells then monitoring infected cells for IE86 expression over 20 days. TRΔgO produced plaques on NHDF and MRC-5 fibroblasts that included reduced numbers of infected cells per plaque: 40-65% compared with wild type TR plaques (Fig. 5). By contrast, on ARPE-19 epithelial cells TRΔgO formed larger plaques that included 350-440% the number of infected cells per plaque compared with wild type TR plaques (Fig. 5). We were unable to obtain plaques with several types of endothelial cells, as the cells did not remain attached to plastic dishes over the course of these long experiments. Given previous results (51, 84, 101), it was not unexpected that TRΔgO would spread poorly on fibroblasts. However, it was surprising that TRΔgO could spread between epithelial cells better compared with wild type TR, especially given that the mutant largely failed to enter these epithelial cells as cell-free virus.

E5. Quantification of TRΔgO virus particles in cell culture supernatants. To determine whether the defects in TRΔgO virus particle entry and subsequent IE86 expression might be related to defects in assembly or release of virus particles, we quantified the numbers of viral genomes in cell culture supernatants. From the studies above, it was clear that some TRΔgO particles were released from cells and it was possible to concentrate these particles and enhance their entry by using PEG. However, these studies did not compare the numbers of particles released. In one set of experiments designed to quantify the numbers of particles present in cell culture supernatants, MRC-5 cells that had been transfected with BAC-TRΔgO or BAC-TR were trypsinized and plated with uninfected MRC-5. After two rounds of expansion of TRΔgO-

infected fibroblasts, the numbers of infected cells were measured by IE86+ staining. In the experiment shown in Fig. 6A, ≈ 10% of the MRC-5 cells were infected with TRΔgO, and 100% of the cells were infected with wild type TR. Cell culture supernatants were harvested from these cells and, following DNase treatment to remove DNA not protected by capsids, HCMV DNA was measured by quantitative PCR. In line with the numbers of infected cells, there were also ≈ 10% the number of TRΔgO genomes compared with wild type TR genomes in these supernatants (Fig 6A). To further determine whether there were defects in assembly or egress of HCMV with loss of gO, we attempted to increase the number of TRΔgO-infected cells by concentrating TRΔgO extracellular particles and used these in conjunction with PEG to enhance entry into cells. We also used fewer wild type TR virions so that after 2 days ≈ 20% of the fibroblasts were infected by both TRΔgO and wild type TR, as assessed by staining with IE86-specific antibodies. Cell culture supernatants were harvested after various times and the quantities of HCMV genomes were measured by quantitative PCR. Given that there were higher input doses of TRΔgO particles, we observed larger quantities of extracellular virus particles (genomes) in TRΔgO supernatants after day 2 (Fig. 6B). This was related to the larger quantities of TRΔgO particles that stuck on cell surfaces (resisting washes), but failed to enter even with PEG enhancement. However, by day 8, differences between the amounts of viral DNA in cell culture supernatants comparing TRΔgO and wild type TR narrowed so that there were comparable amounts of wild type TR and TRΔgO DNA in supernatants. We concluded that TRΔgO assembles particles and these particles are released into cell culture supernatants in relatively normal numbers.

E6. Electron microscopic analyses of TRΔgO-infected fibroblasts. To further examine TRΔgO assembly and egress, we infected fibroblasts with wild type TR or TRΔgO using PEG enhancement. Only 10-20% of the cells were infected with TRΔgO, whereas all cells were infected with wild type TR. We harvested infected cells after 7 days and then fixed and stained the cells for electron microscopy. Enveloped virions were observed in the cytoplasm and on the surfaces of both wild-type TR- and TRΔgO-infected NHDFs (Fig. 7). Due to the differences in the numbers of infected cells, it was difficult to compare (count) the absolute numbers of enveloped virions. However, among the TRΔgO-infected cells, cells that possessed capsids in the nucleus, there was no apparent reduction in the numbers of enveloped versus unenveloped capsids in the cytoplasm and numerous TRΔgO virus particles were observed on cell surfaces. Together with the results in the previous section involving quantification of viral DNA (Fig. 6), we concluded that TR gO is not required for assembly of enveloped virions or egress to cell surfaces.

E7. Loss of gO reduces incorporation of gH/gL complexes into the virion envelope but increases quantities of UL130. In the accompanying paper (184) we showed that gO promotes ER export of gH/gL. Thus, it was of special interest to determine whether loss of gO altered the quantities of gH/gL complexes present in extracellular virus particles. Fibroblasts were infected with wild type TR or TRΔgO using PEG enhancement and cell culture supernatants were harvested and concentrated by pelleting the particles through 20% sorbitol cushions. Viral proteins in these particles were analyzed by western blotting. Again, we attempted to equalize the quantities of wild type TR versus TRΔgO particles by using quantitative PCR to determine virus genomes. Several of the major structural proteins of HCMV particles, including the major capsid

protein (MCP), tegument protein pp65, and glycoproteins gB and gN, were present in wild type TR particles, as expected (Fig. 8). TRΔgO exhibited reduced quantities of gB, MCP, and pp65, although these proteins were reduced by two-fold or less likely reflecting differences in measuring viral DNA and proteins (Fig. 8). gO was detected in TR-infected cells, but not in wild type TR particles, consistent with observations in the accompanying paper (184). Surprisingly, TRΔgO virions contained little gH and gL, despite being present in infected cell lysates at a level comparable to gB (Fig. 1). In lighter exposures, no gH or gL was observed, but darker exposures revealed a small quantity of gH and gL in TRΔgO particles. Even taking into account the reduced quantities of gB and MCP in TRΔgO particles, we estimate that gH and gL were reduced by ≈90-95% in TRΔgO particles compared with TR particles. Despite this decreased quantity of gH/gL, UL130 was increased in TRΔgO virions by 2-3 fold compared with wild type TR. These studies were further confirmation that virus particles were released from TRΔgO-infected cells. We concluded that loss of TR gO leads to the production of virions with much less gH/gL, although the small quantities of gH/gL that are present contain more UL130 and, likely, gH/gL/UL128-131.

F. Discussion

Our studies of HCMV TR gO were prompted by observations that clinical versus laboratory strains of HCMV differ with respect to the expression of gH/gL/UL128-131 which mediates entry into epithelial and endothelial cells (186). Given these results and work with other herpesviruses, it seemed likely that gH/gL/gO might be involved in entry into fibroblasts. Consistent with a role for gH/gL/gO in HCMV entry into fibroblasts, we recently found that expression of gH/gL/gO in fibroblasts effectively interfered with

HCMV entry into fibroblasts, while expression of gB, gH/gL and gH/gL/UL128-131 produced much less or no interference (A. Vanarsdall, M. Chase, and D. Johnson, in press). Thus our working model suggested that HCMV uses gH/gL/UL128-131 to enter epithelial/endothelial cells, gH/gL/gO to enter fibroblasts, and that adaptation to fibroblasts favors the gH/gL/gO entry pathway.

To test the model, a HCMV TR gO-null mutant was constructed using BAC mutagenesis. The use of BACs substantially reduces the chances of second site mutations involving distant genes (17). However, the possibility of second site mutations (affecting neighboring or more distant genes) was made extremely unlikely by three sets of observations. First, the deletion of the gO coding sequences did not affect either the promoters or polyadenylation sites for the adjacent gH and gN genes, and gH and gN proteins were expressed at levels comparable to gB. Second, two separate BAC clones were used to produce two different viruses and both were found to be unable to enter fibroblasts and epithelial cells unless PEG enhancement was used. Third, our TR gO-null mutant was phenotypically similar to the previously characterized HCMV AD169, Towne and TB40/E gO-null mutants with respect to production of small plaques on fibroblasts and relatively normal (or better) spread on epithelial and endothelial cells (84, 101). It is very hard to conceive of the possibility that secondary mutations, i.e., in genes other than the gO gene, would produce this exact phenotype on all cell types.

TRΔgO produced very little infectious virus into cell culture supernatants, i.e., virus that could initiate infection of fibroblasts. When TRΔgO particles from supernatants were concentrated and applied to fibroblast monolayers, only a few cells per dish (0 - 0.04%) were infected. However, when PEG was used to chemically promote membrane fusion, as many as 40% of the fibroblasts could be infected with TRΔgO. The striking

increase in rates of infection with PEG demonstrated that a fraction of TRΔgO particles were markedly defective for entry into fibroblasts. However, PEG enhancement of TRΔgO entry failed to produce rates of infection similar to wild type HCMV. When 10 times as many TRΔgO particles were used (compared to TR at 1 PFU/cell, a dose that infected ≈75% of cells) there was infection of only 5-40% of fibroblasts. These results also suggested that another fraction of TRΔgO particles possessed defects either preceding or following entry fusion.

Quantification of HCMV genomes in extracellular virus particle preparations demonstrated relatively normal numbers of TRΔgO particles compared with wild type TR, when numbers of infected cells were taken into account. These studies were complicated to perform related to defects in TRΔgO infection and spread between fibroblasts. With fewer fibroblasts infected by TRΔgO versus wild type TR, it was difficult to compare extracellular particles. PEG was used to boost the entry of TRΔgO and using lower inputs of TR we could roughly match infections so that after 2 days there was ≈ 20% infection with both mutant and wild type HCMV. After 8 days, TRΔgO produced comparable quantities of HCMV particles (measured by viral DNA). Immunoblotting of extracellular TRΔgO particles also showed that there were relatively normal quantities of gB, the tegument protein pp65 and MCP. Furthermore, electron microscopy of TRΔgO-infected fibroblasts revealed morphologically normal enveloped virus particles in the cytoplasm and on cell surfaces.

Our analyses of extracellular TRΔgO particles derived from fibroblasts by immunoblotting demonstrated marked reductions in the amounts of gH and gL, compared with gB, pp65 and MCP. Related to these observations, in the accompanying

paper (184) we showed that TR gO is largely ER retained but acts as a molecular chaperone to facilitate ER export of gH and gL. Previous studies had shown that co-expression of UL128-131 with gH/gL (without other HCMV proteins) also increases ER export of gH/gL, but, importantly, the UL128-131 proteins remain bound onto gH/gL and become incorporated into virions as gH/gL/UL128-131 (186). The markedly reduced quantities of gH/gL present in extracellular TRΔgO virions probably explains the observed defects in entry into fibroblasts. It appears that gH/gL, and not gH/gL/gO, mediates entry into fibroblasts. This conclusion is partially based on the observations that HCMV UL128-131-null mutants can efficiently enter fibroblasts (70, 185), indicating that a second gH/gL complex must function in fibroblast entry. Furthermore, the highly reduced amounts of gH/gL present in TRΔgO particles might explain our observations that these particles are morphologically normal, yet cannot enter cells efficiently, even with PEG treatment. Without normal amounts of gH/gL, these virions might be less able to bind onto cell surfaces. Reduced binding to cells would not be overcome by PEG treatment. This point is under investigation in ongoing studies.

Entry into epithelial and endothelial cells by TRΔgO (produced by fibroblasts) was also extremely poor. Concentrated stocks of extracellular TRΔgO particles infected only a few cells in the entire monolayer. PEG treatment increased TRΔgO infection to 5-10% of these cells. This inhibition of entry into epithelial and endothelial cells was not predicted from our working model in which gH/gL/UL128-131, rather than some other gH/gL complex, is required for entry into epithelial and endothelial cells. Instead, the highly reduced quantities of gH/gL in the envelope of TRΔgO apparently reduced entry into these cells. This supports a modified hypothesis in which HCMV entry into epithelial and endothelial cells requires both gH/gL and gH/gL/UL128-131.

Spread of TRΔgO in fibroblast monolayers was reduced, as plaques included 35-60% the number of cells compared with wild type TR. By contrast, the defect in entering fibroblasts was much more pronounced, involving 10^4-10^5 fewer cells infected. Cell-to-cell spread of TRΔgO between epithelial cells was increased by 2.5 to 4 fold, compared with wild type TR. Again, this spread was compared with 10^4-10^5 fewer cells infected by TRΔgO. These results were similar to those involving the TB40/E gO-null mutant that could spread normally on endothelial cells (101). We found that there were increased amounts of UL130 present in TRΔgO extracellular virions. It seems probable that this increased UL130 was accompanied by increased UL128 and UL131 as well, although this was not directly tested. If this is the case, the increased quantities of gH/gL/UL128-131 found in TRΔgO virions were insufficient for entry into epithelial/endothelial cells, but apparently promoted increased movement of TRΔgO particles between epithelial and endothelial cells.

Observations of enhanced HCMV cell-to-cell spread with virus particles that were highly defective for entry into cells were quite astonishing. These results underscore major differences between these two processes. One possibility is that cell-to-cell spread is much less sensitive to deficiencies in the quantities of gH/gL, compared with entry of extracellular particles. This may relate to the relative concentration of herpesvirus receptors at cell junctions where cell-to-cell spread likely occurs (reviewed in (102)). Extracellular virus particles might require higher quantities of gH/gL complexes to find and bind receptors on cell surfaces. However, it is important to note that we did not purify extracellular TRΔgO virus particles from epithelial cells. Thus, we do not know whether these extracellular particles, or particles spreading between epithelial cells, have deficiencies in gH/gL, but this is likely.

Previous characterization of an HCMV strain TB40/E gO-null mutant by Jiang et al. (101) produced some similar results compared with ours, but also some different conclusions about how gO functions. They observed very few infectious virus particles (100-1000 fold decreased) produced into fibroblast culture supernatants, similar to our results with TRΔgO. The very small amounts of infectious TB40/E gO-null in cell culture supernatants were concentrated then applied to fibroblasts using 0.001 PFU/cell comparing to the same amount of wild type TB40/E. Both mutant and wild type produced rare cells expressing early and late HCMV genes. From this, they concluded that there were not defects in virus entry associated with loss of gO. However, because TB40/E gO-null particles in supernatants were not enumerated, it was not clear whether there were large quantities of noninfectious TB40/E gO-null (based on the 100-1000 fold decrease in infectious virus) that failed to enter these cells. Certainly, our studies involving PEG treatment directly demonstrated defects in TRΔgO entry into all cells tested. We also observed no defects in virus egress, normal numbers of virus particles were found in cell culture supernatants, and enveloped particles were observed in infected cells in normal numbers by electron microscopy. Jiang et al. observed substantial accumulation of unenveloped capsids in the cytoplasm and few enveloped particles produced in TB40/E gO-null infected fibroblasts by electron microscopy and concluded that gO is required for virus assembly (101). The different conclusions about how TB40/E and TR gO function might also reflect strain differences, as gO is highly variable (49, 152, 160). In this regard, TB40/E was passaged extensively on endothelial cells, whereas TR is a low passage strain. Whether our results involving TR gO apply to other HCMV strains is not clear. TR is a low passage isolate that we consider “wild type.” Alternatively, the concept of “wild type” may be misplaced here, so that different strains

of HCMV make use of gO for different functions. Resolving these issues will require the characterization of other clinical strains of HCMV (e.g., strain Merlin).

In summary, the studies herein and in the accompanying paper (184) demonstrated that TR gO is not present in virions and, instead, acts to increase gH/gL export from the ER (184) and incorporation into extracellular virions (this study). Thus, it appears that gH/gL, rather than gH/gL/gO, mediates HCMV entry into fibroblasts. However, entry into epithelial and endothelial cells appears to require both gH/gL and gH/gL/UL128-131. These observations provide a molecular picture for why loss of UL128-131 proteins promotes adaptation of HCMV to propagation in fibroblasts. The increased levels of UL130 in TRΔgO particles suggest that gO and UL128-131 compete for binding to gH/gL. Consistent with this notion, co-expression of gO with gH/gL decreases binding of UL128-131 to gH/gL in experiments involving Ad vectors (B. Ryckman, unpublished results). By this model, loss of UL128-131 might favor the production of gH/gL and the fibroblast entry pathway. It seems possible that adaptation to passage in fibroblasts produces changes in gO. Observations that both HCMV gH/gL and gH/gL/UL128-131 are required for entry into cells differ from models involving EBV and HHV-6, which use one of two gH/gL complex to enter different cell types (91, 148).

G. Acknowledgments

This work was supported by grants from the National Institutes of Health R01AI081517 (to DCJ) and RO1AI21640 (to JAN). We thank Michael Webb of the OHSU EM Core for excellent technical assistance with the electron microscopy experiments, and Craig Kreklywich for assistance with qPCR. We are grateful to Dr. William J. Britt (University of Alabama-Birmingham) for supplying important monoclonal antibodies. Tiffani Howard prepared the graphics. Finally, we appreciate advice and

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H. Figures

Figure 1. Construction of a HCMV TR gO-null mutant. (A) TR-BAC containing the entire genome of HCMV clinical strain TR (except for where the BAC sequence replaces US2-US5) was previously described (152). The UL73 (gN), UL74 (gO) and UL75 (gH) genes are depicted. The N-terminal 1,141 nucleotides of UL74 (beginning at the gO start codon and extending to codon 380) were replaced by homologous recombination with a kanamycin resistance (kan^R) cassette flanked by FRT sites producing BAC-TRΔgO kan^R. The replacement did not affect the UL73 or UL75 promoters, coding sequences, or polyA sites. Following induction of flp recombinase in bacteria, the kan^R cassette was excised, leaving a single FRT site in place of the N-terminal UL74 sequences (BAC-TRΔgO). Illustration by Tiffani Howard. **(B)** Southern blot analyses of BAC clones. TR-BAC (WT), BAC-TRΔgO kan^R clones 2 and 3, and BAC-TRΔgO clones 2.1 and 3.1 were digested with EcoRI. This produces a 32.4 kilobase fragment for wild type UL74 but a 4.5 kilobase fragment when the kan^R cassette is inserted. Flp recombination produced clones 2.1 and 3.1 that lacked both kan^R and UL74 sequences. The blots were probed with either gO sequences or kan^R sequences. **(C)** Expression of gH, gL, gB, gO and gN proteins in wild type TR and TRΔgO-infected NHDF fibroblasts after 8 days infection by western blotting.

Figure 2-1

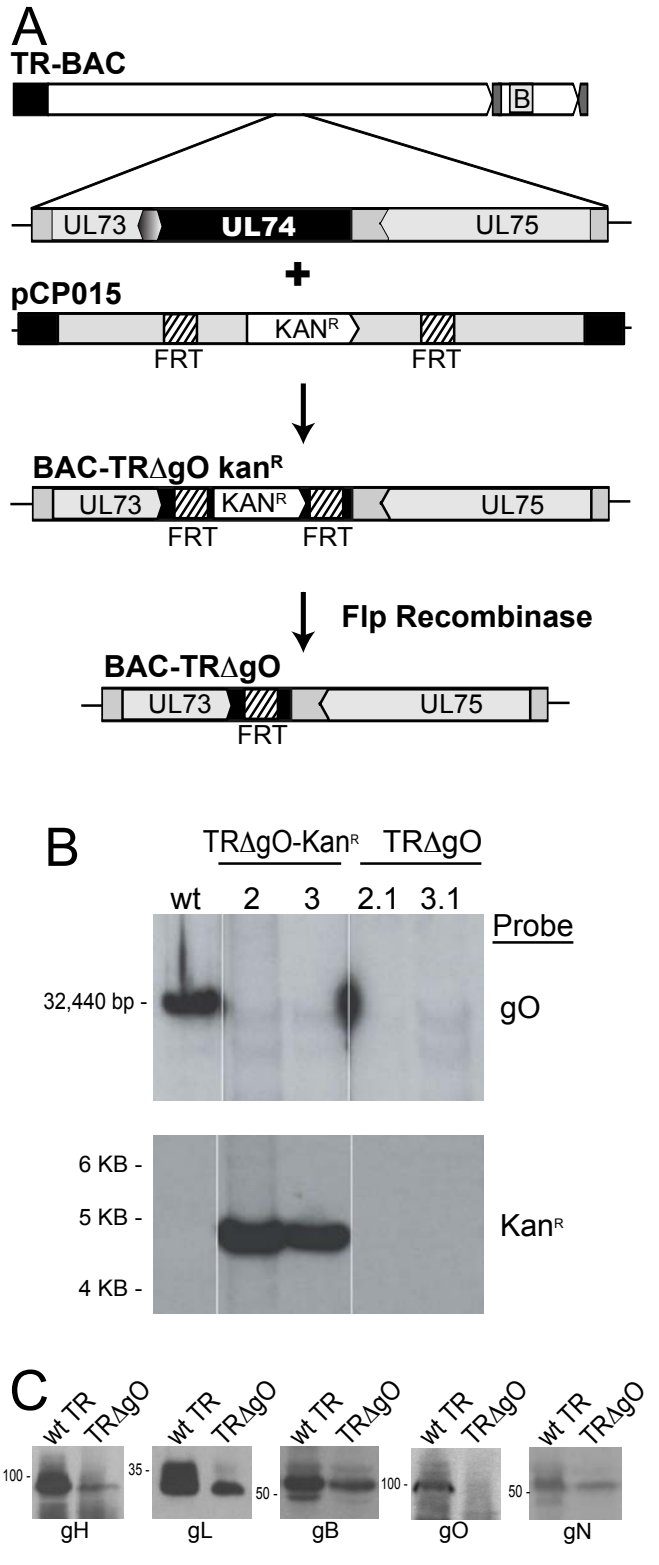

A
TR-BAC
B
UL73
UL74
UL75
pCP015
KANR
FRT
FRT
BAC-TRΔgO kanR
UL73
KANR
UL75
FRT
FRT
Flp Recombinase
BAC-TRΔgO
UL73
UL75
FRT
B
TRΔgO-KanR
TRΔgO
wt
2
3
2.1
3.1
Probe
32,440 bp -
gO
6 KB -
5 KB -
4 KB -
KanR
C
wt TR
TRΔgO
100 -
gH
35 -
gL
50 -
gB
100 -
gO
50 -
gN

Figure 2. Entry of TRΔgO into fibroblasts with and without PEG treatment.

Multi-well dishes of NHDFs were incubated with extracellular HCMV particles concentrated by pelleting from fibroblast culture supernatants. Wild type (wt) TR and TRΔUL131 were used at 1 PFU/cell. A similar quantity of TRΔgO extracellular virions (based on quantifying genomes using qPCR) or 10 times (10X) that amount of TRΔgO were also incubated with cells. These viruses were centrifuged with cells at 800 x g for 1 h. Some of the wells were subsequently treated with 44% PEG for 30 sec then immediately washed (+PEG). After 48 h, cells were fixed, permeabilized and stained for HCMV immediate-early protein IE86. Numbers indicate the average number of IE86+ cells in three replicate wells.

Figure 2-2

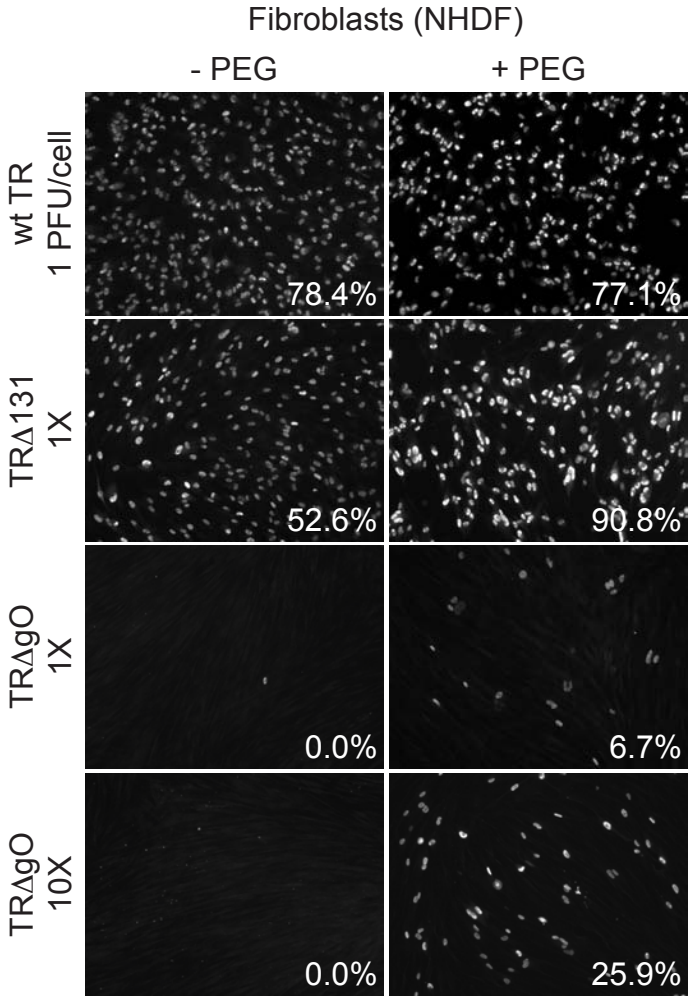

Fibroblasts (NHDF)
- PEG
+ PEG
wt TR
1 PFU/cell
78.4%
77.1%
TRΔ131
1X
52.6%
90.8%
TRΔgO
1X
0.0%
6.7%
TRΔgO
10X
0.0%
25.9%

Figure 3. Entry of TRΔgO into epithelial cells with and without PEG treatment. Multi-well dishes of ARPE-19 epithelial cells were incubated with extracellular HCMV particles: wild-type (wt) TR corresponding to 10 PFU (defined using fibroblasts)/ARPE-19 cell or a similar quantity of TRΔgO virus particles (based on quantifying genomes using qPCR). The virus and cells were centrifuged at 800 × g for 1 h at 15˚C. Some of the wells were subsequently incubated with 44% PEG for 30 sec then immediately washed. After 48 h, cells were fixed, permeabilized and stained for IE86. Numbers indicate the average number of IE86+ cells in three wells.

