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Cytoplasmic assembly of human cytomegalovirus.

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CYTOPLASMIC ASSEMBLY OF HUMAN CYTOMEGALOVIRUS

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ABSTRACT

Human cytomegalovirus (HCMV) is the largest and most structurally complex member of the herpesvirus family. This virus is an important human pathogen, and is a major cause of disease in the immunocompromised host. The assembly of this large DNA virus remains incompletely defined. Capsid assembly of the infectious particle is well studied and conserved for all herpesviruses, whereas the cytoplasmic assembly and final envelopment is poorly understood. Several studies have shown that viral tegument proteins and viral envelope glycoproteins accumulate in the cytoplasmic assembly compartment (AC) late in infection and tegument layer-coated particles bud into this site. These findings suggest that tegument proteins participate in the cytoplasmic phase of HCMV assembly. However, the specific role of tegument proteins in HCMV assembly remains to be determined.

In this study, we have examined the role of a major tegument protein, pp28 in the final envelopment of this virus. We produced recombinant viruses in which pp28 gene was deleted or mutated using a linear recombination system, and characterized growth phenotypes of the recombinant viruses. Our results indicated that localization of pp28 to the AC was essential for the production of infectious virus. Moreover, an investigation of the sequence requirements for intracellular trafficking of pp28 showed that the first 50 aa as well as myristoylation of pp28 were required for its authentic localization within virus-infected cells, and for assembly of infectious virions. Importantly, our characterization of a recombinant virus exhibiting a growth-impaired phenotype provided direct evidence for

the role of pp28 in envelopment and emphasized the importance of postlocalization functions of pp28 in the AC. Finally, we demonstrated that pp28 multimerized within the AC during viral infection. Moreover, the primary sequence requirements for pp28 multimerization within the AC were also necessary for the production of infectious virus. These findings argued that pp28 multimerization within the AC represented an essential step in virus replication. Taken together, our data indicated that further understanding of the role of pp28 in the assembly of the virion would provide important clues about HCMV morphogenesis.

DEDICATION

To my parents.

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
ac	acidic cluster
AC	assembly compartment
ART	active retroviral treatment
BAC	bacterial artificial chromosomes
C-ternimal	carboxy-terminal
DBs	dense bodies
EC	extracellular
EM	electron microscopy
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
EBV	Epstein-Barr virus
FITC	fluorescein isothiocyanate
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
gC	glycoprotein complex
gp	glycoprotein
GST	glutathione S-transferase
HCMV	human cytomegalovirus

HHV	human herpesvirus
HSV	herpes simplex virus
IC	intracellular
IE	immediate early
Ig	immunoglobulin
kb	kilobase
kDa	kilodaltons
mAb	monoclonal antibody
MW	molecular weight
N-terminal	amino-terminal
PACS-1	phosphofurin acidic cluster sorting protein-1
ofr	open reading frame
PBS	phosphate-buffered saline
PP	phosphoprotein
PRV	pseudorabies virus
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TGN	trans-Golgi network
UL	unique long
US	unique short
VZV	varicella-zoster virus

INTRODUCTION

Herpesviridae

Human cytomegalovirus (HCMV) is the largest and structurally most complex member of the herpesvirus family. Herpesviruses are subdivided into three subfamilies, alpha, beta, and gamma, based on the biological properties. The alpha herpesviruses such as herpes simplex virus (HSV) types 1 and 2, varicella-zoster virus (VZV), and pseudorabies virus (PRV), have a short replicative cycle and variable host ranges, and induce cytopathology in monolayer cell cultures. The beta herpesviruses contain all cytomegaloviruses including HCMV as the prototype virus of beta herpesvirus genus, and human herpesviruses 6 and 7. These viruses have a prolonged replicative cycle and restricted host range. The gamma herpesviruses are further divided into the Lymphocryptovirus (Epstein-Barr virus) and Rhadinovirus (human herpesvirus 8, rhesus rhadinovirus) and exhibit a very restricted host range (129).