Figure 2-3

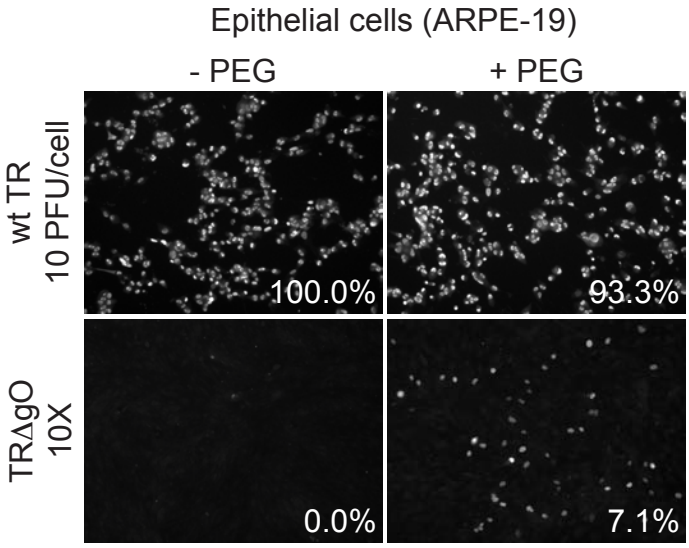

Epithelial cells (ARPE-19)
- PEG
+ PEG
wt TR
10 PFU/cell
100.0%
93.3%
TRΔgO
10X
0.0%
7.1%

Figure 4. Entry of TRΔgO into endothelial cells with and without PEG treatment. Multi-well dishes of HPV-AEC endothelial cells were incubated with extracellular HCMV particles: wild type (wt) TR or TRΔ131 using 1 PFU (defined using fibroblasts)/endothelial cell or a similar quantity (1X) of TRΔgO virus particles (based on quantifying genomes using qPCR) or 10 times that quantity of TRΔgO particles (10X). The cells and viruses were centrifuged at 800 × g for 1 h at 15˚C. Some of the wells were subsequently incubated with 44% PEG for 30 sec then immediately washed. After 48 h, cells were stained for IE86. Numbers indicate the average number of IE86+ cells in three wells.

Figure 2-4

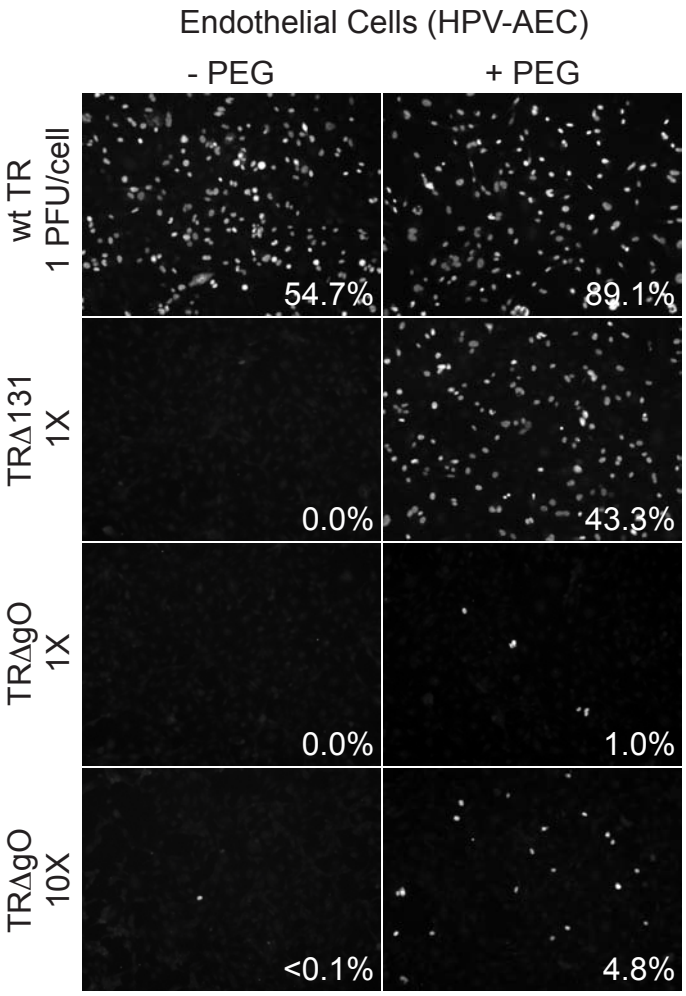

Endothelial Cells (HPV-AEC)
- PEG
+ PEG
wt TR 1 PFU/cell
54.7%
89.1%
TRΔ131 1X
0.0%
43.3%
TRΔgO 1X
0.0%
1.0%
TRΔgO 10X
<0.1%
4.8%

Figure 5. Cell-to-cell spread of TRΔgO is in fibroblasts and epithelial cells. NHDF and MRC-5 fibroblasts and ARPE-19 epithelial cells were infected with wild type (wt) TR or TRΔgO using ≈100-200 PFU/well. Cells and virus were centrifuged at 800 × g for 1 h at 15˚C, all wells treated with 44% PEG for 30 sec, and then the cells washed and incubated in the presence of HCMV neutralizing antibodies for 10 or 20 days. Cells were then fixed and stained for IE86. The numbers of infected cells in 10 representative plaques were counted and the average numbers are shown. Bar represents 100 μm.

Figure 2-5

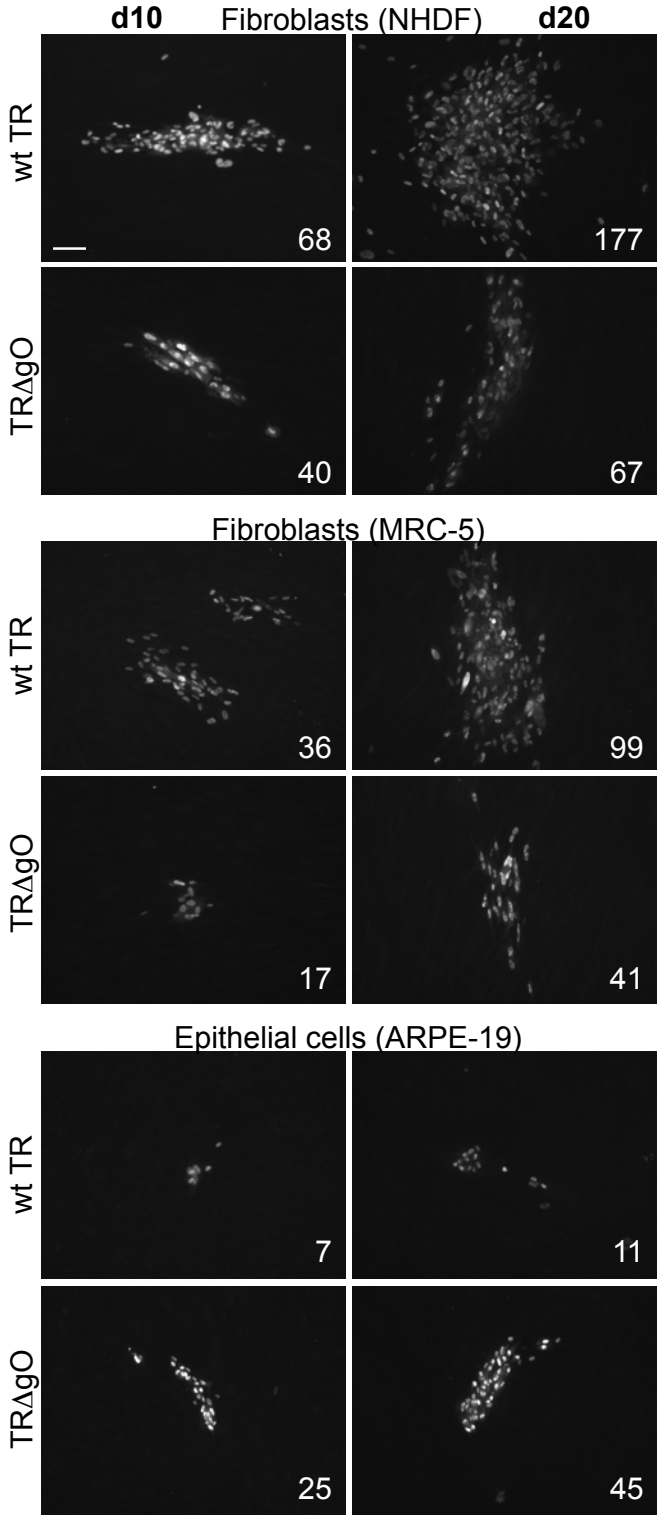

d10
Fibroblasts (NHDF)
d20
wt TR
TRΔgO
68
177
40
67
Fibroblasts (MRC-5)
wt TR
TRΔgO
36
99
17
41
Epithelial cells (ARPE-19)
wt TR
TRΔgO
7
11
25
45

Figure 6. Release of TRΔgO from fibroblasts into extracellular supernatants.

(A) MRC-5 fibroblasts that had been transfected with BAC-TRΔgO or BAC-TR were trypsinized and plated with other MRC-5 cells. This allowed for spread of TRΔgO, such that ≈10% of these cells showed CPE and expressed IE86. After 10 days, culture supernatants from these TRΔgO-infected fibroblasts and supernatants from wild type TR-infected fibroblasts (in which all the cells were infected) were subjected to quantitative PCR to enumerate HCMV genomes. **(B)** NHDF were infected with wild type TR or TRΔgO using low speed centrifugation followed by PEG enhancement of entry, such that approximately 20% of the cells were infected by both viruses following 2 days of infection. Infected cell supernatants were collected 2, 4, 6, and 8 days post infection, treated with DNase, and viral DNA was isolated and quantified by qPCR.

Figure 2-6

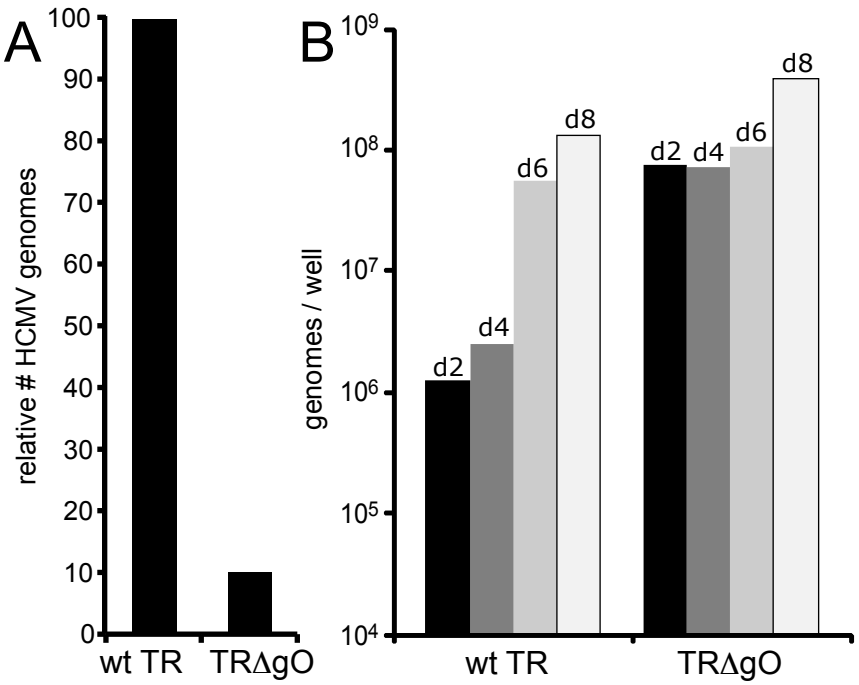

A
relative # HCMV genomes
100
90
80
70
60
50
40
30
20
10
0
wt TR
TRΔgO
B
genomes / well
10^9
10^8
10^7
10^6
10^5
10^4
d2
d4
d6
d8
d2
d4
d6
d8
wt TR
TRΔgO

Figure 7. Electron microscopy of wild type TR- and TRΔgO-infected fibroblasts. NHDF were infected with wild type TR or TRΔgO by using low speed centrifugation and PEG treatment. Under these conditions, ≈10-20% of TRΔgO-infected cells displayed IE86 expression by day 2, whereas all the cells were infected with wild type TR. After 7 days, cells were fixed, stained and analyzed by transmission electron microscopy. cp, cytoplasm; pm and arrow, plasma membrane; arrowheads, enveloped virus particles.

Figure 2-7

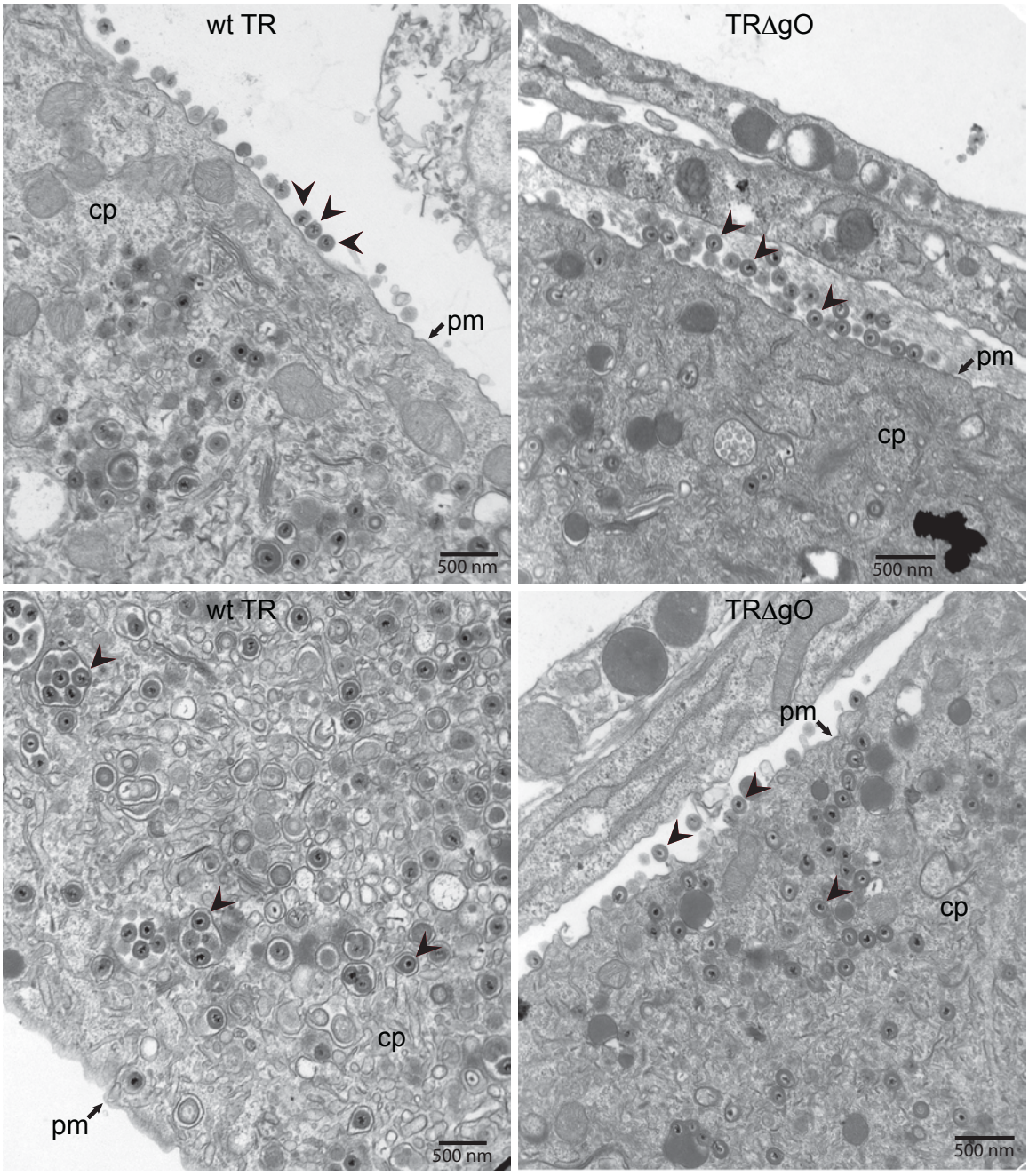
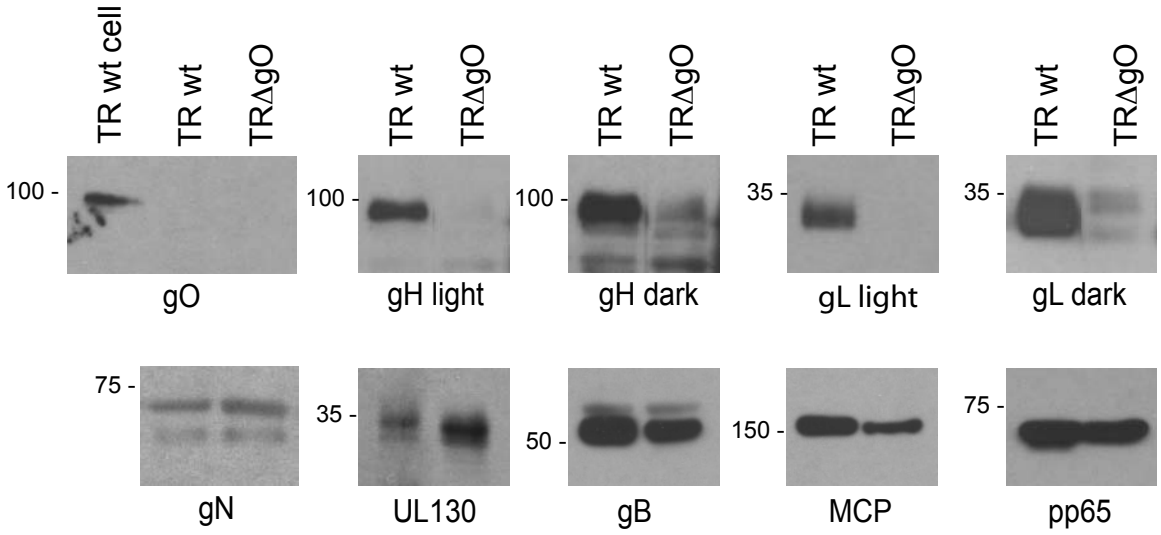

wt TR
cp
pm
500 nm
TRΔgO
pm
cp
500 nm
wt TR
cp
pm
500 nm
TRΔgO
pm
cp
500 nm

Figure 8. Analyses of HCMV proteins in TRΔgO extracellular virus particles.

NHDF were infected with wild type TR or TRΔgO using low speed centrifugation and PEG to enhance entry. After 8 days, virus particles were prepared from cell culture supernatants by centrifugation through 20% sorbitol cushions. The quantities of the virus particles per sample were made similar by measuring viral genomes using qPCR. A cell lysate from wild type TR-infected cells was loaded as a positive control for gO immunoblotting (TR wt cell). Proteins were separated by SDS-PAGE, transferred to Immobilon membranes and western blotted for gO, gH, gL, gN, UL130, gB, the major capsid protein (MCP) or tegument protein pp65. Lighter and darker exposures are shown for gH and gL.

Figure 2-8


TR wt cell
TR wt
TRΔgO
100 -
gO
TR wt
TRΔgO
100 -
gH light
TR wt
TRΔgO
100 -
gH dark
TR wt
TRΔgO
35 -
gL light
TR wt
TRΔgO
35 -
gL dark
75 -
gN
35 -
UL130
50 -
gB
150 -
MCP
75 -
pp65

CHAPTER 3: HCMV PARTICLES LACKING gB CAN ENTER FIBROBLASTS EXPRESSING gB

Paul T. Wille, Marie C. Chase, Brent J. Ryckman, Catherine C. Wright, Marisa K. Isaacson, Teresa Compton, David C. Johnson

A. Statement of Author Contributions

P.T.W. and D.C.J. designed research; P.T.W. performed research; M.C.C. and C.C.W. prepared reagents; B.J.R, C.C.W., M.K.I., and T.C. contributed reagents; P.T.W. and D.C.J. analyzed data; and P.T.W. and D.C.J. wrote chapter.

B. Abstract

All herpesviruses use multiple viral membrane glycoproteins to mediate virus entry. Two universally required glycoprotein components are gB and the heterodimer gH/gL. For human cytomegalovirus (HCMV), we have shown that expression of gB and gH/gL in cells was sufficient to cause cell-cell fusion. Surprisingly, cells expressing only gH/gL fused with cells expressing only gB. This cell-cell fusion *in trans* suggested that the putative fusion protein, gB, can be activated by interactions with gH/gL that occur across the spaces separating cells. Here, we tested whether HCMV mutants deleted for gB (HCMVΔgB) or gH (HCMVΔgH) were able to enter cells expressing gB or gH. Neither glycoprotein appeared to be required for assembly or virus release, and gB-null and gH-null particles were incapable of entering cells except when fusion was enhanced in the presence of polyethylene glycol (PEG). However, HCMVΔgB entered fibroblasts and

epithelial cells expressing gB. We named this novel phenomenon entry *in trans*. HCMVΔgH did not enter gH/gL expressing cells, but could enter gB-expressing cells, albeit to a much lesser extent compared to HCMVΔgB. This phenomenon does not appear to extend to HSV gB-null or gH-null mutants. Because gB is expressed in cells and oriented toward the extracellular space, whereas gH/gL is present in the virion envelope and oriented toward cells, we believe this is strong evidence supporting a model in which gB is the HCMV fusion protein and gH/gL binds cellular receptors to activate gB.

C. Introduction

HCMV infects a broad array of cell types (15, 153, 236). Several different cell types appear to be required for invasion, dissemination, and transmission to new hosts: epithelial cells, endothelial cells, monocyte/macrophages and fibroblasts are all utilized by the virus during the course of infection (143, 153). HCMV uses distinct mechanisms to initiate entry fusion into different cell types. Entry into fibroblasts involves virus fusion with the plasma membrane (40), while entry into epithelial and endothelial cells involves endocytosis and requires low pH (185). These separate portals of entry appear to require unique envelope glycoprotein complexes, as mutants that are unable to assemble the gH/gL/UL128/UL130/UL131 complex are defective for entry into epithelial and endothelial cells, but show no defects for entry into fibroblasts (4, 64, 70, 184, 185, 231, 237). In Chapter 1, I described the other gH/gL complexes, gH/gL and gH/gL/gO, that participate in HCMV entry into both fibroblasts and epithelial and endothelial cells.

One simplified model for how herpesviruses enter cells suggests three steps: i) adhesion or adsorption onto cell surfaces, ii) receptor binding, and iii) fusion between the virion envelope and cellular membranes. Which viral glycoproteins are responsible for

each step is becoming clearer, especially in the case of Epstein-Barr virus (EBV) and herpes simplex virus (HSV) (reviewed in (74, 91)). HCMV adheres to cells through interactions between glycoproteins gB or gM and heparan sulfate glycosaminoglycans (GAGs) (39, 108). These interactions are not sufficient for entry, but are required prior to binding of downstream receptors, and likely increase cell surface levels of virus (41). Following this adsorption, HCMV binds receptors that trigger entry fusion. The two known routes of entry described above apparently require different receptors. Evidence for this involves interference, in which viral glycoproteins are expressed *in trans* and cells are subsequently infected with HCMV. If the viral glycoprotein binds saturable receptors required for entry and this binding prevents incoming virus from interacting with the cell protein, then HCMV cannot enter. Expression of gH/gL/UL128-131 in epithelial cells interfered with HCMV entry via the endosomal pathway (183). By contrast, gH/gL/UL128-131 expression in fibroblasts did not interfere with HCMV entry via fusion with the plasma membrane (183). However, expression of gH/gL/gO caused interference in fibroblasts, but not epithelial cells (A. Vanarsdall, M. Chase, D. Johnson, in press). These studies were consistent with the hypothesis that there are different cellular proteins acting as HCMV receptors in fibroblasts and epithelial cells and that these proteins are bound by different gH/gL complexes. Based on the studies below, we believe that the third step in this pathway, fusion between the virion envelope and cell membranes, is carried out by HCMV gB.

Studies of EBV have demonstrated that different gH/gL complexes can act as receptor-binding proteins for entry into different cell types. EBV gH/gL/gp42 binds to HLA class II molecules to mediate entry into B cells, while gH/gL complexes lacking gp42 bind integrins $\alpha_v\beta_6$ or $\alpha_v\beta_8$ on epithelial cells to mediate fusion (125, 224, 242). Therefore,

our model of HCMV gH/gL complexes binding different receptors on fibroblasts and epithelial cells parallels the studies of EBV entry. However, with EBV the receptors have been identified.

The other universal herpesvirus glycoprotein, gB, appears to be required for a step subsequent to adsorption and receptor binding. The structure of HSV and EBV gB argues very strongly that gB is a class III fusion protein analogous to vesicular stomatitis virus (VSV) G protein (8, 75, 181). Soluble HSV gB is also able to directly associate with lipid vesicles *in vitro*, while soluble HSV gH/gL only associates with vesicles in the presence of gB (31, 72). These observations are consistent with a model in which herpesvirus gB molecules act directly in membrane fusion during entry, i.e., to mix lipids in the virion envelope with those in cell membranes.

Much less is known about how HCMV gB functions, but since all herpesviruses express gB molecules, it seems likely that HCMV gB functions in membrane fusion similar to EBV and HSV gB. A HCMV gB-null was constructed to address its role in HCMV replication (94). This mutant produced normal numbers of virus particles, consistent with no defects in assembly. Moreover, these virus particles could adsorb onto cells, but not enter (94). These studies were consistent with defects in either the receptor binding or membrane fusion steps in entry. The best evidence that HCMV gB participates in membrane fusion involves cell-cell fusion assays. In human epithelial cells, expression of HCMV gB and gH/gL caused fusion. Initially, this involved expression of both gB and gH/gL in the same cells (fusion *in cis*) (227). However, we also observed a phenomenon we denoted cell-cell fusion *in trans*, in which cells expressing HCMV gB alone were mixed with cells expressing HCMV gH/gL alone and, again, efficient cell-cell fusion was observed (227). Thus, it appeared that gB in one cell could be activated by

interactions with gH/gL in an apposing cell. However, the model of HCMV gB as the fusion protein was still largely based on studies of HSV and EBV gB. Further studies of how HCMV gB mediates fusion *in trans* were warranted to extend our knowledge of HCMV entry.

Here, we demonstrated that expression of HCMV gB in fibroblasts promoted the entry of an HCMV gB-null mutant. We denoted this process entry *in trans*. HCMV lacking gH/gL did not enter cells expressing gH/gL complexes. By contrast, we were unable to demonstrate entry *in trans* involving HSV mutants lacking gB or gH. Given the orientation of gB, pointed toward the virion envelope during entry *in trans*, gB cannot act as a receptor binding protein in this process. Given that gH/gL must be oriented toward the cell during entry *in trans*, it appears that gH/gL is acting to bind receptors. These results suggest that HCMV gB is triggered by gH/gL complexes across the space between membranes and causes fusion between the virion envelope and cell membranes.

D. Materials and Methods

D1. Cells. Neonatal normal human dermal fibroblasts (NHDF) were obtained from Cascade Biologics (Portland, OR) and were grown in Dulbecco's Minimum Essential Medium (DMEM, HyClone) that was supplemented with 12% fetal bovine serum (FBS, HyClone). NHDF-gB and -gH were made as described (94, 111). Briefly, NHDF were infected with retrovirus constructs encoding either the HCMV gB or gH gene in a bicistronic cassette with green fluorescent protein (GFP), and were maintained in the same manner as NHDF. The retinal pigment epithelial cell line ARPE-19 was obtained from the ATCC and was grown in a 1:1 mix of DMEM and Ham’s F-12 medium (HyClone) supplemented with 10% FBS. Vero cells were maintained in DMEM

supplemented with 8% FBS. VD60 cells were maintained in DMEM lacking histidine (HyClone) supplemented with 8% FBS and 0.2-0.4 mM histidinol (Sigma) (127). F6 cells (a gift from Tony Minson, Cambridge University) were maintained in DMEM supplemented with 8% FBS and 150 μg/ml G418 (Mediatech) (61).

D2. Viruses. Laboratory strain AD169 (not derived from BAC clones) that has been passaged extensively on fibroblasts was obtained from Jay Nelson. Construction of pAD/CreΔUL55 (HCMVΔgB) was previously described (94). HCMV strain TR was derived from the ocular vitreous fluid of an AIDS patient (198) and was stabilized in the form of a bacterial artificial chromosome (BAC) (152). Generation of TRΔUL75 (HCMVΔgH) is described below. AD169 and TR were propagated on NHDF by infection at a multiplicity of 0.1 in DMEM supplemented with 5% FBS for 10 to 16 days. Complemented stocks of HCMVΔgB or HCMVΔgH containing gB or gH in the virus envelope were generated on NHDF-gB or -gH cells by infecting the cells at an MOI of 0.2-0.5 in DMEM supplemented with 5% FBS for 10 days. gB- or gH-null stocks of HCMVΔgB or HCMVΔgH were generated on NHDF cells by infecting at an MOI of 2-5 for 2 h, washing the cells with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 30 sec to neutralize and remove uninternalized HCMV, and collecting virus after 7-10 days. Virus particles were concentrated from infected cell supernatants by centrifugation at 50,000 × g for 1 h through a cushion of 20% sorbitol in PBS. Pellets were resuspended in DMEM/F12 supplemented with 10% FBS and frozen at -70°C. The number of infectious units (IU) was determined by plating serial dilutions on NHDF confluent monolayers. 1 d post-infection the number of infected cells was determined by immunofluorescence detection of HCMV IE86, and the numbers of IU/mL were defined as the numbers of IE86+ cells per mL.

HSV mutants lacking gB and gH (F-BAC gB- and F-BAC gH-) were previously described (55, 56). F-BAC mutants were propagated and titered on Vero cells.

D3. Replication-defective adenovirus vectors. Non-replicating (E1-) adenovirus (Ad) vectors induced by the tet-transactivator protein (supplied by Ad tet-trans) expressing HCMV TR gB, gH and gL and AD169 gB have been described (73, 98, 183, 186).

D4. Construction of recombinant HCMV TRΔgH. The UL75 gene of BAC-TR was mutated by using λ-phage linear recombination as previously described (23, 123, 244). Briefly, a 1.4-kb PCR product containing a kanamycin resistance (kan^R) gene cassette flanked by Flp recombination target (FRT) sites and with HCMV sequences at the 5' end of the UL75 gene (63 nt, corresponding to nt 147098 to 147160 of published TR sequence, accession no. AC146906) and sequences near the 3' end of the UL75 gene (60 nt, corresponding to nt 146981 to 147040) was generated with primers: CGCAGGAAGCGGATGGGTCTCCCGTAGGTGTTGAGTAGTAGGTGAAACGCGTGAG GGTCCGTGTAGGCTGGAGCTGCTTC and CCAGCGGCGCGGCCGCGCTAAACGGCCCGGCCTCCCCTCCTAGCTCACCGTCTT CGCCGTCTAAATGGGAATTAGCCATGGTCC using a PCR template from pKD4 (46). DH10B bacteria containing TR-BAC and pKD46 plasmid encoding the λ-Red recombination genes were transformed with the kan^R PCR product. Clones were selected for growth on kanamycin and were confirmed by restriction analysis and direct DNA sequence analysis. Infectious HCMV TRΔgH was derived by electroporation of BAC DNA (2 μg) into NHDF-gH cells (5×10^6 cells). Following electroporation, the NHDF-gH cells were plated on 100-mm dishes in DMEM containing 10% serum and fresh medium was added after 24 h. When these cells became confluent, the cells were

trypsinized and replated at a lower cell density three times, until TRΔgH produced cytopathic effect in ~20% of the cells after 28 days.

D5. Quantitative PCR (qPCR) of HCMV DNA. Cell culture supernatants from HCMV-infected cells were treated with DNase-I (Roche) to remove any DNA not protected within viral capsids. Capsids were disrupted by using the detergents and proteinase K in the QIAamp MinElute Virus Spin Kit (Qiagen). DNA was resuspended in 50 μL nuclease-free water and genomes were quantified by real-time qPCR using AD169 UL115-derived primers and TaqMan probes (Applied Biosystems): forward GAGGTGTTTCAGGGTGACAAGTATGA, reverse GGACGGTAACGAATAAGTTGCGATAG, probe 6FAM-CTGCGCCCGTTGGTGAATGTTACCAG. PCR samples were made to a total volume of 20 μL with 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 2 μL viral DNA, 600 nM forward and reverse primers, 150 nM probe, and nuclease-free water. Sequences were detected using an Applied Biosystems Step One Plus. Samples were quantified compared to a HCMV BAC DNA dilution series.

D6. Antibodies. Mouse monoclonal antibodies (MAb) specific for the HCMV major capsid protein (28-4), tegument protein pp65 (28-19), and glycoproteins gB (27-156) and gH (AP86-SA4) were kindly provided by Bill Britt (University of Alabama, Birmingham) (27, 28, 34, 226). Rabbit polyclonal anti-IE86 antibody (R683) has been described previously (99). Rabbit polyclonal antisera directed against HCMV gH was previously described (186). Mouse MAb 58S directed against HSV ICP4 (192) was collected from mouse ascites. Rabbit polyclonal antisera R68 directed against HSV gB and R137 directed against HSV gH were kindly provided by Roselyn Eisenberg and Gary Cohen (95, 161).

D7. Immunofluorescence. Cells were washed 2-3 times with phosphate buffered saline containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS+/+) (HyClone), fixed with 2% formaldehyde for 10 min, washed 2-3 times with PBS+/+, and then permeabilized and blocked using IF buffer (0.5% Triton X-100, 0.5% deoxycholate, 1% BSA, 0.05% sodium azide in PBS). For cell-surface staining, primary antibody was added in PBS+/+ at 4˚C for 2 h prior to fixation. Fixed cells were stained with the appropriate antibodies in IF buffer, washed with PBS+/+, and then incubated with Alexa 594-conjugated goat anti-rabbit or 488-conjugated goat anti-mouse secondary antibodies obtained from Molecular Probes (Eugene, OR). In some experiments, cells were also stained with SYTO 13 green fluorescent nucleic acid stain (Invitrogen) in PBS for 10 min. The cells were analyzed using a Nikon Eclipse TS100 fluorescent microscope.

D8. Immunoblotting. Concentrated virus derived from NHDF, NHDF-gB, or NHDF-gH cell culture supernatants were measured for genomes by qPCR, and equivalent genomes for each virus were pelleted at 100,000 × g for 30 min. Virus pellets were lysed in 1 × sample buffer (50 mM Tris-HCl, pH 6.8 buffer containing 10% glycerol, 2% SDS and 2% β-mercaptoethanol). Proteins were separated using SDS-polyacrylamide gels. Proteins were electrophoretically transferred to Immobilon membranes (Millipore) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. HCMV proteins were detected with rabbit polyclonal antibodies or mouse monoclonal antibodies, horseradish peroxidase-conjugated secondary antibodies (Sigma), and enhanced chemiluminescence (ECL) substrate (Pierce). Images were collected using the ImageQuant detection system (GE).

D9. Entry in trans assay. NHDF or ARPE-19 were plated on 48- or 96-well plates and grown to touch confluence. Cells were infected with adenoviruses at the

MOIs described in figures for 4 h in the appropriate medium for each cell type supplemented with 2-5% FBS. 24 to 48 h later, cells were inoculated with the appropriate viruses. For HCMV, plates were immediately centrifuged at 800 × g for 30 min at 10˚C. In some wells, virus entry was enhanced using polyethylene glycol (PEG) treatment. Cells were washed once with warm PBS+/+, treated with 44% (w/v) PEG (Fluka) in PBS+/+ at 37°C for 30 sec, and then washed immediately ten times with warm PBS and incubated with the appropriate medium for each cell type supplemented with 2-5% FBS. Untreated wells were incubated for 1 to 1.5 h at 37˚C following either centrifugation and then the medium was replaced with the appropriate medium for each cell type supplemented with 2-5% FBS. All wells were fixed with 2% formaldehyde in PBS+/+ 24 to 48 h post-infection and assayed for IE86 expression by immunofluorescence.

For HSV, virus inoculum was incubated with cells for 2 h at 37˚C. Where PEG was used, cells were treated with 44% (w/v) PEG (Fluka) in PBS+/+ at 37°C for 30 sec, and then washed immediately ten times with warm PBS and incubated with DMEM supplemented with 5% FBS. Medium was changed in untreated wells at the same time. 6 h post-treatment, all wells were fixed with 2% formaldehyde in PBS+/+ and assayed for ICP4 expression by immunofluorescence.

D10. HCMV binding assay. NHDF, NHDF-gB, and NHDF-gH were plated to touch confluence. NHDF were infected with HCMVΔgB or HCMVΔgH, NHDF-gB were infected with HCMVΔgB and NHDF-gH were infected with HCMVΔgH at a MOI of 2 IU/cell. 1 d post-infection, medium was supplemented with ^{3}H-thymidine. 7 d post-infection, supernatants were harvested, cell debris was pelleted at 5,000 × g, and virus was pelleted at 50,000 × g through a 20% sorbitol cushion. Virus was resuspended in

DMEM supplemented with 5% FBS and tritium was quantified in CytoScint (ICN) using a LS6500 scintillation counter (Beckman-Coulter). To measure binding, 3,000, 10,000, and 30,000 counts/well of each virus were incubated with NHDF in a 24-well plate in DMEM supplemented with 5% FBS. Virus was bound to cells at 4˚C and plates were centrifuged for 30 min at 800 × g. Inoculum was removed from cells on ice following centrifugation and saved as an input sample. Cells were washed three times with ice-cold medium and each wash was collected as an input sample. Cells were lysed in PBS supplemented with 1% Triton X-100 and this lysate was collected as a bound sample. Wells were washed once with PBS supplemented with 1% Triton X-100 and this was collected as a bound sample. Tritium was quantified, and the four unbound input samples and two bound samples were added together to determine ratio of bound versus unbound virus.

D11. HCMV cell-to-cell spread assay. NHDF, NHDF-gB, and NHDF-gH were plated to touch confluence and infected with 100-200 IU AD169, HCMVΔgB, TR or HCMVΔgH. Cells were maintained in media containing 0.2% pooled human immunoglobulin (a source of HCMV neutralizing antibodies) for 10 days then fixed and analyzed by immunofluorescence for IE86.

D12. HCMV release assay. NHDF, NHDF-gB, and NHDF-gH were plated to touch confluence in a 12-well dish (Falcon) and infected with HCMVΔgB or HCMVΔgH at a MOI of 1 IU/cell in DMEM supplemented with 5% FBS for 2 h then washed three times with DMEM supplemented with 5% FBS. Cell culture medium was changed 1 day post-infection with 1 mL fresh DMEM supplemented with 5% FBS. Cell culture supernatants were collected after 2, 4, 6, and 8 days from replicate plates. Cell debris was removed from supernatants by centrifugation at 20,000 × g for 10 min. 200 μL of each sample was DNase I treated and viral DNA was subsequently harvested with the QIAamp MinElute

Virus Spin Kit (Qiagen). Genomes were measured by qPCR. At these same times, the cells were fixed and analyzed by immunofluorescence for IE86 expression, and data was expressed as genomes per 1000 infected cells.

E. Results

E1. Characterization of HCMV gB- and gH-null virus particles. To study HCMV glycoproteins gB and gH/gL further, we produced viral null mutants unable to express gB or gH. Since gH/gL is a constitutive heterodimer, deletion of the gH gene is sufficient to extinguish gH/gL expression. Previously, an HCMV gB-null mutant was constructed using the HCMV strain AD169 BAC, and virus was propagated in human fibroblasts transduced with a retrovirus expressing gB (94). I used a BAC containing the HCMV TR genome to disrupt the UL75 (gH) gene by homologous recombination, as described previously (23, 123, 244). The initiator methionine codon was mutated from ATG to AAA, and 58 nucleotides near the 5' end of the UL75 (gH) open reading frame were replaced with a cassette encoding kanamycin resistance. Bacterial clones resistant to kanamycin were selected and screened by restriction digest and sequence analysis to determine the replacement of the gH gene with the kan^R cassette (data not shown). An isolate denoted HCMVΔgH-BAC was identified and characterized further.

As HCMV gH/gL is expected to be essential for entry, it was necessary to complement gH expression before rescuing the HCMV gH-null virus. A retrovirus vector expressing HCMV TR gH was obtained from Teresa Compton and used to infect neonatal human dermal fibroblasts (NHDF). Integration of the retrovirus genome into the cell genome transduced the NHDFs to become a cell line expressing gH (NHDF-gH) and these cells could be passaged approximately 20 population doublings following transduction. NHDF-gH cells constitutively express a bicistronic mRNA encoding gH and

green fluorescent protein (GFP), which is translated from an internal ribosomal entry site (IRES). Thus, to indirectly test for expression of gH, cells were assayed for GFP fluorescence. I also used another retrovirus provided by Teresa Compton to produce a gB-expressing cell line (NHDF-gB) in order to propagate HCMVΔgB, as described (94).

NHDF transduced with retroviruses expressing gB or gH were then electroporated with either HCMVΔgB-BAC or HCMVΔgH-BAC (Fig. 1A). Focal, virus-induced cytopathic effect was observed by bright-field microscopy 10-14 days post-electroporation with both HCMVΔgB-BAC or HCMVΔgH-BAC. The virus was subsequently propagated by serial passage; infected cells were split 1:3 every 4-9 days depending on cell density. Once infected cells were greater than 20% of the culture, HCMV was allowed to spread and infect the entire monolayer, and both cell- and supernatant-derived stocks were collected. The HCMV mutant lacking gH, but grown on gH-expressing fibroblasts (complemented), was denoted HCMVΔgH+gH and the mutant lacking gB, but grown on gB-expressing cells, was denoted HCMVΔgB+gB. These complemented viruses were titered by an infectious unit (IU) assay, where fibroblasts were infected with a dilution series of virus, and the number of HCMV immediate-early protein IE86-positive nuclei 24 h post-infection were quantified by immunofluorescence. Titered, complemented virus was used to infect untransduced fibroblasts at approximately 2-5 IU/cell (Fig. 1A). Virus particles lacking gH (HCMVΔgH-gH) or gB (HCMVΔgB-gB) were collected 7 days post-infection from cell culture supernatants.

The quantities of complemented and null virus particles produced into infected-cell supernatants were measured by qPCR 1, 2, 4, 6, and 8 days following infection of either complementing or untransduced fibroblasts. Relatively high levels of input contaminated the samples produced by HCMVΔgB (day 1) but *de novo* virus production

was observed by day 4 (Fig. 1B). Lower levels of input were evident for HCMVΔgH. Production of virus in cell culture supernatants was reduced when gB or gH was absent. However, there was significant production of virus particles without gB or gH, and my results demonstrate that neither glycoprotein is required for assembly and release of genome-containing particles. This is consistent with previous reports describing HCMVΔgB and for an HCMVΔgL mutant that assembled normally (20, 94).

Released particles were also characterized for whether they contained similar quantities of HCMV structural proteins (Fig. 1C). These comparisons involved attempts to load similar amounts of the major capsid protein (MCP) in each lane, although it was not always possible to determine exactly equal quantities of each virus, partly related to problems in quantifying non-infectious viruses. All viruses contained comparable levels of the tegument protein pp65 compared to their parental strain. As expected, HCMVΔgH-gH virus did not contain gH. HCMVΔgH+gH was reduced for gH incorporation compared to parental strain TR, but a significant gH band was observed (Fig. 1C). HCMVΔgH+gH and -gH particles contained relatively normal amounts of gB compared with parental strain TR. HCMVΔgB-gB lacks gB, as previously reported. The relative incorporation of gB into HCMVΔgB+gB was low compared with parental strain AD169. In contrast to gB incorporation into HCMVΔgH particles, there was a substantial loss of gH incorporation into both HCMVΔgB+gB and -gB compared to AD169. It is not clear how reduced gH/gL incorporation into particles lacking gB (or with reduced gB) comes about. This might be related to observations by Adam Vanarsdall that gB and gH/gL can coprecipitate from HCMV cell extracts (227), and thus, incorporation of gH/gL may rely on interactions with

gB. Regardless, this reduced gH/gL in gB-null particles may influence the interpretation of the data below.

Spread of each virus with and without gB or gH was assessed in order to further characterize complementation (Fig. 1D). HCMVΔgH and HCMVΔgB produced plaques on complementing cells, with both viruses producing smaller plaques than those produced by their parental virus (Fig. 1D). However, plaque formation, similar to virus release (Fig. 1B), was good considering the very low levels of gB and gH incorporated into complemented virus. There were no plaques produced by HCMVΔgH and HCMVΔgB on normal human fibroblasts (NHDF); instead, single infected cells were observed.

HCMVΔgB-gB and HCMVΔgH-gH were assessed for their ability to enter fibroblasts (Fig. 2). Cells were infected with equivalent numbers of genomes as measured by qPCR, in either the presence or absence of polyethylene glycol (PEG), a chemical fusogen that overcomes entry defects. Neither virus was able to enter fibroblasts as determined by immunofluorescence staining for HCMV immediate-early protein IE86 24-48 hours post-infection (Fig. 2, panel a, and c). In fact, there was not a single IE+ cell in the entire monolayer of cells. However, when virus particles were applied to cells for 30 minutes, then briefly treated with 44% PEG, both HCMVΔgB-gB and HCMVΔgH-gH entered 47% of cells (Fig. 2, panels b & d). These results demonstrated that both gB and gH/gL are absolutely essential for entry into human fibroblasts, as expected.

E2. HCMVΔgB and HCMVΔgH entry *in trans*. HCMV gB and gH/gL can mediate cell-cell fusion when expressed in apposing membranes (227) and thus we tested whether a similar process could occur between the virion envelope and cell

membranes. Fibroblasts were transduced to express gB, gH/gL, or GFP by using non-replicating Ad vectors. The same number of HCMVΔgB-gB and HCMVΔgH-gH particles as were used in Figure 2 were incubated with these cells and IE+ cells were quantified after 24 hours. Neither gB-null nor gH-null virus particles entered any control Ad GFP-transduced cells (Fig. 3A, panels a-b), i.e., there were zero cells infected. HCMVΔgB-gB also did not enter fibroblasts expressing gH/gL (Fig. 3A, panel e). Cells expressing HCMV gB, however, facilitated entry of HCMVΔgB-gB, and these particles entered 5-14% of these cells (Fig. 3A, panel c). Importantly, HCMVΔgH-gH particles did not enter cells expressing gH/gL (Fig. 3A, panel f), gH/gL/gO (data not shown), or gH/gL/UL128-131 (data not shown), There was some low level entry of HCMVΔgH-gH into fibroblasts expressing gB (Fig. 3A, panel d). We denoted this unique phenomenon entry *in trans*, as gB in the plasma membrane of cells apparently mediates the entry of virus that lacks gB.

We also tested whether other cell types were capable of mediating entry *in trans*. ARPE-19 epithelial cells were transduced with either gB or gH/gL (Figure 3B). Again, HCMVΔgB-gB and HCMVΔgH-gH entered gB-expressing cells (2.4% and 1.1%, Fig 3B panels c and e, respectively), but not GFP- or gH/gL-expressing cells (Fig. 3B, panels a and e, b and f, respectively). Entry into ARPE-19 cells with wild-type HCMV TR is less efficient compared with entry into fibroblasts, and this observation may partly explain the lower level of entry *in trans*. However, poor entry *in trans* may also be explained by the absence of gH/gL/UL128-131 complexes from HCMVΔgB-gB. Entry into epithelial cells requires gH/gL/UL128-131, and this complex is not present on HCMVΔgB-gB because this virus was derived from the UL131-deficient strain AD169.

We tested whether HCMV entry *in trans* was specifically mediated only by HCMV gB, or whether other class III fusion proteins could mediate HCMV entry *in trans* (Fig.

3C). Fibroblasts were infected with an adenovirus vector expressing HSV gB (Fig. 3C, panel b) and compared to HCMV gB (Fig. 3C, panel a). HSV gB was incapable of mediating HCMVΔgB entry *in trans*.

E3. HSV glycoproteins do not mediate entry *in trans* of HSV glycoprotein-null mutants. Given that HSV glycoproteins have also been found to mediate cell-cell fusion *in trans* (7), we tested whether HSV gB or gH/gL could mediate entry *in trans* of HSVΔgB and HSVΔgH. First, cell surface and total expression levels of HCMV and HSV gB and gH expressed from Ad vectors were compared by immunofluorescence microscopy (Fig. 4A). Cell surface expression of HSV glycoproteins was much higher than HCMV glycoproteins, and likely to be sufficient to cause entry *in trans* if this was possible. Both HSVΔgB and HSVΔgH were inoculated on cells that had been previously transduced with either no Ad or Ad expressing gB or gH/gL. The HSV doses were calculated to be the amount of virus necessary to infect most or all cells in the presence of PEG. Neither HSV gB or gH/gL could mediate entry *in trans* of HSVΔgB or HSVΔgH mutants (Fig. 4B and 4C).

E4. Adsorption of HCMVΔgB-gB and HCMVΔgH-gH particles onto fibroblasts is normal. The efficiency of entry *in trans* by HCMVΔgB-gB (5-14%) was reduced compared with entry of HCMVΔgB-gB following PEG treatment (47%) or entry of a comparable number of wild-type AD169 particles (100%). To determine whether this was related to reduced virus adsorption, I measured virus binding to cells. Extracellular virus particles labeled with ^{3}H-thymidine were prepared and incubated with fibroblasts. Both HCMVΔgB+gB and HCMVΔgB-gB bound to fibroblasts (Fig. 5). In this experiment there was a small increase in the binding of HCMVΔgB-gB particles compared with

HCMVΔgB+gB. This might be explained by a higher specific activity of the HCMVΔgB-gB particles, i.e., higher ^{3}H-thymidine incorporation during the production of these particles. Regardless, it was clear that the absence of gB in the virion envelope does not decrease virus adsorption onto cells, as this process likely involves binding of multiple glycoproteins to heparan sulfate GAGs. HCMVΔgH+gH and HCMVΔgH-gH also bound to cells, but the binding of these particles was lower compared with HCMVΔgB+gB and HCMVΔgB-gB particles (Fig. 5). The reasons for these differences are not clear, but may again reflect the specific activities of these particles. Thus, it appears that particles lacking gH can bind to cells, although perhaps less well.

F. Discussion

F1. HCMV virions lacking gB can enter cells expressing gB. Previously, Adam Vanarsdall showed that expression of HCMV glycoproteins gB and gH/gL in cells produced cell-to-cell fusion (227). Importantly, gB and gH/gL could be expressed in apposing membranes and fusion was similarly efficient (227). The development of methods for constructing and complementing HCMV gB- and gH-null mutants (94) allowed me to test whether virus particles lacking gB or gH/gL could enter cells expressing gB or gH/gL *in trans*. HCMVΔgB was able to enter as many as 14% of gB-expressing fibroblasts. When HCMV gB was absent, there was not a single infected cell in wells containing 2×10^{4} cells and incubated with the same number of HCMVΔgB-gB virus particles. HCMVΔgB-gB particles also did not enter cells expressing HSV gB. Moreover, there was no entry of HCMVΔgH-gH into cells expressing gH/gL. However, there was some low level entry of HCMVΔgH-gH into cells expressing gB. This may be

related to the notion that gB is the fusion protein and can be triggered (but to a much lesser extent) in cell membranes missing gH/gL.

HCMVΔgB entry *in trans* was less efficient compared with HCMVΔgB entry mediated by the chemical fusogen PEG. This is likely related to the low levels of gH/gL in the HCMVΔgB particles and the lower concentration of gB in cell membranes compared to gB in the virion envelope. Clearly, gB was expressed very poorly on cell surfaces (Fig. 4) and this is likely related to endocytosis motifs in the gB cytoplasmic domain (12, 43). To address this possibility, I have made two HCMV gB mutants with these endocytosis motifs removed, in the hopes that these mutants will be more extensively expressed on cell surfaces and mediate more efficient entry *in trans* compared to wild-type gB.

F2. Conclusions about gB from entry *in trans* observations. My results showed that when HCMV gB is oriented in cell membranes toward the extracellular space, this protein can mediate entry of HCMV particles that contain gH/gL in the virion envelope. By contrast, expression of gH/gL oriented toward the virion envelope did not allow entry of virus particles lacking gH/gL. Therefore, it appears that gH/gL must be oriented toward the cells. This supports the hypothesis that HCMV gB acts to fuse membranes, whereas gH/gL complexes act to bind cellular receptors and to trigger gB. Second, because gB and gH/gL were in separate membranes, the extracellular domains of gB and gH/gL must have interacted across the space between the cell membrane and the virion envelope so that gB was triggered for fusion. This is similar to cell-cell fusion *in trans*, in which gB and gH/gL were oriented head-to-head, apposing one another, rather than anchored together and running parallel to one another. Our studies add to the

structural information on HSV and EBV gB (8, 75) and studies of liposome binding (31, 72) to strongly support the conclusion that gB is the fusion protein for all herpesviruses.

F3. Conclusions about the role of gH/gL from these studies. The HCMVΔgH mutant I constructed did not enter cells, which demonstrates (as previously shown for HCMVΔgL (20)) that gH/gL is essential for HCMV entry into cells. The gH-null mutant was not blocked in assembly, and normal numbers of virus particles were released from cells. The gH-null particles could also adsorb onto cell surfaces. Unlike HCMVΔgB entry *in trans* into gB-expressing cells, HCMVΔgH did not enter cells expressing gH/gL complexes (i.e., gH/gL, gH/gL/gO, or gH/gL/UL128-131), demonstrating that gH/gL oriented toward the virion envelope fails to promote entry. Thus, gH/gL must be present in the virion envelope and oriented toward cellular membranes in order to mediate entry. These results are consistent with our previous conclusions that gH/gL complexes are involved in binding cellular receptors ((183), A. Vanarsdall, M. Chase and D. Johnson, in press) and these interactions trigger gB for fusion.

F4. Can gB-mediated entry *in trans* function for other herpesviruses? We tested whether HSVΔgB or HSVΔgH could enter cells expressing HSV gB or gH/gL *in trans* and did not observe any entry. Several different cell lines were tested including a melanoma cell line expressing relatively high levels of nectin-1 (C10) that can undergo cell-cell fusion *in trans* (7). However, even with C10 cells there was no evidence that either virus could enter a single cell in the dish (data not shown). This result may be related to the fact that HSV uses three glycoproteins that are all essential for entry: gD, gH/gL and gB. In entry *in trans* assays, cells expressing HSV gB were incubated with HSV lacking gB but expressing gH/gL and gD in virion envelopes. However, the triggering of gD and gH/gL to cause gB-mediated fusion might be much less efficient

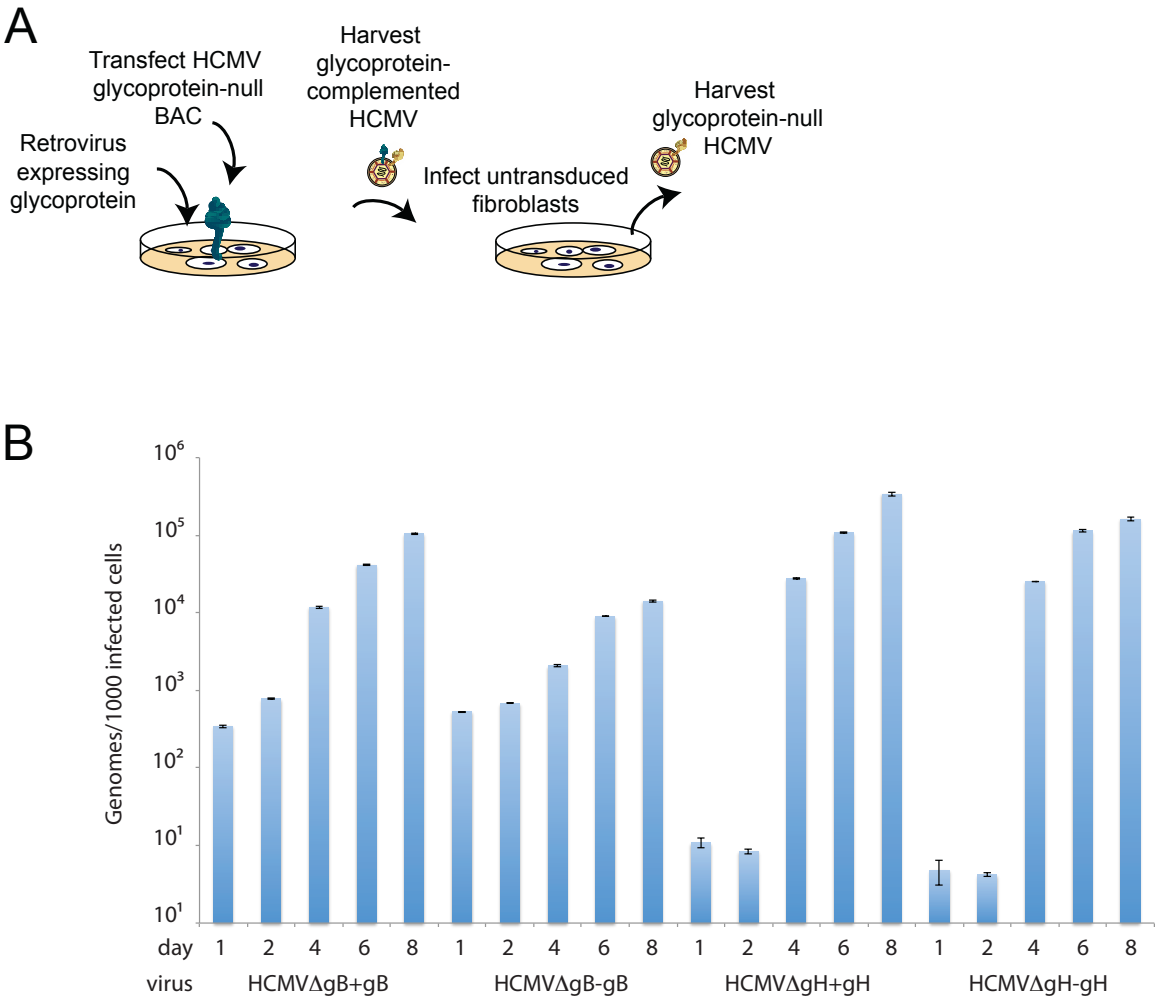
compared with HCMV gH/gL triggering gB, as HCMV apparently does not require a third protein in its core fusion machinery (227). I also initiated efforts to test EBV entry *in trans*. This involved a previously described EBV gB-null mutant (155), and we constructed Ad vectors expressing EBV gB and gH/gL. Unfortunately, I was unable to prepare sufficient quantities of extracellular EBV particles lacking gB to test entry *in trans*. There are experiments ongoing in Lindsey Hutt-Fletcher's laboratory to examine this point further.

F5. Summary. These entry *in trans* observations are novel, as there has never been a description of a viral fusion protein that can act in cell membranes to mediate entry of virus particles lacking that fusion protein. Moreover, these observations hold great potential to expand our understanding of how gB functions in fusion and how gH/gL triggers gB. Thus, cell-cell fusion and entry *in trans* assays can be used to further define the mechanism and regulation of HCMV glycoproteins required for fusion.

G. Figures

Figure 1. Characterization of HCMV gB- and gH-null. (A) A schematic of the derivations of HCMVΔgB and HCMVΔgH. Neonatal human dermal fibroblasts (NHDF) are the primary cells for HCMV replication in cell culture. These cells can support the replication and spread of HCMV mutants deleted for gB and gH expression when they are transduced with retroviruses that constitutively express gB or gH, respectively. Virus collected from these cells is used to infect untransduced fibroblasts, and the resulting progeny virus does not contain the deleted glycoprotein. Illustration by Tiffani Howard. **(B)** HCMVΔgB-gB and HCMVΔgH-gH virus release from complementing or untransduced cells. NHDF-gB, NHDF-gH or NHDF were infected with HCMVΔgB+gB or HCMVΔgH+gH at an MOI of 1 IU/cell. HCMVΔgB+gB was collected from NHDF-gB, HCMVΔgH+gH was collected from NHDF-gH, and HCMVΔgB-gB and HCMVΔgH-gH were collected from untransduced NHDFs. 1, 2, 4, 6, and 8 days post-infection, supernatants were collected from replicate wells. Viral DNA was harvested from supernatants and copy number was determined by qPCR. Wells at each time point were stained for IE86+ cells. Genomes per 1000 infected cells are shown.

Figure 3-1


A
Transfect HCMV glycoprotein-null BAC
Retrovirus expressing glycoprotein
Harvest glycoprotein-complemented HCMV
Infect untransduced fibroblasts
Harvest glycoprotein-null HCMV
B
Genomes/1000 infected cells
10^6
10^5
10^4
10^3
10^2
10^1
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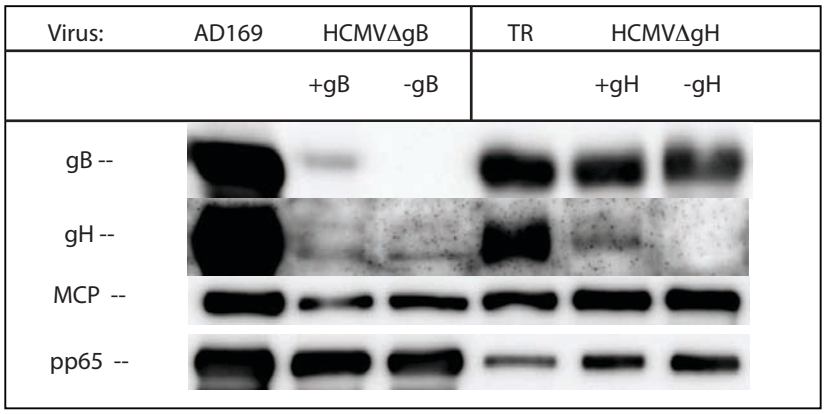
day 1 2 4 6 8 1 2 4 6 8 1 2 4 6 8 1 2 4 6 8
virus HCMVΔgB+gB HCMVΔgB-gB HCMVΔgH+gH HCMVΔgH-gH

Figure 1. Characterization of HCMV gB- and gH-null. (C) Supernatant virus structural protein content. Each virus was quantified by qPCR. Equal numbers of particles were concentrated by ultracentrifugation and then extracted in SDS-containing sample buffer. Proteins were separated by SDS-PAGE, transferred to Immobilon membranes, and western blotted for gB, gH, major capsid protein (MCP), and pp65. **(D)** HCMVΔgB and HCMVΔgH plaque size on complementing and untransduced cells. NHDF-gB, NHDF-gH, and NHDF were infected with either HCMVΔgB+gB or HCMVΔgH+gH. Plaques were fixed at 10 d and assayed for IE86 expression by immunofluorescence.

Figure 3-1

C


Virus:
AD169
HCMVΔgB
TR
HCMVΔgH
+gB
-gB
+gH
-gH
gB --
gH --
MCP --
pp65 --

D

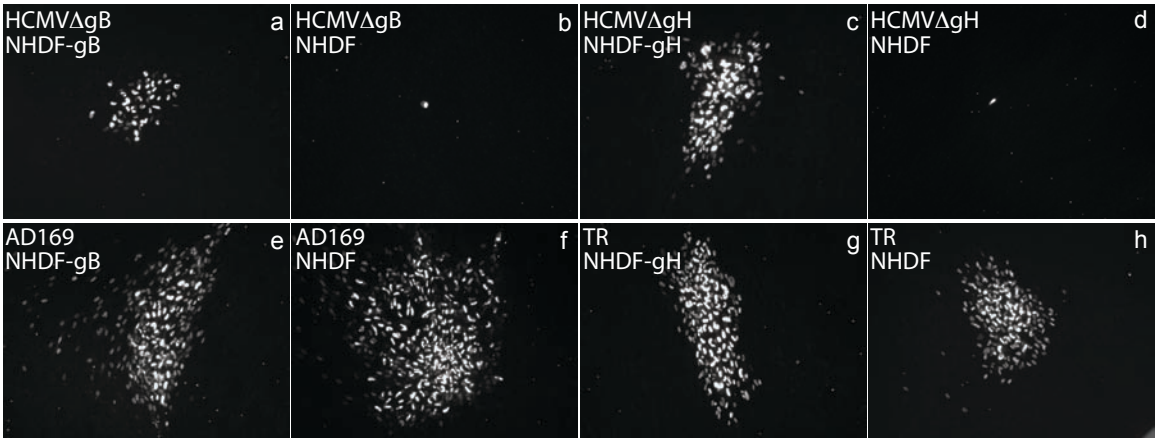

HCMVΔgB
NHDF-gB
a
HCMVΔgB
NHDF
b
HCMVΔgH
NHDF-gH
c
HCMVΔgH
NHDF
d
AD169
NHDF-gB
e
AD169
NHDF
f
TR
NHDF-gH
g
TR
NHDF
h

Figure 2. HCMVΔgB-gB and HCMVΔgH-gH entry in the presence or absence of PEG. An equivalent number of genomes of HCMVΔgB-gB and HCMVΔgH-gH (approximately 1×10^5 genomes/cell as measured by qPCR) were inoculated onto untransduced fibroblasts. The cells and viruses were centrifuged at 800 × g for 30 min at 10˚C. Some of the wells were subsequently incubated with 44% PEG for 30 sec then immediately washed. 24 h post-infection, cells were assayed for IE86 expression by immunofluorescence. The percent of cells infected in the representative image is indicated.

Figure 3-2

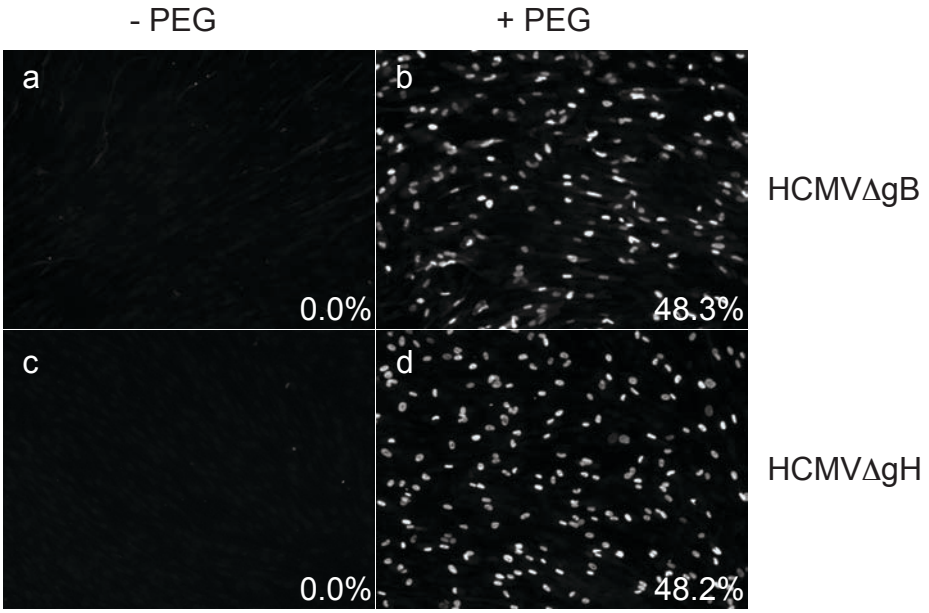


Figure 3. HCMVΔgB-gB and HCMVΔgH-gH entry *in trans*. (A) NHDF were infected with Ad GFP, Ad gB, or Ad gH and Ad gL at an MOI of 100 PFU/cell. 24-48 h after adenovirus infection, cells were inoculated with a volume of HCMVΔgB-gB or HCMVΔgH-gH such that ~50% of the cells were infected in the presence of PEG, and 24 h post-infection, cells were assayed for IE86 expression by immunofluorescence. The percent of cells infected in the representative image is indicated. **(B)** Conditions for (A) were repeated with ARPE-19 cells. **(C)** Fibroblasts were infected with Ad HSV or HCMV gB at a MOI of 100 PFU/cell, and 24 h later cells were inoculated with a volume of HCMVΔgB-gB such that ~50% of the cells were infected in the presence of PEG. 24 h post-infection, cells were assayed for IE86 expression by immunofluorescence. The percent of cells infected in the representative image is indicated. Illustrations by Tiffani Howard.

Figure 3-3

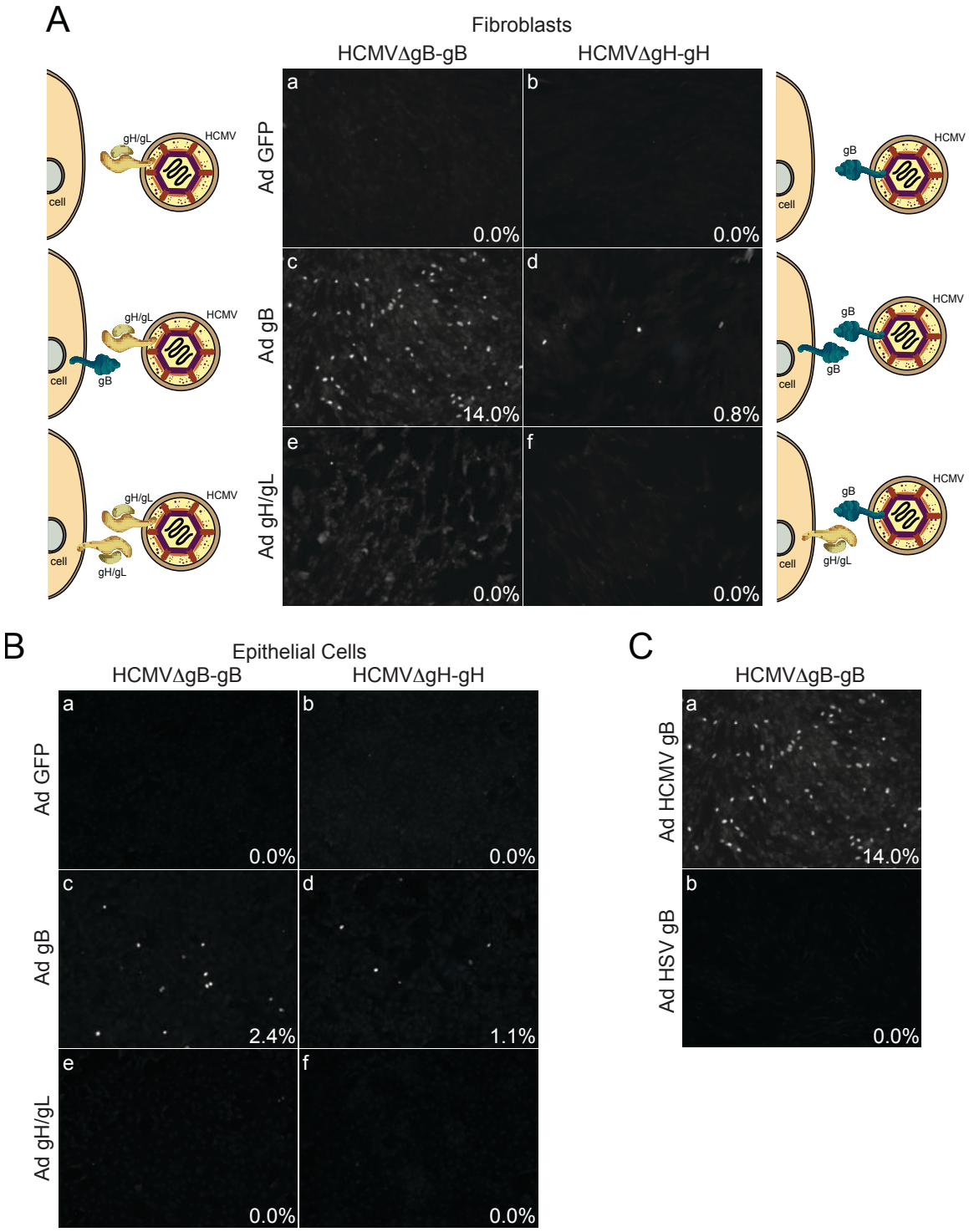

A
Fibroblasts
HCMVΔgB-gB
HCMVΔgH-gH
Ad GFP
Ad gB
Ad gH/gL
a
b
c
d
e
f
0.0%
0.0%
14.0%
0.8%
0.0%
0.0%
cell
gH/gL
HCMV
gB
B
Epithelial Cells
HCMVΔgB-gB
HCMVΔgH-gH
Ad GFP
Ad gB
Ad gH/gL
0.0%
0.0%
2.4%
1.1%
0.0%
0.0%
C
HCMVΔgB-gB
Ad HCMV gB
Ad HSV gB
14.0%
0.0%

Figure 4. HSVΔgB and HSVΔgH entry *in trans*. (A) Expression of HCMV and HSV gB and gH by immunofluorescence. Permeabilized and non-permeabilized cells were stained to demonstrate the relative level of cell surface expression compared to total expression. NHDF were infected at a MOI of 100 PFU/cell with Ad HCMV gB, Ad HCMV gH and gL, Ad HSV gB, or Ad HSV gH and gL. 48 h post-infection, cells were assayed for glycoprotein expression by immunofluorescence. **(B)** NHDF were infected with adenovirus expressing HSV gB at a MOI of 100 PFU/cell. 24 h later, cells were inoculated with HSVΔgB (such that all cells were infected in the presence of PEG), and 2 h post-infection one well was treated with 44% PEG to enhance entry. 8 h after HSV infection, cells were fixed and stained for HSV immediate early protein ICP4. **(C)** Cells were treated similar to (B), except cells were infected with Ad HSV gH and Ad HSV gL at a MOI of 100 PFU/cell and subsequently infected with HSVΔgH.

Figure 3-4

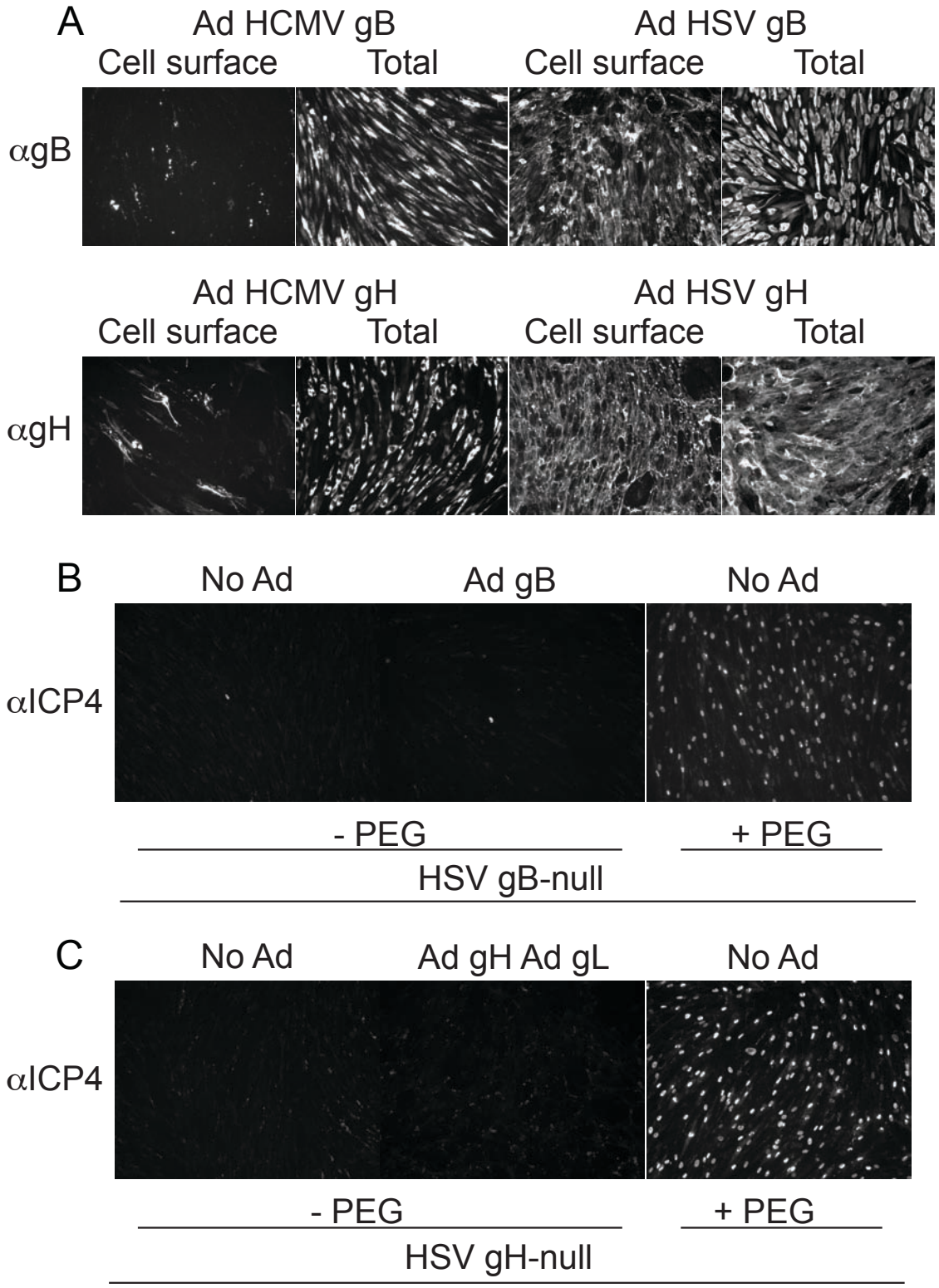

A
Ad HCMV gB
Ad HSV gB
Cell surface
Total
Cell surface
Total
αgB
Ad HCMV gH
Ad HSV gH
Cell surface
Total
Cell surface
Total
αgH
B
No Ad
Ad gB
No Ad
αICP4
- PEG
+ PEG
HSV gB-null
C
No Ad
Ad gH Ad gL
No Ad
αICP4
- PEG
+ PEG
HSV gH-null

Figure 5. Adsorption of HCMVΔgB-gB and HCMVΔgH-gH onto fibroblasts.

To test whether the absence of gB or gH reduced the ability of HCMV to adsorb onto fibroblasts, HCMVΔgB+gB or -gB and HCMVΔgH+gH or -gH supernatant virus particles that replicated in the presence of ^{3}H-thymidine were collected from NHDF-gB, NHDF-gH or NHDF. 3,000, 10,000, and 30,000 cpm/well of each labeled virus were then incubated with untransduced NHDF. The cells and viruses were centrifuged at 800 × g for 30 min at 10˚C. Supernatants and washes were collected as the unbound samples, lysed cells and subsequent washes were collected as the bound samples, and data are expressed as the ratio of the total bound versus unbound counts.

Figure 3-5

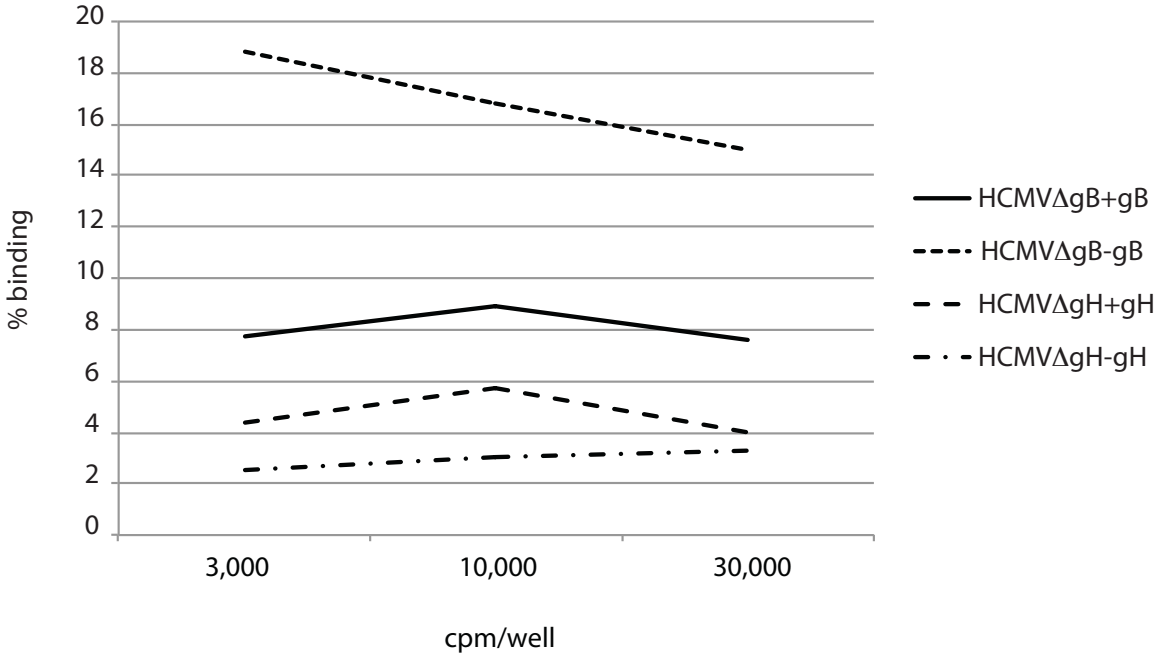
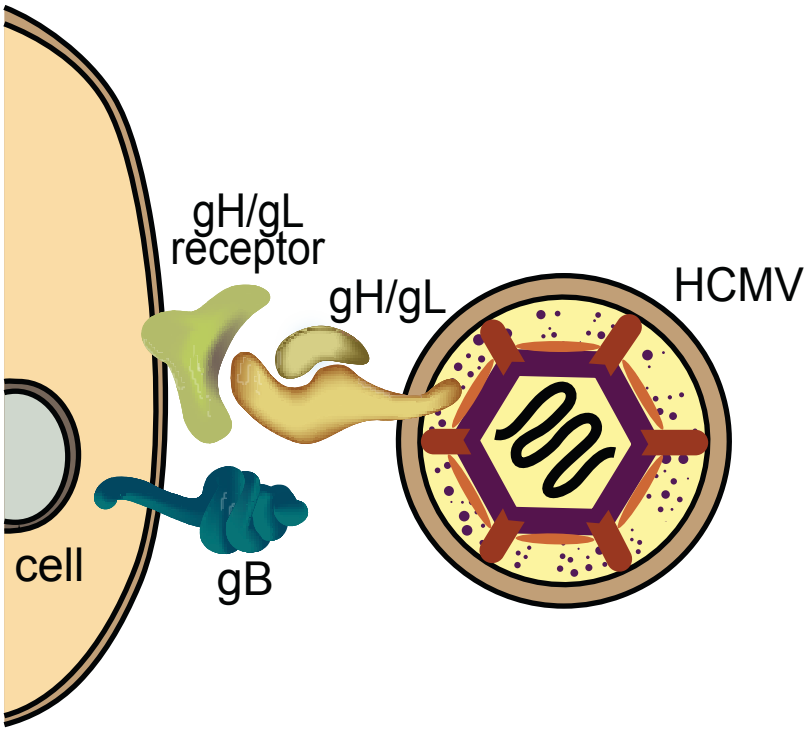

20
18
16
14
12
10
8
6
4
2
0
% binding
3,000
10,000
30,000
cpm/well
HCMVΔgB+gB
HCMVΔgB-gB
HCMVΔgH+gH
HCMVΔgH-gH

Figure 6. Model of HCMV entry *in trans*. HCMV lacking gB in the virion envelope is able to enter fibroblasts expressing gB on the cell surface. The model suggests that this is triggered by gH/gL engagement of cellular receptors. Entry *in trans* appears to be specifically mediated by gB, as gH/gL complexes expressed in fibroblasts do not mediate entry of HCMV gH-null particles. Illustration by Tiffani Howard.

Figure 3-6



CHAPTER 4: DISCUSSION

The studies herein have focused on the role of HCMV envelope glycoproteins in entry. Using a genetic and biochemical approach, I advanced our understanding of the role of gO as a member of a gH/gL complex and the function of gB as the HCMV fusion protein. Here, I will interpret my studies in the context of previous work on HSV, EBV, and HCMV, and discuss how further studies might advance our understanding of gO and gB.

A. General summary of herpesvirus envelope glycoprotein function during entry

The main function of herpesvirus envelope glycoproteins is to mediate fusion between the virion envelope and cellular membranes so viruses can enter cells. Entry can be broken down into three steps: i) attachment, ii) receptor binding, and iii) membrane fusion. Detailed understanding of each of these events differs between herpesviruses.

A1. Attachment. For α- and β-herpesviruses, attachment is a relatively non-specific event involving cell surface heparan sulfate glycosaminoglycans (GAGs) (41, 191, 241, 246). Attachment onto these GAGs does not lead to entry, as GAG binding does not lead to the conformational changes in viral fusion proteins needed to mediate membrane fusion. Instead, GAG binding likely increases the local concentration of virus particles on cell surfaces, promoting downstream interactions between receptors and receptor binding proteins.

A2. Receptor binding. These downstream interactions involve viral receptor binding envelope glycoproteins interacting with cellular receptors. The best described herpesvirus receptor binding proteins are HSV gD and EBV gH/gL and gH/gL/gp42 (37, 103, 125, 146). These interactions are different from attachment to GAGs because they

cause conformational changes in the receptor binding protein that trigger the fusion protein.

A3. Membrane fusion. Conformational changes triggered by receptor interactions lead the fusion protein to penetrate hydrophobic domains into the cell membrane, causing lipid mixing (i.e., fusion) between the virion envelope and cell membrane. Once the membranes have fully fused, content mixing occurs and the capsid is released into the cytosol. There is strong evidence that the herpesvirus protein responsible for fusion is gB, based on structural data from EBV and HSV gB (8, 75) and *in vitro* penetration of HSV gB into lipid vesicles (72).

B. HSV and EBV entry

In order to better understand how HCMV entry might occur, it is instructive to understand the similarities and differences between the role of HSV, EBV, and HCMV envelope glycoproteins in entry.

B1. HSV. In terms of comparing HSV to other herpesviruses, the most important information we have learned about HSV entry is the cascade of events leading to fusion: gD binds a receptor, this triggers an interaction between gH/gL and gB, and gB apparently promotes fusion between the virus and cell membranes.

gD is clearly the HSV receptor binding protein. In virus particles lacking gD, entry does not progress past attachment (127). gD interacts with nectin-1 or HVEM, and these interactions initiate fusion (117, 146). gD does not directly signal gB for fusion, however. Instead, gH/gL is also required for entry (61). gD appears to trigger an interaction between gH/gL and gB, as shown by bimolecular complementation studies that determined that the gH/gL and gB cytoplasmic domains interacted only after gD receptor engagement (6). While gH/gL has not been formally ruled out as a fusion protein, three

sets of data suggest that this is not the case and that gB is the fusion protein: (i) the structure of gB is similar to other class III fusion proteins such as the vesicular stomatitis virus G protein (75, 107, 181), (ii) gB penetrates lipid vesicles in the absence of other envelope glycoproteins (72), but (iii) gH/gL does not directly associate with lipid vesicles unless gB is also present, where it is believed that a complex of gB and gH/gL is formed, but only gB enters the lipid bilayer (31). These data suggest that gH/gL does not directly participate in lipid mixing, but instead conveys the receptor binding signal from gD to gB, which then inserts hydrophobic fusion loops into the cell membrane to draw the cell and virion membranes together, causing fusion (reviewed in (74)).

B2. EBV. For purposes of comparison to HCMV, three important conclusions have been drawn from studies of EBV entry: (i) gH/gL complexes are receptor binding proteins, (ii) separate gH/gL complexes bind unique receptors to mediate entry into different cell types, and (iii) virus entry can occur at the plasma membrane or by endocytosis (reviewed in (91).

EBV forms two separate gH/gL complexes, gH/gL and gH/gL/gp42. gH/gL mediates entry into epithelial cells via interactions with integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ (37) and does not require endocytosis (140). In contrast, entry into B cells involves EBV gp350 binding the CR2 complement receptor and gH/gL/gp42 subsequently binding to HLA class II molecules (125, 126), which triggers endocytosis and fusion with endosomes (140). At least for entry into epithelial cells, gH/gL appears to directly convey the signal produced by integrin binding to gB, triggering the conformational changes in gB that cause fusion. As with HSV, the structure for EBV gB has been solved (8), and clearly demonstrates that gB is a class III fusion protein.

C. Comparing HCMV entry to HSV and EBV

HCMV entry has similarities to both HSV and EBV. Like HSV, HCMV adsorbs onto heparan sulfate GAGs, involving gB and gM/gN (41, 108). Like EBV, two different HCMV gH/gL complexes appear to be the receptor binding proteins ((183), A. Vanarsdall, M. Chase, and D. Johnson, in press), and mediate entry via two different pathways: at the plasma membrane and following endocytosis (40, 185). It appears from my studies that, like HSV and EBV, HCMV gB is the fusion protein (Chapter 3).

D. Two different gH/gL protein complexes and two different entry pathways

HCMV entry into different cell types occurs by at least two different pathways, with different gH/gL complexes involved in each pathway. Entry into fibroblasts occurs by fusion of the virion envelope with the plasma membrane at neutral pH (40). Entry into epithelial and endothelial cells involves endocytosis followed by fusion with endosomes, and is inhibited when endosomal pH is increased (185). This endosomal pathway is correlated with the requirement for the HCMV five protein complex gH/gL/UL128-131. This complex, however, is not required for entry into fibroblasts. Another complex, involving gH/gL/gO, is also formed in infected cells. My studies described in Chapter 2 and those of Brent Ryckman (184) showed that gO is not present in the envelope of HCMV clinical strain TR, and thus, it appears that gH/gL is the complex required for entry into fibroblasts. However, as shown in Chapter 2, gO is required in order for gH/gL to be exported from the ER, reach the TGN, and incorporate into the virion envelope. Models that gH/gL/UL128-131 and gH/gL interact with receptors are based on using these complexes to interfere with entry into various cells types. Our laboratory demonstrated that gH/gL/UL128-131 expression in epithelial cells *in trans* is sufficient to block entry into these cells (185), but that gH/gL, gH/gL/gO, and gB cannot interfere with

epithelial cell entry (183). When gH/gL/gO is expressed in human fibroblasts there is interference with HCMV entry, while gH/gL/UL128-131 and gB have no effect (A. Vanarsdall, M. Chase, D. Johnson, in press). These studies indicated that different HCMV gH/gL complexes bind unique, saturable receptors in fibroblasts and epithelial cells, and that gB does not bind a saturable receptor on either cell type. While this does not exclude the possibility that gB binds a fusion receptor, instead, it is more likely that gH/gL complexes interact with saturable receptors and then trigger gB to promote membrane fusion, similar to HSV and EBV.

E. Previously described HCMV receptors

Several cellular proteins have been proposed to bind HCMV gH/gL or gB, including the epidermal growth factor receptor (EGFR), certain integrins, and the platelet derived growth factor receptor PDGFRα, but it is still unclear whether these proteins act as fusion receptors similar to: (i) nectin-1 and HVEM for HSV and (ii) HLA class II and integrins for EBV. It was reported that gB binds to EGFR and acts as an HCMV entry mediator (234). As well, it was proposed that EGFR signaling was necessary for either entry or to prepare cells for HCMV replication. However, this was challenged by other studies showing that HCMV can enter EGFR-null fibroblasts and cells treated with EGFR-neutralizing antibodies, and that HCMV binding did not activate signals downstream of EGFR (38, 92). Integrins have also been implicated in post-attachment steps of HCMV entry, and gB contains an integrin binding motif called a disintegrin-like domain (DLD). Both peptides derived from this domain and β_1-integrin antibodies partially block HCMV entry at a step between attachment and delivery of the tegument protein pp65 into the cytosol (57, 58), but it is unclear whether the relatively high concentrations of these peptides and antibodies selectively block entry or some other

stage of HCMV replication. A second report suggested that gH/gL interacts with integrins (233), but these interactions were analyzed by co-precipitation of integrins with gH/gL and by changes in signaling, rather than directly assessing virus entry. Another cell surface signaling molecule, platelet-derived growth factor-α receptor (PDGFRα) was demonstrated to bind HCMV gB and be activated by either HCMV infection or soluble gB (201). In this case, however, blocking signaling events downstream from PDGFRα only delayed gene expression and did not prevent entry. It is still possible, though, that PDGFRα acts as an entry mediator in a manner distinct from signal transduction. Recent studies by Adam Vanarsdall have demonstrated that PDGFRα can mediate HCMV entry into a variety of cell types, but there is good evidence that PDGFRα does not explain how HCMV enters epithelial and endothelial cells.

PDGFRα, EGFR, and integrins do not fit the criteria for HCMV entry receptors for a number of biologically relevant cell types. These molecules were all defined using the HCMV laboratory strain AD169. While AD169 can infect fibroblasts, this virus cannot infect a list of relevant cell types including epithelial and endothelial cells and monocyte-macrophages. Moreover, the suggestion that gB is the receptor binding protein does not fit with observations that AD169 and TR gB sequences are very nearly identical. It is unlikely that gB would be the receptor binding protein for entry into fibroblasts, but not epithelial or endothelial cells. As well, EGFR and integrins are present in all of these cells. What is more likely, based on our interference studies, is that HCMV entry into epithelial and endothelial cells and monocyte-macrophages involves proteins bound by gH/gL/UL128-131, whereas other proteins are used to enter fibroblasts, likely involving the binding of gH/gL. This is a better model of entry in part because AD169 enters epithelial and endothelial cells when UL131 expression, and thus gH/gL/UL128-131

expression, is restored (231). Thus, HCMV entry into these cell types could not have relied on a missing gB receptor.

It is clear that HCMV glycoproteins—gH/gL, gB and perhaps others—can potentially interact with many cellular proteins to promote virus entry. However, at present, we know little about which cellular receptors trigger gH/gL and gB for entry fusion. Adam Vanarsdall in our laboratory is attempting to use a cDNA library from epithelial cells to find a receptor that binds to gH/gL/UL128-131 and specifically triggers fusion.

F. HCMV gO and entry

F1. Previous studies of gO. Prior to my studies of HCMV gO, our knowledge of how gO functions relied on the work of three laboratories, those of Teresa Compton, Ulrich Koszinowski, and Christian Sinzger. Huber and Compton initially described gO as the third member of the gH/gL complex from HCMV strain AD169 (87). Hobom et al. subsequently discovered that loss of AD169 gO led to decreased plaque size and single-step growth curve kinetics on fibroblasts (84). More recently, a gO-null mutant in the endotheliotropic HCMV strain TB40/E was demonstrated to have a similar defect in replicating in, and spreading between, fibroblasts, but also spread normally between endothelial cells (101). Defects in the TB40/E gO-null mutant in fibroblasts were attributed to a reduction in virus assembly, and electron microscopy revealed high levels of cytosolic unenveloped capsids. Aside from the reduction in plaque size and titer, no assays performed with either AD169 or the two mutants demonstrated a direct role for gO in entry. There are several limitations to these sets of studies. First, the Compton and Koszinowski laboratories relied on strain AD169. It is clear that extensive passage of HCMV in fibroblasts leads to modifications of gH/gL complexes, e.g. UL128-UL131

expression is lost. It may also be that AD169 gO function has changed during long-term AD169 propagation in fibroblasts. These changes could potentially have made AD169 gO function differently from wild-type gO. Thus, the AD169 gO cannot be used to understand how wild-type gO functions during entry into both fibroblasts and other clinically relevant cell types, especially cells that AD169 does not enter. Studies from the Sinzger laboratory were limited by their approach. Loss of infectivity in their gO-null mutant was attributed to loss of particle production into the supernatant, but they did not directly address whether uninfectious virus particles were released from cells. Related to this, they did not address whether released particles might be diminished in their capacity to bind to or enter cells. For these reasons, I characterized HCMV TR gO and directly determined whether gO is necessary for HCMV entry into a variety of cell types.

F2. Two approaches to studying the function of TR gO. Based on the interference studies performed by Brent Ryckman, our model suggested that entry into epithelial and endothelial cells is mediated by gH/gL/UL128-131 and involves binding to "gH/gL/UL128-131 receptors" (183). Entry into fibroblasts appeared to be mediated by the other gH/gL complex, gH/gL/gO. The first half of this model is now well supported by studies from our laboratory and other laboratories (4, 62, 70, 183, 185, 186, 231, 232). We took two different approaches toward testing the second half of this model, i.e., determining the function of gO. Brent Ryckman studied the assembly and intracellular transport of the gH/gL/gO complex in the context of viral infection and by expressing individual proteins or complexes of proteins by using non-replicating adenovirus (Ad) vectors. I took a genetic approach, producing and characterizing an HCMV TR gO-null mutant. This mutant, TRΔgO, was derived from a bacterial artificial chromosome (BAC)

containing the TR genome, and was constructed by Amber Knoche and Michael Jarvis to contain a mutation deleting the gO gene.

F3. gO is a chaperone that transiently associates with gH/gL, mediating its incorporation into the HCMV virion envelope. Brent Ryckman's approach to studying gO function immediately altered our model of the role of gH/gL complexes in HCMV entry. First, gO was discovered to be absent from the envelope of extracellular HCMV TR particles, clearly indicating that gO could not have a direct role in entry. Thus, a gH/gL complex other than gH/gL/gO and gH/gL/UL128-131 must be responsible for TR entry into fibroblasts. Second, gH/gL relies on either UL128-131 or gO for ER export. But, unlike the gH/gL/UL128-131 complex that is stably incorporated into the virion envelope, gH/gL/gO is a transient complex. When gH, gL, and gO were expressed by using Ad vectors, gH/gL became endoglycosidase H resistant (indicating maturation in the Golgi apparatus), but gO did not. This means that gO dissociates from gH/gL during intracellular transport from the ER to the Golgi apparatus. These studies also showed that gO is a chaperone, because gH/gL expressed without gO is not exported from the ER and does not acquire endo H resistance. Thus, this work demonstrated that gO plays a critical role in the intracellular transport of gH/gL, but that gO does not remain bound to gH/gL when gH/gL is incorporated into the virion envelope.

F4. gO is necessary for assembly of HCMV particles capable of entering all cell types. In my studies of gO, I characterized the TRΔgO mutant for defects in assembly and entry. TRΔgO was successfully rescued as an infectious virus using fibroblasts, although this mutant was very difficult to rescue and grew very poorly on fibroblasts. As with the AD169 and TB40/E gO-null mutants, TRΔgO formed very small plaques compared to wild-type and produced very little infectious virus associated with

cells and in cell culture supernatants (Chapter 2). However, I concentrated supernatant-derived TRΔgO and examined whether there were particles that could be forced to enter fibroblasts in the presence of polyethylene glycol (PEG). PEG acts to chemically fuse virus particles into cells, overcoming defects in virus entry. PEG treatment dramatically enhanced the entry of TRΔgO derived from cell culture supernatants, indicating that the loss of gO did not prevent the accumulation of virus particles in extracellular supernatant, but rather made them incapable of entry. This explained the results of Jiang, et al. (101), who failed to find infectious virus particles in cell culture supernatants, but did not look for non-infectious virus. Our model of entry suggested that the absence of gO would affect entry into fibroblasts but not other cell types, so I tested whether TRΔgO would enter epithelial and endothelial cells. To our surprise, TRΔgO was also largely incapable of infecting these cells. Again, these defects could be overcome by PEG treatment indicating that the gO-null mutant was blocked in virus entry. I also determined that defects in entry were not due to gross alterations in virus assembly or release. Similar numbers of wild type and gO-null HCMV particles were released, as measured by quantifying virus genomes. Moreover, electron microscopy demonstrated that TRΔgO particles were found on cell surfaces and that there were no obvious defects in assembly of these particles. Instead, an examination of the protein content of supernatant-derived gO-null particles revealed that there was a substantial decrease (>90%) in gH/gL incorporated into the virion envelope. I also demonstrated that TRΔgO was capable of spreading more quickly between epithelial cells. We believe that this enhanced spread in epithelial monolayers is due to the increased amount of gH/gL/UL128-131 in the virion envelope. Therefore, spread between these cells relies largely or entirely on

gH/gL/UL128-131 and not gH/gL in the virion envelope, yet entry into epithelial cells absolutely requires gH/gL as well as gH/gL/UL128-131.

My studies extended conclusions about how HCMV gO functions, showing for the first time that gO is necessary for entry into all cells tested. Rather than acting directly during entry as part of a gH/gL/gO complex in the virion, TR gO chaperones gH/gL out of the ER to the Golgi apparatus and presumably to sites of assembly in the TGN ((184), Chapter 2). This is dramatically different from AD169 that forms a complex with gH/gL and remains part of that complex as gH/gL/gO is incorporated into extracellular virions. This may represent changes in gO or gH/gL that relate to the loss of gH/gL/UL128-131 assembly. The gH/gL complexes chaperoned by TR gO apparently do not contain UL128-131 based on the studies of Wang and Shenk (232) and my observations that gH/gL/UL128-131 is increased in gO-null virus particles. Thus, we believe that these data suggest a new model in which gO is a binding partner of gH/gL, chaperones gH/gL from the ER, and dissociates from gH/gL in the endoplasmic reticulum-Golgi-intermediate compartment (ERGIC) or cis-Golgi apparatus, prior to envelopment. This chaperone activity appears to be necessary for HCMV to incorporate gH/gL, as well as gH/gL/UL128-131 into the envelope of mature HCMV virions.

G. Future exploration of gO functions

Several questions remain about how HCMV gO functions. First among these questions involves mechanisms of how gO assembles with gH/gL and later dissociates from gH/gL. Second, is the question of why AD169 particles contain gO, while TR particles do not contain gO. Third, it is not clear whether AD169 gO contributes to the entry of AD169 into human fibroblasts, given that the gH/gL/gO complex is present in

AD169 virions. Fourth, it is not known whether other low-passage, clinical HCMV strains also behave like TR and fail to incorporate gO into the virus particle.

G1. Assembly and stability of different gH/gL complexes. Further studies of the role of gO in the assembly and transport of gH/gL are important because: (i) we do not understand whether gO competes with UL128-131 for binding to gH/gL or (ii) where gO dissociates from gH/gL and how this promotes ER export of gH/gL but not gO. Our current model of gH/gL assembly suggests that gH and gL assemble in the ER quickly and then bind either gO or UL128-131. We believe this is the case because: (i) Wang and Shenk determined that gO is not detected in gH/gL complexes containing UL128-131, and UL128-131 is not found in gH/gL complexes containing gO (232), and (ii) I demonstrated that TR gH/gL/UL128-131 complexes are increased in the virion envelope in the absence of gO (Chapter 2). The Wang and Shenk study is the most direct demonstration of separate complexes; however, this study is limited by the fact that it involved HCMV strain AD169 that was repaired so that gH/gL/UL128-UL131 was expressed. My experiments showed that TR gO-null mutant virus collected from cell culture supernatants had increased levels of gH/gL/UL128-131, but this reflected released virus particles and not events in the ER. Thus, it is possible that TR and other low-passage strains create gH/gL complexes containing all six proteins during assembly. Arguing against this, studies by Brent Ryckman involving characterization of different immunoprecipitated gH/gL complexes have not shown any evidence for the presence of gO in gH/gL/UL128-131 complexes.

Our current knowledge of TR gH/gL complex formation is limited to results from two experimental conditions: (i) expression of proteins that allow gH/gL complexes to exit the ER and become endo H resistant and (ii) expression of gH/gL complexes using

Ad vectors and measuring function in interference assays. Either gO or UL128-131 allow gH/gL to exit the ER. It appears that gH/gL (without gO) interferes with HCMV entry into fibroblasts while gH/gL/UL128-131 interferes with HCMV entry into epithelial cells. These experiments involve downstream readouts of function and, thus, do not directly address the assembly process itself.

Two important questions remain. First is whether gO competes with UL128-131 in binding to gH/gL in the ER. Second is whether gH/gL is incorporated into the virion envelope or whether gH/gL/gO is incorporated, followed by loss of gO as the virus particles are secreted from cells. Loss of gO following assembly is still formally possible because Brent Ryckman only characterized mature, extracellular TR particles that lacked gO. One reasonable approach to addressing competition and the specific forms of gH/gL present in cells and virion envelopes is the development of monoclonal antibodies that recognize unique epitopes. For example, we need antibodies to gH that only recognize gH when it is part of the gH/gL/UL128-131 complex, as well as antibodies that recognize gH/gL but only when UL128-131 or gO are absent. These antibodies would be made by injecting purified protein complexes of gH/gL or gH/gL/UL128-131 into mice, harvesting B cell clones, making hybridomas, and screening the antibodies from these hybridomas for their ability to recognize the different proteins and complexes. In order to assess which complexes are formed during infection, these antibodies would be used for immunoprecipitations of metabolically labeled, infected cell lysates, and we could quantify by pulse-chase analysis the kinetics of formation of specific complexes. For example, in a short chase we might observe gH/gL alone, such that antibodies that only recognize gH/gL/UL128-131 would not recognize the gH/gL complex present in these samples. In later chase samples, we might discern gH/gL/gO that subsequently

dissociates to form gH/gL. In order to determine which complexes are present in the virion envelope, we would simply need to immunoprecipitate virion lysates with these conformational antibodies and then perform immunoblot analysis to look for which other proteins are present. For example, an antibody that recognizes gH/gL alone (i.e., without UL128-131 or gO bound) could be used to prove for the first time that gH/gL lacking gO and UL128-131 is present in the virion envelope. At present this is just a hypothesis and not proven. Thus, we believe the addition of these antibodies would provide us with the means to better assess gH/gL complex formation and incorporation into the virus particle. Moreover, a panel of conformation-dependent antibodies would be a very powerful tool towards understanding function. It is believed that gH/gL receptor binding alters gH/gL conformation and then triggers gB, but this cannot be understood without antibodies as molecular probes.

G2. Incorporation of gO into AD169 but not TR. It is currently unclear why AD169 gO is incorporated into extracellular virus particles but this is not the case with HCMV clinical strain TR. It is important to understand why these differences arose because we do not know at this point whether there is a significant functional difference between AD169 gO and TR gO or if this represents a difference between all clinical strains and laboratory strains. If indeed AD169 is aberrant in this respect, this could have resulted from mutations in AD169 gO or mutations in gH/gL.

Two types of research are needed to address these questions. First, it is important to address whether other clinical strains of HCMV possess gO in the virion envelope. Brent Ryckman attempted to address this question using two rabbit polyclonal antibodies generated with peptides derived from TR gO; however, he found that the very large amino acid sequence variability of gO between strains (up to 25% of the residues)

caused TR gO antibodies to not recognize gO produced by other strains (184). Antibodies specific for gO from these other HCMV strains could be developed in a similar manner to those raised against TR gO: small peptides derived from each gO would be coupled to Keyhole Limpet Hemocyanin (KLH, a high molecular weight protein and potent immunogen) and injected into rabbits to produce serum able to recognize other gO molecules. Alternatively, each gO molecule from several different low passage strains could be epitope-tagged at their amino- or carboxy-termini. These constructs could then be cloned into the BACs containing their respective strain's genomes and used to produce HCMV with epitope-tagged gO molecules.

The second related question asks whether AD169 gO is mutant compared with TR gO and whether this accounts for incorporation of AD169 gO into the virion. This question can be addressed by swapping gO between strains, i.e., replace AD169 gO with TR gO and vice versa. Replacing the gO coding sequence in AD169 BAC and TR BAC using standard recombineering methods would generate these viruses. Initial characterization of virus particles released into the supernatant by immunoblot would determine whether gO was present. Based on a simple model of gO incorporation, two results are possible: incorporation of gO is intrinsic to gO; i.e., differences in incorporation are due entirely to differences in the gO polypeptide sequences. If this is the case, TR gO would not incorporate into AD169 virions, but AD169 gO would incorporate into TR. However, gO incorporation might depend upon mutations in AD169 gH/gL. In that case, TR gO might be incorporated into AD169 particles and AD169 gO would not be incorporated into TR particles.

G3. Participation of AD169 gO in entry. Previous studies with wild type and gO-null mutant AD169 have not addressed whether the AD169 gO protein that is

incorporated in virions is directly involved in entry. Again, this is an important question because it will reveal whether the stable association of AD169 gH/gL and gO represents an innocuous difference between strains or whether AD169 gO plays a different role in entry compared with TR gO. A similar strategy to that used to characterize TRΔgO could be employed to help answer this question. As part of the efforts to swap gO between AD169 and TR and vice versa, we would construct an AD169 gO-null mutant. Like TRΔgO, this AD169ΔgO virus would be characterized for: (i) its ability to assemble and release particles, (ii) its ability to enter fibroblasts, (iii) plaque formation on fibroblasts, and, (iv) protein content of released virus. One possibility would be that noninfectious AD169ΔgO particles would be released into cell culture supernatants and that these particles would be incapable of entering fibroblasts. Coupled with the study of gH/gL transport in cells infected with AD169ΔgO, we might conclude that the loss of AD169 gO prevents gH/gL ER export and, thus, prevents incorporation of gO into the virion envelope. If this is the case, then AD169 relies on gO for incorporation of gH/gL into the virion envelope as with HCMV strain TR. These experiments with AD169ΔgO, then, will not have determined if AD169 gO has a direct role in entry. However, it is possible that AD169 gH/gL would be incorporated into the virion envelope in the absence of gO, and that the loss of gO directly prevents gH/gL function during entry. This result would indicate that AD169 gO has been modified to play a different role than TR gO, and thus would likely not be representative of wild-type gO.

Another tool at our disposal for addressing whether AD169 gO participates in entry is the swaps. Swapping TR gO in place of AD169 gO gives us a genetic means to separate the chaperone function of AD169 gO from its role in entry. There are four scenarios possible in this experiment: TR gO is incorporated into AD169 virus particles,

and entry is either restored or not, or gO from TR is not incorporated and entry is either restored or not. (1) If TR gO is incorporated into AD169 virions and entry does occur, then none of the differences between AD169 gO and TR gO has made AD169 gO uniquely necessary for entry. (2) If entry does not occur when TR gO has been incorporated this would likely indicate that mutations in AD169 gO have a direct role in entry and this function cannot be substituted for by TR gO. (3) If TR gO is not incorporated into AD169 virions and gH/gL is present at wild-type levels in the virion, entry into cells would indicate that AD169 gO is coincidentally incorporated into the particle while facilitating incorporation of gH/gL, and thus does not have a direct role in entry. (4) If TR gO is not incorporated and gH/gL is incorporated into virions, but entry does not occur, this would demonstrate AD169 gO has a specific role in entry beyond incorporation of gH/gL for strain AD169.

In summary, there are several differences in the behavior of AD169 and TR gO and understanding these differences is crucial to determining whether our conclusions about the function of TR gO represents wild-type gO. We believe that there are several experiments that would help answer this question and that building the reagents for these experiments is important and reasonable based on our previous experience working with HCMV.

H. The functions of HCMV gB

For reasons discussed above, it is relatively clear that HSV gB is the viral protein that mediates entry fusion. It would seem likely that HCMV gB is also the fusion protein, and that gB is triggered by gH/gL complexes that bind cellular receptors. Supporting the notion that HCMV gB is the fusion protein and does not bind saturable receptors, our interference studies showed that gB expression in cells does not block virus entry.

H1. Cell-cell fusion assays. Besides the interference data indicating that gH/gL complexes bind receptors, there is cell-cell fusion data as described by Adam Vanarsdall that indicate that HCMV gB and gH/gL are sufficient for cell-cell fusion. Cell-cell fusion assays have been a critical tool for defining the constituents of each herpesvirus' fusion machinery (69, 165, 225). Early studies of cell-cell fusion by Teresa Compton's laboratory concluded that gH/gL was sufficient for cell-cell fusion (111). However, these studies were compromised by the use of CHO cells, which have a substantial background of syncytia regardless of HCMV protein expression, and by relatively low levels of HCMV protein expression from retrovirus constructs. Moreover, only a small percentage of cells formed syncytia. Adam Vanarsdall in our laboratory used Ad vectors that produce higher levels of TR gB, gH, and gL and expressed these proteins in the epithelial cell line ARPE-19, demonstrating high levels of cell-cell fusion that only occurred when both gB and gH/gL were expressed (227). Thus, the minimal fusion machinery of HCMV is gB and gH/gL. Remarkably, though, gB and gH/gL did not need to be expressed in the same cell in order for cell-cell fusion to occur. In a phenomenon we denoted cell-cell fusion *in trans*, cells expressing gB can fuse with cells expressing gH/gL. This was the first report of cell-cell fusion of this type. Later, similar results were reported for HSV (7). We concluded from these results that gB and gH/gL can interact across the space between apposing membranes to mediate fusion. However, these data did not demonstrate conclusively which HCMV glycoprotein, gB or gH/gL, was producing the membrane fusion.

H2. Entry in trans. Despite the utility of cell-cell fusion assays, they are limited by the possibility that cell-to-cell fusion is governed by different parameters (e.g., kinetics and mechanism) compared with the fusion that occurs between the virion envelope and

cellular membranes when HCMV enters cells. For example, HCMV entry into ARPE-19 cells occurs by fusion with endosomal membranes and requires gH/gL/UL128-131 and gB (183, 185, 186, 227). By contrast, ARPE-19 cell-cell fusion is much slower than entry fusion, requires only gH/gL and gB, and occurs at neutral pH involving the plasma membrane (227). Thus, when our laboratory observed the unique phenomenon of cell-cell fusion *in trans*, we were very interested in testing whether this could occur in the context of virus infection. This is a phenomenon known as entry *in trans*, in which gB or gH/gL is expressed in cells and the cells are subsequently incubated with HCMV mutants lacking gB or gH/gL. In this case, gB and gH/gL must interact across the space between the virion envelope and the cell membrane expressing gB or gH/gL. The availability of an HCMV mutant unable to express gB, HCMVΔgB, constructed by Isaacson and Compton (94) allowed me to test whether gB could mediate entry *in trans.* HCMVΔgB was derived from AD169 using a BAC and propagated in fibroblasts transduced with gB. I also constructed a TR BAC in which gH was deleted and used fibroblasts transduced with a gH-expressing retrovirus (from Teresa Compton) to propagate HCMVΔgH.

I initially characterized both mutant viruses for their ability to be released from fibroblasts expressing either gB or gH (NHDF-gB or NHDF-gH). The complemented virus particles were denoted: HCMVΔgB+gB and HCMVΔgH+gH. There were only slight decreases in the quantities of the complemented virus particles released into cell culture supernatants for both HCMVΔgB+gB and HCMVΔgH+gH, compared to wild type viruses. I could also concentrate extracellular particles. Characterization of these extracellular virus particles showed that both mutants were significantly different compared with wild type AD169 or TR. This was apparently related to incomplete complementation, i.e.,

there were reduced levels of gB or gH expressed by the retroviruses compared to the levels present during wild-type infection. Unlike what was previously reported by Isaacson, et al. (94), HCMVΔgB+gB virus particles also appeared to contain significantly less gH compared with their parent AD169. By contrast, HCMVΔgH+gH were complemented better and contained wild-type levels of gB. It is unclear at this point as to why the loss of gB prevents gH/gL incorporation into AD169 virus particles.

The complemented virus particles HCMVΔgB+gB and HCMVΔgH+gH were used to produce virus particles lacking gB (HCMVΔgB-gB) or gH (HCMVΔgH-gH). These particles were quantified by using quantitative real-time PCR (qPCR) to detect virus genomes. Neither of these viruses were capable of infecting any cell type tested. In order to determine whether this block was at the stage of entry (as had been reported for the gB-null (94)), I used PEG enhancement. If the particles were able to attach to cells but defective for fusion, PEG would overcome this defect, allowing the gB and gH mutant viruses to enter cells. By concentrating virus and using PEG enhancement, I could produce stocks of HCMVΔgB-gB and HCMVΔgH-gH that infected approximately 50% of fibroblasts. HCMVΔgB-gB and HCMVΔgH-gH were tested for whether they could enter fibroblasts or ARPE-19 epithelial cells expressing either gB or gH/gL. In this case, the fibroblasts or epithelial cells were transduced with Ad vectors to express gB or gH/gL *in trans*. Remarkably, virus particles lacking gB (HCMVΔgB-gB) could enter fibroblasts and epithelial cells expressing gB. As many as 14% of fibroblasts in these dishes became IE+ when incubated with HCMVΔgB-gB, while up to 3% of epithelial cells became IE+. There was also some low level entry of HCMVΔgH-gH into cells expressing gB. There is no background entry by either null mutant, i.e., not a single cell in the entire

monolayer became HCMV IE+, when cells were not transduced with gB. Moreover, expression of HCMV gH/gL did not facilitate entry of HCMVΔgH-gH or HCMVΔgB-gB. As well, transduction of fibroblasts with HSV gB did not facilitate the entry of HCMV gB-null. Entry *in trans* did not work, in my hands, when several types of cells were transduced with HSV gB and then infected with HSV particles lacking gB. Although not all cells were infected when HCMVΔgB-gB was induced to enter cells expressing gB, the result was still quite amazing. No one has described an example in which a viral entry mediator can be expressed in cells and promote entry of a virus.

H3. Future experiments involving gB entry in trans. To extend these results, I am planning to try to increase the entry *in trans* by HCMVΔgB-gB mediated by gB expressing cells. Note that 5-14% of the gB-expressing cells were infected by HCMVΔgB-gB but there were many other HCMVΔgB-gB particles that could be caused to enter cells by PEG treatment. One reason may be that HCMV gB reaches cell surfaces, but is rapidly internalized by endocytosis. In fact, the vast majority of gB appears to be present in cytoplasmic membranes, the Golgi apparatus, trans-Golgi network and endosomes (43). This internalization is mediated largely by the cytoplasmic domain of HCMV gB that contains several obvious endocytosis motifs (43, 170). Thus, I have constructed a number of gB mutant molecules that delete or mutate portions of the gB cytoplasmic domain. In preliminary experiments, an HCMV mutant truncated at residue 882, denoted gB 882 stop, which removes the C-terminal 25 residues, appears to be present on the cell surface to a greater extent than wild-type gB and mediates cell-cell fusion at lower expression levels than wild-type gB. Experiments to determine whether gB 882 stop increases entry *in trans* are currently underway.

In order to further test our hypothesis that HCMV gB functions directly in the fusion process during HCMVΔgB-gB entry *in trans*, I have constructed a second panel of mutant gB molecules. These were based on the structure of HSV gB which contains internal bipartite "fusion loops" composed of both hydrophobic and hydrophilic residues (74). Mutation of these fusion loops blocks cell-cell fusion (72). These gB fusion loops are thought to interact with host membranes during fusion, i.e., dive into the membranes to promote lipid mixing (72). Based on sequence alignments between HSV and HCMV gB, Martin Muggeridge helped us identify two similar fusion loops in HCMV gB. My aim was to mutate these fusion loops in HCMV gB, producing a protein that was able to fold and be transported to cell surfaces but unable to promote membrane fusion. Initially, I produced three HCMV gB mutants, Y153K, Y157K, and W240R, constructed Ad expression vectors, and tested the gB mutants for ER export (which measures folding of the glycoprotein) and for cell-cell fusion. Unfortunately, two (Y153K, W240R) were improperly folded and did not escape the ER, and one (Y157K) was exported from the ER to cell surfaces but retained the capacity to mediate cell-cell fusion. A second set of fusion loop mutants, Y155K, L241R, and Y242K are currently being tested for ER export and cell-cell fusion.

H4. Conclusions about gB function from entry in trans. My observations that gB can mediate entry *in trans*, but gH/gL cannot, provides an important new understanding about how gB and gH/gL function in entry. The results show that gB expressed in cells and thus pointed toward the HCMV virion envelope can mediate entry, apparently by fusing the virion envelope with the plasma membrane. This demonstrates that gB does not require interactions with cellular proteins in the host membrane. This argues strongly against the notion that gB is a receptor binding protein or is needed to

cause signaling in host cells. One could argue that this process is inefficient and not related to what normally occurs when HCMV enters cells. But infection of 5-14% of all the cells was not insignificant and PEG only increased this entry by 3-10 fold. There were also observations that particles lacking gB were also deficient for gH/gL, and this deficiency (similar to the loss of gH/gL from gO-null virus) likely reduces the entry capacity of these particles mediated by gB *in trans*. The results suggest that gB expressed in cells is mediating entry of HCMV particles that lack gB. These results strongly support the hypothesis that gB is the fusion protein and gH/gL acts as a receptor binding protein to trigger gB for fusion. Put another way, gB recognized a protein in the viral membrane, likely gH/gL, and gH/gL recognized a molecule in the cell membrane, i.e., gH/gL receptors. I believe that these results provide the best functional data to date to support this model for HCMV entry.

I. Overall summary

The two projects described in Chapters 2 and 3 have illuminated the functions of two HCMV glycoproteins, gB and gH/gL, that are necessary for virus entry. My work on gO coupled with that of Brent Ryckman demonstrated that the previous model of how gO functions was inaccurate. The prevailing model was that gH/gL/gO was formed, incorporated into the virion, and gH/gL/gO acted directly in entry into fibroblasts. We showed that gO acts as a chaperone to promote ER export of gH/gL. Without gO, there was much less gH/gL present in extracellular virions and this reduction prevented entry into fibroblasts and epithelial and endothelial cells. Thus, gO's chaperone function is essential and gO does not play a direct role in virus entry.

My demonstration of gB-mediated entry *in trans* provides the best functional evidence to date that HCMV gB is the fusion protein. Moreover, this is the first

description of any viral fusion protein acting in a cellular membrane to trigger entry of the virus. We believe that this has not been shown before because other viral fusion proteins (e.g., VSV-G protein) must be triggered by binding to cellular receptors. However, in the case of HCMV, gH/gL in the virion envelope can bind receptors, reach across the space between virus particles and cells expressing gB, and then trigger that gB for fusion. This assay also provides a means by which the function and mechanism of gB can be defined and genetically separated from the function of gH/gL complexes.

Based on my results and many previous results, our model of HCMV entry is as follows: entry that occurs at the cell surface (e.g., into fibroblasts) is mediated by gH/gL engagement with an unknown receptor and this interaction leads to gB being triggered for fusion. With epithelial and endothelial cells, entry involves endocytosis and requires both gH/gL and gH/gL/UL128-131. There is evidence that gH/gL/UL128-131 engages with an unknown receptor (different from those on fibroblasts), either on cell surfaces or in endosomes, and this triggers gB for fusion. This entry pathway is disrupted when the low pH of endosomes is raised.

Finally, this work has important implications for how all herpesvirus gB and gH/gL molecules function. Although the structures of HSV and EBV gB were consistent with gB being a fusion protein, the structures of HSV and EBV gH/gL did not prove that they were not fusion proteins. My data is consistent with the notion that gB molecules are the fusion protein, while gH/gL molecules are not. It seems possible that the entry *in trans* assay can be extended to other herpesviruses in order to further study function, e.g., domains important for gB interactions with gH/gL. To date, my efforts to adapt this assay to HSV have failed. This may relate to the fact that HSV expresses gD, which binds receptors to initiate interactions between gH/gL and gB. The addition of gD makes the

interactions much more complicated, although it is important to note that cell-cell fusion *in trans* occurs with HSV glycoproteins (7). Other attempts to adapt these assays to EBV in collaboration with the Hutt-Fletcher laboratory are underway and may help us determine whether this phenomenon is unique to CMV. Determining how different herpesvirus gB molecules can function *in trans* will likely substantially illuminate how gB functions.

REFERENCES

1. **Adler, B., L. Scrivano, Z. Ruzcics, B. Rupp, C. Sinzger, and U. Koszinowski**. 2006. Role of human cytomegalovirus UL131A in cell type-specific virus entry and release. J Gen Virol **87**:2451-2460.
2. **Adler, S. P**. 1992. Cytomegalovirus transmission and child day care. Adv Pediatr Infect Dis **7**:109-122.
3. **Adler, S. P., and B. Marshall**. 2007. Cytomegalovirus infections. Pediatr Rev **28**:92-100.
4. **Akter, P., C. Cunningham, B. P. McSharry, A. Dolan, C. Addison, D. J. Dargan, A. F. Hassan-Walker, V. C. Emery, P. D. Griffiths, G. W. Wilkinson, and A. J. Davison**. 2003. Two novel spliced genes in human cytomegalovirus. J Gen Virol **84**:1117-1122.
5. **Alford, C. A., S. Stagno, R. F. Pass, and W. J. Britt**. 1990. Congenital and perinatal cytomegalovirus infections. Rev Infect Dis **12 Suppl 7**:S745-53.
6. **Atanasiu, D., J. C. Whitbeck, T. M. Cairns, B. Reilly, G. H. Cohen, and R. J. Eisenberg**. 2007. Bimolecular complementation reveals that glycoproteins gB and gH/gL of herpes simplex virus interact with each other during cell fusion. Proc Natl Acad Sci U S A **104**:18718-18723.
7. **Atanasiu, D., W. T. Saw, G. H. Cohen, and R. J. Eisenberg**. 2010. Cascade of events governing cell-cell fusion induced by herpes simplex virus glycoproteins gD, gH/gL, and gB. Journal of virology **84**:12292-12299.
8. **Backovic, M., R. Longnecker, and T. S. Jardetzky**. 2009. Structure of a trimeric variant of the Epstein-Barr virus glycoprotein B. Proc Natl Acad Sci USA **106**:2880-2885.

9. **Baines, J. D., E. Wills, R. J. Jacob, J. Pennington, and B. Roizman**. 2007. Glycoprotein M of herpes simplex virus 1 is incorporated into virions during budding at the inner nuclear membrane. J Virol **81**:800-812.

10. **Baldanti, F., S. Paolucci, G. Campanini, A. Sarasini, E. Percivalle, M. G. Revello, and G. Gerna**. 2006. Human cytomegalovirus UL131A, UL130 and UL128 genes are highly conserved among field isolates. Arch Virol **151**:1225-1233.

11. **Batterson, W., D. Furlong, and B. Roizman**. 1983. Molecular genetics of herpes simplex virus. VIII. further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. Journal of Virology **45**:397-407.

12. **Beitia Ortiz de Zarate, I., K. Kaelin, and F. Rozenberg**. 2004. Effects of mutations in the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B on intracellular transport and infectivity. Journal of virology **78**:1540-1551.

13. **Benson, C. A., J. E. Kaplan, H. Masur, A. Pau, K. K. Holmes, CDC, N. I. o. Health, and I. D. S. o. America**. 2004. Treating opportunistic infections among HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association/Infectious Diseases Society of America. MMWR Recomm Rep **53**:1-112.

14. **Billstrom, M. A., and W. J. Britt**. 1995. Postoligomerization folding of human cytomegalovirus glycoprotein B: identification of folding intermediates and importance of disulfide bonding. J Virol **69**:7015-7022.

15. **Bissinger, A. L., C. Sinzger, E. Kaiserling, and G. Jahn**. 2002. Human cytomegalovirus as a direct pathogen: correlation of multiorgan involvement and

cell distribution with clinical and pathological findings in a case of congenital inclusion disease. J Med Virol **67**:200-206.

16. **Boehme, K.W., and T. Compton**. 2006. Virus Entry and Activation of Innate Immunity, p. 111-130. *In* M. J. Reddehase (ed.), Cytomegaloviruses: Molecular Biology and Immunology, Caister Academic Press, Norfolk, UK.
17. **Borst, E. M., G. Hahn, U. H. Koszinowski, and M. Messerle**. 1999. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in Escherichia coli: a new approach for construction of HCMV mutants. J Virol **73**:8320-8329.
18. **Borza, C. M., and L. M. Hutt-Fletcher**. 2002. Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus. Nat Med **8**:594-599.
19. **Botto, S., D. N. Streblow, V. Defilippis, L. White, C. N. Kreklywich, P. P. Smith, and P. Caposio**. 2011. IL-6 in human cytomegalovirus secretome promotes angiogenesis and survival of endothelial cells through the stimulation of survivin. Blood **117**:352-361.
20. **Bowman, J. J., J. C. Lacayo, P. Burbelo, E. R. Fischer, and J. I. Cohen**. 2011. Rhesus and human cytomegalovirus glycoprotein L are required for infection and cell-to-cell spread of virus but cannot complement each other. Journal of virology **85**:2089-2099.
21. **Brack, A. R., J. M. Dijkstra, H. Granzow, B. G. Klupp, and T. C. Mettenleiter**. 1999. Inhibition of virion maturation by simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. Journal of Virology **73**:5364-5372.
22. **Britt, W. J., and D. Auger**. 1986. Synthesis and processing of the envelope gp55-116 complex of human cytomegalovirus. J Virol **58**:185-191.

23. **Britt, W. J., M. Jarvis, J. Y. Seo, D. Drummond, and J. Nelson**. 2004. Rapid genetic engineering of human cytomegalovirus by using a lambda phage linear recombination system: demonstration that pp28 (UL99) is essential for production of infectious virus. J Virol **78**:539-543.

24. **Britt, W. J., and L. G. Vugler**. 1989. Processing of the gp55-116 envelope glycoprotein complex (gB) of human cytomegalovirus. J Virol **63**:403-410.

25. **Britt, W. J., and L. G. Vugler**. 1992. Oligomerization of the human cytomegalovirus major envelope glycoprotein complex gB (gp55-116). J Virol **66**:6747-6754.

26. **Britt, W.J., and C. A. Alford**. 1996. Cytomegaloviruses, p. 2493-2523. *In* Fields, B. N., Knipe, D. M., and P. M. Howley (eds.), Fields Virology, 3rd ed. ed. Lippincott-Raven Press, Philadelphia.

27. **Britt, W. J**. 1984. Neutralizing antibodies detect a disulfide-linked glycoprotein complex within the envelope of human cytomegalovirus. Virology **135**:369-378.

28. **Britt, W. J., and D. Auger**. 1985. Identification of a 65 000 dalton virion envelope protein of human cytomegalovirus. Virus Res **4**:31-36.

29. **Bruggeman, C. A., W. H. Debie, A. D. Muller, B. Schutte, and M. C. van Dam-Mieras**. 1988. Cytomegalovirus alters the von Willebrand factor content in human endothelial cells. Thromb Haemost **59**:264-268.

30. **Buchkovich, N. J., T. G. Maguire, and J. C. Alwine**. 2010. Role of the endoplasmic reticulum chaperone BiP, SUN domain proteins, and dynein in altering nuclear morphology during human cytomegalovirus infection. J Virol **84**:7005-7017.

31. **Cairns, T. M., J. C. Whitbeck, H. Lou, E. E. Heldwein, T. K. Chowdary, R. J. Eisenberg, and G. H. Cohen**. 2011. Capturing the herpes simplex virus core fusion complex (gB-gH/gL) in an acidic environment. Journal of virology **85**:6175-6184.

32. **Cepeda, V., M. Esteban, and A. Fraile-Ramos**. 2010. Human cytomegalovirus final envelopment on membranes containing both trans-Golgi network and endosomal markers. Cell Microbiol **12**:386-404.

33. **Cha, T. A., E. Tom, G. W. Kemble, G. M. Duke, E. S. Mocarski, and R. R. Spaete**. 1996. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. J Virol **70**:78-83.

34. **Chee, M., S. A. Rudolph, B. Plachter, B. Barrell, and G. Jahn**. 1989. Identification of the major capsid protein gene of human cytomegalovirus. J Virol **63**:1345-1353.

35. **Cheeran, M. C., J. R. Lokensgard, and M. R. Schleiss**. 2009. Neuropathogenesis of congenital cytomegalovirus infection: disease mechanisms and prospects for intervention. Clin Microbiol Rev **22**:99-126, Table of Contents.

36. **Cherepanov, P. P., and W. Wackernagel**. 1995. Gene disruption in Escherichia coli: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158**:9-14.

37. **Chesnokova, L. S., S. L. Nishimura, and L. M. Hutt-Fletcher**. 2009. Fusion of epithelial cells by Epstein-Barr virus proteins is triggered by binding of viral glycoproteins gHgL to integrins alphavbeta6 or alphavbeta8. Proceedings of the National Academy of Sciences of the United States of America **106**:20464-20469.

38. **Cobbs, C. S., L. Soroceanu, S. Denham, W. Zhang, W. J. Britt, R. Pieper, and M. H. Kraus**. 2007. Human cytomegalovirus induces cellular tyrosine kinase signaling and promotes glioma cell invasiveness. J Neurooncol **85**:271-280.

39. **Compton, T**. 1993. An immortalized human fibroblast cell line is permissive for human cytomegalovirus infection. J Virol **67**:3644-3648.

40. **Compton, T., R. R. Nepomuceno, and D. M. Nowlin**. 1992. Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. Virology **191**:387-395.

41. **Compton, T., D. M. Nowlin, and N. R. Cooper**. 1993. Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. Virology **193**:834-841.

42. **Cranage, M. P., G. L. Smith, S. E. Bell, H. Hart, C. Brown, A. T. Bankier, P. Tomlinson, B. G. Barrell, and T. C. Minson**. 1988. Identification and expression of a human cytomegalovirus glycoprotein with homology to the Epstein-Barr virus BXLF2 product, varicella-zoster virus gpIII, and herpes simplex virus type 1 glycoprotein H. Journal of Virology **62**:1416-1422.

43. **Crump, C. M., C. H. Hung, L. Thomas, L. Wan, and G. Thomas**. 2003. Role of PACS-1 in trafficking of human cytomegalovirus glycoprotein B and virus production. J Virol **77**:11105-11113.

44. **Crump, C. M., B. Bruun, S. Bell, L. E. Pomeranz, T. Minson, and H. M. Browne**. 2004. Alphaherpesvirus glycoprotein M causes the relocalization of plasma membrane proteins. J Gen Virol **85**:3517-3527.

45. **Das, S., A. Vasanji, and P. E. Pellett**. 2007. Three-dimensional structure of the human cytomegalovirus cytoplasmic virion assembly complex includes a reoriented secretory apparatus. J Virol **81**:11861-11869.

46. **Datsenko, K. A., and B. L. Wanner**. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proceedings of the National Academy of Science **97(12)**:6640-6645.

47. **Dechat, T., K. Pfleghaar, K. Sengupta, T. Shimi, D. K. Shumaker, L. Solimando, and R. D. Goldman**. 2008. Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin. Genes Dev **22**:832-853.

48. **Döhner, K., and B. Sodeik**. 2005. The role of the cytoskeleton during viral infection. Curr Top Microbiol Immunol **285**:67-108.

49. **Dolan, A., C. Cunningham, R. D. Hector, A. F. Hassan-Walker, L. Lee, C. Addison, D. J. Dargan, D. J. McGeoch, D. Gatherer, V. C. Emery, P. D. Griffiths, C. Sinzger, B. P. McSharry, G. W. Wilkinson, and A. J. Davison**. 2004. Genetic content of wild-type human cytomegalovirus. J Gen Virol **85**:1301-1312.

50. **Drew, W. L., G. Tegtmeier, H. J. Alter, M. E. Laycock, R. C. Miner, and M. P. Busch**. 2003. Frequency and duration of plasma CMV viremia in seroconverting blood donors and recipients. Transfusion **43**:309-313.

51. **Dunn, W., C. Chou, H. Li, R. Hai, D. Patterson, V. Stolc, H. Zhu, and F. Liu**. 2003. Functional profiling of a human cytomegalovirus genome. Proc Natl Acad Sci U S A **100**:14223-14228.

52. **Eggers, M., E. Bogner, B. Agricola, H. F. Kern, and K. Radsak**. 1992. Inhibition of human cytomegalovirus maturation by brefeldin A. The Journal of general virology **73**:2679-2692.

53. **Eickmann, M., D. Gicklhorn, and K. Radsak**. 2006. Glycoprotein Trafficking in Virion Morphogenesis, p. 245-264. *In* M. J. Reddehase (ed.), Cytomegaloviruses: Molecular Biology and Immunology, Caister Academic Press, Norfolk, UK.

54. **Farnsworth, A., K. Goldsmith, and D. C. Johnson**. 2003. Herpes simplex virus glycoproteins gD and gE/gI serve essential but redundant functions during acquisition of the virion envelope in the cytoplasm. J Virol **77**:8481-8494.

55. **Farnsworth, A., and D. C. Johnson**. 2006. Herpes simplex virus gE/gI must accumulate in the trans-Golgi network at early times and then redistribute to cell junctions to promote cell-cell spread. J Virol **80**:3167-3179.

56. **Farnsworth, A., T. W. Wisner, M. Webb, R. Roller, G. Cohen, R. Eisenberg, and D. C. Johnson**. 2007. Herpes simplex virus glycoproteins gB and gH function in fusion between the virion envelope and the outer nuclear membrane. Proc Natl Acad Sci U S A **104**:10187-10192.

57. **Feire, A. L., H. Koss, and T. Compton**. 2004. Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. Proc Natl Acad Sci U S A **101**:15470-15475.

58. **Feire, A. L., R. M. Roy, K. Manley, and T. Compton**. 2010. The glycoprotein B disintegrin-like domain binds beta 1 integrin to mediate cytomegalovirus entry. Journal of Virology **84**:10026-10037.

59. **Fish, K. N., C. Soderberg-Naucler, and J. A. Nelson**. 1998. Steady-state plasma membrane expression of human cytomegalovirus gB is determined by the phosphorylation state of Ser900. J Virol **72**:6657-6664.

60. **Fisher, S., O. Genbacev, E. Maidji, and L. Pereira**. 2000. Human cytomegalovirus infection of placental cytotrophoblasts in vitro and in utero: implications for transmission and pathogenesis. J Virol **74**:6808-6820.

61. **Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson**. 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J Virol **66**:341-348.

62. **Gerna, G., E. Percivalle, D. Lilleri, L. Lozza, C. Fornara, G. Hahn, F. Baldanti, and M. G. Revello**. 2005. Dendritic-cell infection by human cytomegalovirus is restricted to strains carrying functional UL131-128 genes and mediates efficient viral antigen presentation to CD8+ T cells. J Gen Virol **86**:275-284.

63. **Gerna, G., F. Baldanti, and M. G. Revello**. 2004. Pathogenesis of human cytomegalovirus infection and cellular targets. Hum Immunol **65**:381-386.

64. **Gerna, G., E. Percivalle, F. Baldanti, S. Sozzani, P. Lanzarini, E. Genini, D. Lilleri, and M. G. Revello**. 2000. Human cytomegalovirus replicates abortively in polymorphonuclear leukocytes after transfer from infected endothelial cells via transient microfusion events. J Virol **74**:5629-5638.

65. **Gibson, W**. 2006. Assembly and Maturation of the Capsid, p. 231-243. *In* M. J. Reddehase (ed.), Cytomegaloviruses: Molecular Biology and Immunology, Caister Academic Press, Norfolk, UK.

66. **Gibson, W**. 2008. Structure and formation of the cytomegalovirus virion. Curr Top Microbiol Immunol **325**:187-204.

67. **Gnann, J. W., J. Ahlmén, C. Svalander, L. Olding, M. B. Oldstone, and J. A. Nelson**. 1988. Inflammatory cells in transplanted kidneys are infected by human cytomegalovirus. Am J Pathol **132**:239-248.

68. **Goodrum, F., C. Jordan, K. High, and T. Shenk**. 2002. Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency. Proc Natl Acad Sci U S A **99**:16255-16260.

69. **Haan, K. M., S. K. Lee, and R. Longnecker**. 2001. Different functional domains in the cytoplasmic tail of glycoprotein B are involved in Epstein-Barr virus-induced membrane fusion. Virology **290**:106-114.

70. **Hahn, G., M. G. Revello, M. Patrone, E. Percivalle, G. Campanini, A. Sarasini, M. Wagner, A. Gallina, G. Milanesi, U. Koszinowski, F. Baldanti, and G. Gerna**. 2004. Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. J Virol **78**:10023-10033.

71. **Hahn, G., R. Jores, and E. S. Mocarski**. 1998. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. Proceedings of the National Academy of Sciences of the United States of America **95**:3937-3942.

72. **Hannah, B. P., T. M. Cairns, F. C. Bender, J. C. Whitbeck, H. Lou, R. J. Eisenberg, and G. H. Cohen**. 2009. Herpes simplex virus glycoprotein B associates with target membranes via its fusion loops. J Virol **83**:6825-6836.

73. **Hegde, N. R., C. Dunn, D. M. Lewinsohn, M. A. Jarvis, J. A. Nelson, and D. C. Johnson**. 2005. Endogenous human cytomegalovirus gB is presented efficiently by MHC class II molecules to CD4+ CTL. J Exp Med **202**:1109-1119.

74. **Heldwein, E. E., and C. Krummenacher**. 2008. Entry of herpesviruses into mammalian cells. Cell Mol Life Sci **65**:1653-1668.

75. **Heldwein, E. E., H. Lou, F. C. Bender, G. H. Cohen, R. J. Eisenberg, and S. C. Harrison**. 2006. Crystal structure of glycoprotein B from herpes simplex virus 1. Science **313**:217-220.

76. **Hensel, G. M., H. H. Meyer, I. Buchmann, D. Pommerehne, S. Schmolke, B. Plachter, K. Radsak, and H. F. Kern**. 1996. Intracellular localization and expression of the human cytomegalovirus matrix phosphoprotein pp71 (ppUL82): evidence for its translocation into the nucleus. The Journal of general virology **77**:3087-3097.

77. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear**. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J Virol **65**:1090-1098.

78. **Herold, B. C., R. J. Visalli, N. Susmarski, C. R. Brandt, and P. G. Spear**. 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J Gen Virol **75**:1211-1222.

79. **Herrold, R. E., A. Marchini, S. Fruehling, and R. Longnecker**. 1996. Glycoprotein 110, the Epstein-Barr virus homolog of herpes simplex virus glycoprotein B, is essential for Epstein-Barr virus replication in vivo. J Virol **70**:2049-2054.

80. **Hileman, R. E., J. R. Fromm, J. M. Weiler, and R. J. Linhardt**. 1998. Glycosaminoglycan-protein interactions: definition of consensus sites in glycosaminoglycan binding proteins. Bioessays **20**:156-167.

81. **Ho, D. D., T. R. Rota, C. A. Andrews, and M. S. Hirsch**. 1984. Replication of human cytomegalovirus in endothelial cells. J Infect Dis **150**:956-957.

82. **Ho, M**. 1977. Virus infections after transplantation in man. Brief review. Arch Virol **55**:1-24.

83. **Ho, M**. 1991. Cytomegalovirus, biology and infection, (ed.), Plenum Publishing Corporation, New York.

84. **Hobom, U., W. Brune, M. Messerle, G. Hahn, and U. H. Koszinowski**. 2000. Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. J Virol **74**:7720-7729.

85. **Holland, G. N., J. S. Pepose, T. H. Pettit, M. S. Gottlieb, R. D. Yee, and R. Y. Foos**. 1983. Acquired immune deficiency syndrome. Ocular manifestations. Ophthalmology **90**:859-873.

86. **Holma, K., T. Törnroth, C. Grönhagen-Riska, and I. Lautenschlager**. 2000. Expression of the cytomegalovirus genome in kidney allografts during active and latent infection. Transpl Int **13 Suppl 1**:S363-5.

87. **Huber, M. T., and T. Compton**. 1997. Characterization of a novel third member of the human cytomegalovirus glycoprotein H-glycoprotein L complex. J Virol **71**:5391-5398.

88. **Huber, M. T., and T. Compton**. 1998. The human cytomegalovirus UL74 gene encodes the third component of the glycoprotein H-glycoprotein L-containing envelope complex. J Virol **72**:8191-8197.

89. **Huber, M. T., and T. Compton**. 1999. Intracellular formation and processing of the heterotrimeric gH-gL-gO (gCIII) glycoprotein envelope complex of human cytomegalovirus. J Virol **73**:3886-3892.

90. **Huber, M. T., T. W. Wisner, N. R. Hegde, K. A. Goldsmith, D. A. Rauch, R. J. Roller, C. Krummenacher, R. J. Eisenberg, G. H. Cohen, and D. C. Johnson**. 2001. Herpes simplex virus with highly reduced gD levels can efficiently enter and spread between human keratinocytes. J Virol **75**:10309-10318.

91. **Hutt-Fletcher, L. M**. 2007. Epstein-Barr virus entry. J Virol **81**:7825-7832.

92. **Isaacson, M. K., A. L. Feire, and T. Compton**. 2007. Epidermal growth factor receptor is not required for human cytomegalovirus entry or signaling. J Virol **81**:6241-6247.

93. **Isaacson, M. K., L. K. Juckem, and T. Compton**. 2008. Virus entry and innate immune activation. Curr Top Microbiol Immunol **325**:85-100.

94. **Isaacson, M. K., and T. Compton**. 2009. Human cytomegalovirus glycoprotein B is required for virus entry and cell-to-cell spread but not for virion attachment, assembly, or egress. J Virol **83**:3891-3903.

95. **Isola, V. J., R. J. Eisenberg, G. R. Siebert, C. J. Heilman, W. C. Wilcox, and G. H. Cohen**. 1989. Fine mapping of antigenic site II of herpes simplex virus glycoprotein D. J Virol **63**:2325-2334.

96. **Jackson, J. O., and R. Longnecker**. 2010. Reevaluating herpes simplex virus hemifusion. J Virol **84**:11814-11821.

97. **Jarvis, M. A., and J. A. Nelson**. 2002. Mechanisms of human cytomegalovirus persistence and latency. Front Biosci **7**:d1575-82.

98. **Jarvis, M. A., T. R. Jones, D. D. Drummond, P. P. Smith, W. J. Britt, J. A. Nelson, and C. J. Baldick**. 2004. Phosphorylation of human cytomegalovirus glycoprotein B (gB) at the acidic cluster casein kinase 2 site (Ser900) is required for localization of gB to the trans-Golgi network and efficient virus replication. J Virol **78**:285-293.

99. **Jarvis, M. A., C. E. Wang, H. L. Meyers, P. P. Smith, C. L. Corless, G. J. Henderson, J. Vieira, W. J. Britt, and J. A. Nelson**. 1999. Human cytomegalovirus infection of caco-2 cells occurs at the basolateral membrane and is differentiation state dependent. J Virol **73**:4552-4560.

100. **Jassal, S. V., J. M. Roscoe, J. S. Zaltzman, T. Mazzulli, M. Krajden, M. Gadawski, D. C. Cattran, C. J. Cardella, S. E. Albert, and E. H. Cole**. 1998. Clinical practice guidelines: prevention of cytomegalovirus disease after renal transplantation. J Am Soc Nephrol **9**:1697-1708.

101. **Jiang, X. J., B. Adler, K. L. Sampaio, M. Digel, G. Jahn, N. Ettischer, Y. D. Stierhof, L. Scrivano, U. Koszinowski, M. Mach, and C. Sinzger**. 2008. UL74 of human cytomegalovirus contributes to virus release by promoting secondary envelopment of virions. J Virol **82**:2802-2812.

102. **Johnson, D. C., and M. T. Huber**. 2002. Directed egress of animal viruses promotes cell-to-cell spread. J Virol **76**:1-8.

103. **Johnson, D. C., and M. W. Ligas**. 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J Virol **62**:4605-4612.

104. **Johnson, D. C., M. Webb, T. W. Wisner, and C. Brunetti**. 2001. Herpes simplex virus gE/gI sorts nascent virions to epithelial cell junctions, promoting virus spread. J Virol **75**:821-833.

105. **Johnson, D. C., and J. D. Baines**. 2011. Herpesviruses remodel host membranes for virus egress. Nature reviews Microbiology **9**:382-394.

106. **Johnson, D. C., T. W. Wisner, and C. C. Wright**. 2011. Herpes Simplex Virus Glycoproteins gB and gD Function in a Redundant Fashion To Promote Secondary Envelopment. Journal of virology **85**:4910-4926.

107. **Kadlec, J., S. Loureiro, N. G. A. Abrescia, D. I. Stuart, and I. M. Jones**. 2008. The postfusion structure of baculovirus gp64 supports a unified view of viral fusion machines. Nature structural & molecular biology **15**:1024-1030.

108. **Kari, B., and R. Gehrz**. 1993. Structure, composition and heparin binding properties of a human cytomegalovirus glycoprotein complex designated gC-II. The Journal of general virology **74**:255-264.

109. **Kaye, J. F., U. A. Gompels, and A. C. Minson**. 1992. Glycoprotein H of human cytomegalovirus (HCMV) forms a stable complex with the HCMV UL115 gene product. J Gen Virol **73**:2693-2698.

110. **Keay, S., T. C. Merigan, and L. Rasmussen**. 1989. Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. Proceedings of the National Academy of Sciences of the United States of America **86**:10100-10103.

111. **Kinzler, E. R., and T. Compton**. 2005. Characterization of human cytomegalovirus glycoprotein-induced cell-cell fusion. J Virol **79**:7827-7837.

112. **Klupp, B., J. Altenschmidt, H. Granzow, W. Fuchs, and T. C. Mettenleiter**. 2008. Glycoproteins required for entry are not necessary for egress of pseudorabies virus. Journal of Virology **82**:6299-6309.

113. **Kondo, K., H. Kaneshima, and E. S. Mocarski**. 1994. Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. Proceedings of the National Academy of Sciences of the United States of America **91**:11879-11883.

114. **Kornfeld, R., and S. Kornfeld**. 1985. Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem **54**:631-664.

115. **Krech, U**. 1973. Complement-fixing antibodies against cytomegalovirus in different parts of the world. Bull World Health Organ **49**:103-106.

116. **Krishnan, H. H., N. Sharma-Walia, L. Zeng, S. J. Gao, and B. Chandran**. 2005. Envelope glycoprotein gB of Kaposi's sarcoma-associated herpesvirus is essential for egress from infected cells. J Virol **79**:10952-10967.

117. **Krummenacher, C., A. V. Nicola, J. C. Whitbeck, H. Lou, W. Hou, J. D. Lambris, R. J. Geraghty, P. G. Spear, G. H. Cohen, and R. J. Eisenberg**. 1998. Herpes simplex virus glycoprotein D can bind to poliovirus receptor-related protein 1 or herpesvirus entry mediator, two structurally unrelated mediators of virus entry. J Virol **72**:7064-7074.

118. **Krzyzaniak, M., M. Mach, and W. Britt**. 2007. The cytoplasmic tail of glycoprotein M (gpUL100) expresses trafficking signals required for human cytomegalovirus assembly and replication. J Virol **81**:10316-10328.

119. **Krzyzaniak, M. A., M. Mach, and W. J. Britt**. 2009. HCMV-encoded glycoprotein M (UL100) interacts with Rab11 effector protein FIP4. Traffic **10**:1439-1457.

120. **Lake, C. M., and L. M. Hutt-Fletcher**. 2000. Epstein-Barr virus that lacks glycoprotein gN is impaired in assembly and infection. J Virol **74**:11162-11172.

121. **Lake, C. M., S. J. Molesworth, and L. M. Hutt-Fletcher**. 1998. The Epstein-Barr virus (EBV) gN homolog BLRF1 encodes a 15-kilodalton glycoprotein that cannot be authentically processed unless it is coexpressed with the EBV gM homolog BBRF3. J Virol **72**:5559-5564.

122. **Landolfo, S., M. Gariglio, G. Gribaudo, and D. Lembo**. 2003. The human cytomegalovirus. Pharmacol Ther **98**:269-297.

123. **Lee, E. C., D. Yu, J. Martinez de Velasco, L. Tessarollo, D. A. Swing, D. L. Court, N. A. Jenkins, and N. G. Copeland**. 2001. A highly efficient Escherichia

coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics **73**:56-65.

124. **Li, L., J. A. Nelson, and W. J. Britt**. 1997. Glycoprotein H-related complexes of human cytomegalovirus: identification of a third protein in the gCIII complex. J Virol **71**:3090-3097.
125. **Li, Q., M. K. Spriggs, S. Kovats, S. M. Turk, M. R. Comeau, B. Nepom, and L. M. Hutt-Fletcher**. 1997. Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes. J Virol **71**:4657-4662.
126. **Li, Q., S. M. Turk, and L. M. Hutt-Fletcher**. 1995. The Epstein-Barr virus (EBV) BZLF2 gene product associates with the gH and gL homologs of EBV and carries an epitope critical to infection of B cells but not of epithelial cells. J Virol **69**:3987-3994.
127. **Ligas, M. W., and D. C. Johnson**. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. J Virol **62**:1486-1494.
128. **Lopper, M., and T. Compton**. 2004. Coiled-coil domains in glycoproteins B and H are involved in human cytomegalovirus membrane fusion. J Virol **78**:8333-8341.
129. **Mach, M., B. Kropff, P. Dal Monte, and W. Britt**. 2000. Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73). J Virol **74**:11881-11892.
130. **Mach, M., K. Osinski, B. Kropff, U. Schloetzer-Schrehardt, M. Krzyzaniak, and W. Britt**. 2007. The carboxy-terminal domain of glycoprotein N of human cytomegalovirus is required for virion morphogenesis. J Virol **81**:5212-5224.

131. **Maciejewski, J. P., E. E. Bruening, R. E. Donahue, E. S. Mocarski, N. S. Young, and S. C. St Jeor**. 1992. Infection of hematopoietic progenitor cells by human cytomegalovirus. Blood **80**:170-178.

132. **Maciejewski, J. P., E. E. Bruening, R. E. Donahue, S. E. Sellers, C. Carter, N. S. Young, and S. St Jeor**. 1993. Infection of mononucleated phagocytes with human cytomegalovirus. Virology **195**:327-336.

133. **Maidji, E., S. McDonagh, O. Genbacev, T. Tabata, and L. Pereira**. 2006. Maternal antibodies enhance or prevent cytomegalovirus infection in the placenta by neonatal Fc receptor-mediated transcytosis. Am J Pathol **168**:1210-1226.

134. **Mettenleiter, T. C**. 2002. Herpesvirus assembly and egress. J Virol **76**:1537-1547.

135. **Mettenleiter, T. C., B. G. Klupp, and H. Granzow**. 2009. Herpesvirus assembly: an update. Virus Res **143**:222-234.

136. **Meyer, G., D. Gicklhorn, T. Strive, K. Radsak, and M. Eickmann**. 2002. A three-residue signal confers localization of a reporter protein in the inner nuclear membrane. Biochem Biophys Res Commun **291**:966-971.

137. **Meyer, G. A., and K. D. Radsak**. 2000. Identification of a novel signal sequence that targets transmembrane proteins to the nuclear envelope inner membrane. The Journal of biological chemistry **275**:3857-3866.

138. **Milbradt, J., S. Auerochs, H. Sticht, and M. Marschall**. 2009. Cytomegaloviral proteins that associate with the nuclear lamina: components of a postulated nuclear egress complex. The Journal of general virology **90**:579-590.

139. **Miller, M. S., and L. Hertel**. 2009. Onset of human cytomegalovirus replication in fibroblasts requires the presence of an intact vimentin cytoskeleton. J Virol **83**:7015-7028.

140. **Miller, N., and L. M. Hutt-Fletcher**. 1992. Epstein-Barr virus enters B cells and epithelial cells by different routes. J Virol **66**:3409-3414.

141. **Milne, R. S., D. A. Paterson, and J. C. Booth**. 1998. Human cytomegalovirus glycoprotein H/glycoprotein L complex modulates fusion-from-without. The Journal of general virology **79**:855-865.

142. **Minton, E. J., C. Tysoe, J. H. Sinclair, and J. G. Sissons**. 1994. Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. J Virol **68**:4017-4021.

143. **Mocarski, E. S., Hahn, G., White, K. L., Xu, J., Slobedman, B., Hertel, L., Aguirre, S. A., and S. Noda**. 2006. Myeloid Cell Recruitment and Function in Pathogenesis and Latency, p. 465-481. *In* M. J. Reddehase (ed.), Cytomegaloviruses: Molecular Biology and Immunology, Caister Academic Press, Norfolk, UK.

144. **Mocarski, E. S., Shenk, T., and R. F. Pass**. 2007. Cytomegaloviruses, p. 2701-2772. *In* Knipe, D. M., and P. M. Howley (eds.), Fields Virology, 5th ed. ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.

145. **Molesworth, S. J., C. M. Lake, C. M. Borza, S. M. Turk, and L. M. Hutt-Fletcher**. 2000. Epstein-Barr virus gH is essential for penetration of B cells but also plays a role in attachment of virus to epithelial cells. J Virol **74**:6324-6332.

146. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear**. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87**:427-436.

147. **Morgello, S., E. S. Cho, S. Nielsen, O. Devinsky, and C. K. Petito**. 1987. Cytomegalovirus encephalitis in patients with acquired immunodeficiency

syndrome: an autopsy study of 30 cases and a review of the literature. Hum Pathol **18**:289-297.

148. **Mori, Y**. 2009. Recent topics related to human herpesvirus 6 cell tropism. Cell Microbiol **11**:1001-1006.

149. **Mori, Y., P. Akkapaiboon, S. Yonemoto, M. Koike, M. Takemoto, T. Sadaoka, Y. Sasamoto, S. Konishi, Y. Uchiyama, and K. Yamanishi**. 2004. Discovery of a second form of tripartite complex containing gH-gL of human herpesvirus 6 and observations on CD46. J Virol **78**:4609-4616.

150. **Muller, W. J., C. A. Jones, and D. M. Koelle**. 2010. Immunobiology of herpes simplex virus and cytomegalovirus infections of the fetus and newborn. Current immunology reviews **6**:38-55.

151. **Muranyi, W., J. Haas, M. Wagner, and U. H. Koszinowski**. 2002. Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina. Science **287**:854-856.

152. **Murphy, E., D. Yu, J. Grimwood, J. Schmutz, M. Dickson, M. A. Jarvis, G. Hahn, J. A. Nelson, R. M. Myers, and T. E. Shenk**. 2003. Coding potential of laboratory and clinical strains of human cytomegalovirus. Proc Natl Acad Sci U S A **100**:14976-14981.

153. **Myerson, D., R. C. Hackman, J. A. Nelson, D. C. Ward, and J. K. McDougall**. 1984. Widespread presence of histologically occult cytomegalovirus. Hum Pathol **15**:430-439.

154. **Nemerow, G. R., C. Mold, V. K. Schwend, V. Tollefson, and N. R. Cooper**. 1987. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-

Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. Journal of Virology **61**:1416-1420.

155. **Neuhierl, B., R. Feederle, D. Adhikary, B. Hub, K. Geletneky, J. Mautner, and H. J. Delecluse**. 2009. Primary B-cell infection with a deltaBALF4 Epstein-Barr virus comes to a halt in the endosomal compartment yet still elicits a potent CD4-positive cytotoxic T-cell response. J Virol **83**:4616-4623.
156. **Noda, S., S. A. Aguirre, A. Bitmansour, J. M. Brown, T. E. Sparer, J. Huang, and E. S. Mocarski**. 2006. Cytomegalovirus MCK-2 controls mobilization and recruitment of myeloid progenitor cells to facilitate dissemination. Blood **107**:30-38.
157. **Ogawa-Goto, K., K. Tanaka, W. Gibson, E. Moriishi, Y. Miura, T. Kurata, S. Irie, and T. Sata**. 2003. Microtubule network facilitates nuclear targeting of human cytomegalovirus capsid. J Virol **77**:8541-8547.
158. **Pass, R. F., C. Hutto, R. Ricks, and G. A. Cloud**. 1986. Increased rate of cytomegalovirus infection among parents of children attending day-care centers. N Engl J Med **314**:1414-1418.
159. **Pass, R. F., S. C. Hutto, D. W. Reynolds, and R. B. Polhill**. 1984. Increased frequency of cytomegalovirus infection in children in group day care. Pediatrics **74**:121-126.
160. **Paterson, D. A., A. P. Dyer, R. S. Milne, E. Sevilla-Reyes, and U. A. Gompels**. 2002. A role for human cytomegalovirus glycoprotein O (gO) in cell fusion and a new hypervariable locus. Virology **293**:281-294.
161. **Peng, T., M. Ponce de Leon, M. J. Novotny, H. Jiang, J. D. Lambris, G. Dubin, P. G. Spear, G. H. Cohen, and R. J. Eisenberg**. 1998. Structural and antigenic

analysis of a truncated form of the herpes simplex virus glycoprotein gH-gL complex. J Virol **72**:6092-6103.

162. **Pereira L., Maidji, E., McDonagh, S., and T. Tabata**. 2006. Routes of Human CMV Transmission and Infection at the Uterine-Placental Interface, p. 29-48. *In* M. J. Reddehase (ed.), Cytomegaloviruses: Molecular Biology and Immunology, Caister Academic Press, Norfolk, UK.
163. **Pereira, L., M. Hoffman, M. Tatsuno, and D. Dondero**. 1984. Polymorphism of human cytomegalovirus glycoproteins characterized by monoclonal antibodies. Virology **139**:73-86.
164. **Pereira, L., E. Maidji, S. McDonagh, O. Genbacev, and S. Fisher**. 2003. Human cytomegalovirus transmission from the uterus to the placenta correlates with the presence of pathogenic bacteria and maternal immunity. J Virol **77**:13301-13314.
165. **Pertel, P. E., A. Fridberg, M. L. Parish, and P. G. Spear**. 2001. Cell fusion induced by herpes simplex virus glycoproteins gB, gD, and gH-gL requires a gD receptor but not necessarily heparan sulfate. Virology **279**:313-324.
166. **Pietropaolo, R., and T. Compton**. 1999. Interference with annexin II has no effect on entry of human cytomegalovirus into fibroblast cells. J Gen Virol **80**:1807-1816.
167. **Plachter, B., C. Sinzger, and G. Jahn**. 1996. Cell types involved in replication and distribution of human cytomegalovirus. Adv Virus Res **46**:195-261.
168. **Powers, C., V. Defilippis, D. Malouli, and K. Früh**. 2008. Cytomegalovirus immune evasion. Current topics in microbiology and immunology **325**:333-359.
169. **Prichard, M. N., M. E. Penfold, G. M. Duke, R. R. Spaete, and G. W. Kemble**. 2001. A review of genetic differences between limited and extensively passaged human cytomegalovirus strains. Rev Med Virol **11**:191-200.

170. **Radsak, K., M. Eickmann, T. Mockenhaupt, E. Bogner, H. Kern, A. Eis-Hübinger, and M. Reschke**. 1996. Retrieval of human cytomegalovirus glycoprotein B from the infected cell surface for virus envelopment. Archives of virology **141**:557-572.

171. **Radsak, K., M. Eickmann, T. Mockenhaupt, E. Bogner, H. Kern, A. Eis-Hübinger, and M. Reschke**. 1996. Retrieval of human cytomegalovirus glycoprotein B from the infected cell surface for virus envelopment. Arch Virol **141**:557-572.

172. **Radsak, K., R. Fuhrmann, R. P. Franke, D. Schneider, A. Kollert, K. H. Brücher, and D. Drenckhahn**. 1989. Induction by sodium butyrate of cytomegalovirus replication in human endothelial cells. Arch Virol **107**:151-158.

173. **Rafailidis, P. I., E. G. Mourtzoukou, I. C. Varbobitis, and M. E. Falagas**. 2008. Severe cytomegalovirus infection in apparently immunocompetent patients: a systematic review. Virology journal **5**:47.

174. **Rasmussen, L., A. Geissler, C. Cowan, A. Chase, and M. Winters**. 2002. The genes encoding the gCIII complex of human cytomegalovirus exist in highly diverse combinations in clinical isolates. J Virol **76**:10841-10848.

175. **Rasmussen, L., J. Mullenax, M. Nelson, and T. C. Merigan**. 1985. Human cytomegalovirus polypeptides stimulate neutralizing antibody in vivo. Virology **145**:186-190.

176. **Rasmussen, L., M. Nelson, M. Neff, and T. C. Merigan**. 1988. Characterization of two different human cytomegalovirus glycoproteins which are targets for virus neutralizing antibody. Virology **163**:308-318.

177. **Reeves, M. B., H. Coleman, J. Chadderton, M. Goddard, J. G. Sissons, and J. H. Sinclair**. 2004. Vascular endothelial and smooth muscle cells are unlikely to be major sites of latency of human cytomegalovirus in vivo. The Journal of general virology **85**:3337-3341.

178. **Reynolds, A. E., B. J. Ryckman, J. D. Baines, Y. Zhou, L. Liang, and R. J. Roller**. 2001. U(L)31 and U(L)34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids. J Virol **75**:8803-8817.

179. **Reynolds, D. W., S. Stagno, T. S. Hosty, M. Tiller, and C. A. Alford**. 1973. Maternal cytomegalovirus excretion and perinatal infection. N Engl J Med **289**:1-5.

180. **Rinaldo, C. R., P. H. Black, and M. S. Hirsch**. 1977. Interaction of cytomegalovirus with leukocytes from patients with mononucleosis due to cytomegalovirus. J Infect Dis **136**:667-678.

181. **Roche, S., S. Bressanelli, F. Rey, and Y. Gaudin**. 2006. Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science (New York, NY) **313**:187-191.

182. **Roop, C., L. Hutchinson, and D. C. Johnson**. 1993. A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. J Virol **67**:2285-2297.

183. **Ryckman, B. J., M. C. Chase, and D. C. Johnson**. 2008. HCMV gH/gL/UL128-131 interferes with virus entry into epithelial cells: evidence for cell type-specific receptors. Proc Natl Acad Sci U S A **105**:14118-14123.

184. **Ryckman, B. J., M. C. Chase, and D. C. Johnson**. 2010. Human Cytomegalovirus TR Strain Glycoprotein O Acts as a Chaperone Promoting gH/gL Incorporation into Virions but Is Not Present in Virions. J Virol **84**:2597-2609.

185. **Ryckman, B. J., M. A. Jarvis, D. D. Drummond, J. A. Nelson, and D. C. Johnson**. 2006. Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. J Virol **80**:710-722.

186. **Ryckman, B. J., B. L. Rainish, M. C. Chase, J. A. Borton, J. A. Nelson, M. A. Jarvis, and D. C. Johnson**. 2008. Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates entry into epithelial and endothelial cells. J Virol **82**:60-70.

187. **Sanchez, V., K. D. Greis, E. Sztul, and W. J. Britt**. 2000. Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly. J Virol **74**:975-986.

188. **Sarov, B., L. Naggan, R. Rosenzveig, S. Katz, H. Haikin, and I. Sarov**. 1982. Prevalence of antibodies to human cytomegalovirus in urban, kibbutz, and Bedouin children in southern Israel. J Med Virol **10**:195-201.

189. **Scholz, M., H. W. Doerr, and J. Cinatl**. 2003. Human cytomegalovirus retinitis: pathogenicity, immune evasion and persistence. Trends Microbiol **11**:171-178.

190. **Scrivano, L., C. Sinzger, H. Nitschko, U. H. Koszinowski, and B. Adler**. 2011. HCMV Spread and Cell Tropism are Determined by Distinct Virus Populations. PLoS Pathogens **7**:e1001256.

191. **Secchiero, P., D. Sun, A. L. de Vico, R. W. Crowley, M. S. Reitz, G. Zauli, P. Lusso, and R. C. Gallo**. 1997. Role of the extracellular domain of human herpesvirus 7 glycoprotein B in virus binding to cell surface heparan sulfate proteoglycans. Journal of virology **71**:4571-4580.

192. **Showalter, S. D., M. Zweig, and B. Hampar**. 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. Infection and Immunity **34**:684-692.

193. **Sinclair, J**. 2008. Human cytomegalovirus: Latency and reactivation in the myeloid lineage. J Clin Virol **41**:180-185.

194. **Sinzger, C., M. Digel, and G. Jahn**. 2008. Cytomegalovirus cell tropism. Curr Top Microbiol Immunol **325**:63-83.

195. **Sinzger, C., and G. Jahn**. 1996. Human cytomegalovirus cell tropism and pathogenesis. Intervirology **39**:302-319.

196. **Sinzger, C., H. Müntefering, T. Löning, H. Stöss, B. Plachter, and G. Jahn**. 1993. Cell types infected in human cytomegalovirus placentitis identified by immunohistochemical double staining. Virchows Arch A Pathol Anat Histopathol **423**:249-256.

197. **Smiley, M. L., E. C. Mar, and E. S. Huang**. 1988. Cytomegalovirus infection and viral-induced transformation of human endothelial cells. J Med Virol **25**:213-226.

198. **Smith, I. L., I. Taskintuna, F. M. Rahhal, H. C. Powell, E. Ai, A. J. Mueller, S. A. Spector, and W. R. Freeman**. 1998. Clinical failure of CMV retinitis with intravitreal cidofovir is associated with antiviral resistance. Arch Ophthalmol **116**:178-185.

199. **Söderberg-Nauclér, C**. 2008. HCMV microinfections in inflammatory diseases and cancer. J Clin Virol **41**:218-223.

200. **Soderberg-Naucler, C., K. N. Fish, and J. A. Nelson**. 1997. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. Cell **91**:119-126.

201. **Soroceanu, L., A. Akhavan, and C. S. Cobbs**. 2008. Platelet-derived growth factor-alpha receptor activation is required for human cytomegalovirus infection. Nature **455**:391-395.

202. **Spaete, R. R., K. Perot, P. I. Scott, J. A. Nelson, M. F. Stinski, and C. Pachl**. 1993. Coexpression of truncated human cytomegalovirus gH with the UL115 gene product or the truncated human fibroblast growth factor receptor results in transport of gH to the cell surface. Virology **193**:853-861.

203. **Spaete, R. R., A. Saxena, P. I. Scott, G. J. Song, W. S. Probert, W. J. Britt, W. Gibson, L. Rasmussen, and C. Pachl**. 1990. Sequence requirements for proteolytic processing of glycoprotein B of human cytomegalovirus strain Towne. J Virol **64**:2922-2931.

204. **Stagno, S., R. F. Pass, and C. A. Alford**. 1981. Perinatal infections and maldevelopment. Birth Defects Orig Artic Ser **17**:31-50.

205. **Stagno, S., R. F. Pass, G. Cloud, W. J. Britt, R. E. Henderson, P. D. Walton, D. A. Veren, F. Page, and C. A. Alford**. 1986. Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. Jama **256**:1904-1908.

206. **Stagno, S., R. F. Pass, M. E. Dworsky, R. E. Henderson, E. G. Moore, P. D. Walton, and C. A. Alford**. 1982. Congenital cytomegalovirus infection: The

relative importance of primary and recurrent maternal infection. N Engl J Med **306**:945-949.

207. **Stagno, S., D. W. Reynolds, R. F. Pass, and C. A. Alford**. 1980. Breast milk and the risk of cytomegalovirus infection. N Engl J Med **302**:1073-1076.

208. **Stannard, L. M., S. Himmelhoch, and S. Wynchank**. 1996. Intra-nuclear localization of two envelope proteins, gB and gD, of herpes simplex virus. Arch Virol **141**:505-524.

209. **Stanton, R., D. Westmoreland, J. D. Fox, A. J. Davison, and G. W. Wilkinson**. 2005. Stability of human cytomegalovirus genotypes in persistently infected renal transplant recipients. J Med Virol **75**:42-46.

210. **Staras, S. A., S. C. Dollard, K. W. Radford, W. D. Flanders, R. F. Pass, and M. J. Cannon**. 2006. Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. Clin Infect Dis **43**:1143-1151.

211. **Stinski, M. F., and J. Meier**. 2006. Immediate early viral gene regulation and function, p. 239-261. *In* Arvin, A. M., Mocarski, E. S., and P. Moore (eds.), Human Herpesviruses: Biology, Therapy and Immunoprophylaxis, Cambridge Press, Cambridge.

212. **Streblow, D. N., S. L. Orloff, and J. A. Nelson**. 2001. Do pathogens accelerate atherosclerosis? J Nutr **131**:2798S-2804S.

213. **Streblow, D. N., S. L. Orloff, and J. A. Nelson**. 2007. Acceleration of allograft failure by cytomegalovirus. Curr Opin Immunol **19**:577-582.

214. **Streblow, D. N., C. Soderberg-Naucler, J. Vieira, P. Smith, E. Wakabayashi, F. Ruchti, K. Mattison, Y. Altschuler, and J. A. Nelson**. 1999. The human

cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. Cell **99**:511-520.

215. **Strive, T., D. Gicklhorn, M. Wohlfahrt, L. Kolesnikova, M. Eickmann, E. Borst, M. Messerle, and K. Radsak**. 2005. Site directed mutagenesis of the carboxyl terminus of human cytomegalovirus glycoprotein B leads to attenuation of viral growth in cell culture. Arch Virol **150**:585-593.
216. **Subramanian, R., I. Sehgal, O. D'Auvergne, and K. G. Kousoulas**. 2010. Kaposi's sarcoma-associated herpesvirus glycoproteins B and K8.1 regulate virion egress and synthesis of vascular endothelial growth factor and viral interleukin-6 in BCBL-1 cells. Journal of Virology **84**:1704-1714.
217. **Subramanian, R. P., and R. J. Geraghty**. 2007. Herpes simplex virus type 1 mediates fusion through a hemifusion intermediate by sequential activity of glycoproteins D, H, L, and B. Proc Natl Acad Sci U S A **104**:2903-2908.
218. **Tanner, J., J. Weis, D. Fearon, Y. Whang, and E. Kieff**. 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. Cell **50**:203-213.
219. **Theiler, R. N., and T. Compton**. 2001. Characterization of the signal peptide processing and membrane association of human cytomegalovirus glycoprotein O. J Biol Chem **276**:39226-39231.
220. **Torrisi, M. R., C. Di Lazzaro, A. Pavan, L. Pereira, and G. Campadelli-Fiume**. 1992. Herpes simplex virus envelopment and maturation studied by fracture label. J Virol **66**:554-561.

221. **Tugizov, S., E. Maidji, J. Xiao, Z. Zheng, and L. Pereira**. 1998. Human cytomegalovirus glycoprotein B contains autonomous determinants for vectorial targeting to apical membranes of polarized epithelial cells. J Virol **72**:7374-7386.

222. **Tugizov, S., D. Navarro, P. Paz, Y. Wang, I. Qadri, and L. Pereira**. 1994. Function of human cytomegalovirus glycoprotein B: syncytium formation in cells constitutively expressing gB is blocked by virus-neutralizing antibodies. Virology **201**:263-276.

223. **Tugizov, S., Y. Wang, I. Qadri, D. Navarro, E. Maidji, and L. Pereira**. 1995. Mutated forms of human cytomegalovirus glycoprotein B are impaired in inducing syncytium formation. Virology **209**:580-591.

224. **Tugizov, S. M., J. W. Berline, and J. M. Palefsky**. 2003. Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells. Nat Med **9**:307-314.

225. **Turner, A., B. Bruun, T. Minson, and H. Browne**. 1998. Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. J Virol **72**:873-875.

226. **Urban, M., W. Britt, and M. Mach**. 1992. The dominant linear neutralizing antibody-binding site of glycoprotein gp86 of human cytomegalovirus is strain specific. J Virol **66**:1303-1311.

227. **Vanarsdall, A. L., B. J. Ryckman, M. C. Chase, and D. C. Johnson**. 2008. Human cytomegalovirus glycoproteins gB and gH/gL mediate epithelial cell-cell fusion when expressed either in cis or in trans. J Virol **82**:11837-11850.

228. **Vey, M., W. Schäfer, B. Reis, R. Ohuchi, W. J. Britt, W. Garten, H. D. Klenk, and K. Radsak**. 1995. Proteolytic processing of human cytomegalovirus

glycoprotein B (gpUL55) is mediated by the human endoprotease furin. Virology **206**:746-749.

229. **Waldman, W. J., W. H. Roberts, D. H. Davis, M. V. Williams, D. D. Sedmak, and R. E. Stephens**. 1991. Preservation of natural endothelial cytopathogenicity of cytomegalovirus by propagation in endothelial cells. Arch Virol **117**:143-164.
230. **Waldman, W. J., J. M. Sneddon, R. E. Stephens, and W. H. Roberts**. 1989. Enhanced endothelial cytopathogenicity induced by a cytomegalovirus strain propagated in endothelial cells. J Med Virol **28**:223-230.
231. **Wang, D., and T. Shenk**. 2005. Human Cytomegalovirus UL131 Open Reading Frame Is Required for Epithelial Cell Tropism. J Virol **79**:10330-10338.
232. **Wang, D., and T. Shenk**. 2005. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. Proc Natl Acad Sci U S A **102**:18153-18158.
233. **Wang, X., D. Y. Huang, S. M. Huong, and E. S. Huang**. 2005. Integrin alphavbeta3 is a coreceptor for human cytomegalovirus. Nat Med **11**:515-521.
234. **Wang, X., S. M. Huong, M. L. Chiu, N. Raab-Traub, and E. S. Huang**. 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature **424**:456-461.
235. **Wentworth, B. B., and E. R. Alexander**. 1971. Seroepidemiology of infectious due to members of the herpesvirus group. Am J Epidemiol **94**:496-507.
236. **Wiley, C. A., and J. A. Nelson**. 1988. Role of human immunodeficiency virus and cytomegalovirus in AIDS encephalitis. Am J Pathol **133**:73-81.
237. **Wille, P. T., A. J. Knoche, J. A. Nelson, M. A. Jarvis, and D. C. Johnson**. 2010. A human cytomegalovirus gO-null mutant fails to incorporate gH/gL into the virion

envelope and is unable to enter fibroblasts and epithelial and endothelial cells. Journal of virology **84**:2585-2596.

238. **Wills, E., F. Mou, and J. D. Baines**. 2009. The U(L)31 and U(L)34 gene products of herpes simplex virus 1 are required for optimal localization of viral glycoproteins D and M to the inner nuclear membranes of infected cells. J Virol **83**:4800-4809.
239. **Wisner, T., C. Brunetti, K. Dingwell, and D. C. Johnson**. 2000. The extracellular domain of herpes simplex virus gE is sufficient for accumulation at cell junctions but not for cell-to-cell spread. J Virol **74**:2278-2287.
240. **Wright, J. F., A. Kurosky, E. L. Pryzdial, and S. Wasi**. 1995. Host cellular annexin II is associated with cytomegalovirus particles isolated from cultured human fibroblasts. J Virol **69**:4784-4791.
241. **WuDunn, D., and P. G. Spear**. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J Virol **63**:52-58.
242. **Xiao, J., J. M. Palefsky, R. Herrera, and S. M. Tugizov**. 2007. Characterization of the Epstein-Barr virus glycoprotein BMRF-2. Virology **359**:382-396.
243. **Yeager, A. S., F. C. Grumet, E. B. Hafleigh, A. M. Arvin, J. S. Bradley, and C. G. Prober**. 1981. Prevention of transfusion-acquired cytomegalovirus infections in newborn infants. J Pediatr **98**:281-287.
244. **Yu, D., H. M. Ellis, E. C. Lee, N. A. Jenkins, N. G. Copeland, and D. L. Court**. 2000. An efficient recombination system for chromosome engineering in Escherichia coli. Proc Natl Acad Sci U S A **97**:5978-5983.
245. **Yu, D., M. C. Silva, and T. Shenk**. 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. Proc Natl Acad Sci U S A **100**:12396-12401.

246. **Zhu, Z., M. D. Gershon, R. Ambron, C. Gabel, and A. A. Gershon**. 1995. Infection of cells by varicella zoster virus: inhibition of viral entry by mannose 6-phosphate and heparin. Proceedings of the National Academy of Sciences of the United States of America **92**:3546-3550.