Herpesvirions have three major structural components, capsid, tegument, and envelope. The 100-nm diameter capsid containing viral DNA consists of 162 capsomers, and its structural features are characteristic of all herpesviruses. The tegument layer represents an amorphous region that lies between the capsid and envelope. The thickness of tegument varies depending on the state of assembly of the virion within the infected cell. The outer virion envelope has a typical trilaminar appearance and appears to be derived from patches of altered cellular membranes. The envelope also contains numerous virus encoded glycoproteins that assume the shape of short spikes in electron

micrographs of cell free virions. The size of herpesvirions varies from 120 to nearly 300 nm. The variation is due to variability in the thickness of tegument and the composition of the envelope. The herpesvirus DNAs are linear and double-stranded in the virion but circularize immediately upon release from capsids into the nuclei of infected cells. The size of the genome of herpesviruses varies from approximately 120 to 230 kbp with HCMV containing the largest genome of all herpesviruses. Herpesviruses also appear to share several unique biological properties. All herpesviruses specify a large array of enzymes involved in nucleic acid metabolism, DNA synthesis, and processing of proteins. The synthesis of viral DNAs and assembly of capsids occurs in the nucleus and production of infectious progeny virus causes the irreversible destruction of the infected cell. Finally, the herpesviruses infections result in virus persistence either through chronic productive infections or as a result of maintainance of a latent infection in their natural hosts. During latency the viral genome takes the form of closed circular molecule, and only few viral genes are expressed. The maintenance of latent infection is not completely understood for most herpesviruses but several mechanisms have been proposed including promoter silencing by methylation, cis acting elements in viral regulatory genes that are recognized by host cell functions, viral transcripts that autoregulate viral gene expression, and most recently micro RNAs (24, 25, 42). In contrast to latency, chronic productive infection is an alternative strategy for virus persistence and is perhaps best illustrated by HCMV. This virus persists for the life of the host and appears to continually replicate, albeit at low levels, as evidenced by recovery of infectious virus from infected hosts and the commitment of a large amount (>10%) of the immunological repertoire for recognition of virion structural proteins (158).

The early events in the replication of herpesviruses are characterized by

attachment to the target cell surface, fusion between viral and cell membrane and entry of the tegumented capsid into the cytoplasm of the host cell. The infection is initiated by attachment of virus to both low affinity glycosoaminoglycans cell surface receptors such as heparan sulfate followed by engagement of high affinity receptors such as the HVEM (herpes virus entry mediator) or CR2 for Ebstein-Barr virus (8, 110, 152, 153). Once attached, fusion of the envelope with the plasma membrane follows. This fusion event is likely dependent on at least two families of conserved glycoproteins, gB and gH/gL/gO for all herpesviruses and four glycoproteins for the alpha-herpesviruses, gB, gH/gL, and gD (an alpha-herpesvirus specific envelope glycoprotein). Once fusion is initiated the envelope dissolves and the partially tegument deenveloped capsid is then transported by minus end microtubules to the nuclear pores where linear DNA is released into the nucleus likely by mechanisms closely related to the injection of nucleic acids by bacteriophage (61, 106, 131). Transcription, replication of viral DNA, and assembly of new capsids take place in the nucleus. Viral DNA is transcribed throughout the reproductive cycle by host RNA polymerase II, but with the participation of viral factors at all stages of infection. The synthesis of viral gene products is tightly regulated and in the case of alpha herpesviruses, accompanied by virion controlled shut down of host cell protein synthesis. Viral gene expression is regulated and sequentially ordered in a cascade fashion with autoregulation of viral gene expression and in some cases feedback regulation by products of viral genes whose expression is initiated by expression of genes upstream in the transcription program. Several of the gene products are enzymes, including the viral DNA polymerase and DNA binding proteins involved in viral DNA replication such as viral proteins with helicase/primase activity as well as terminase activity. The bulk of viral DNA is synthesized by a rolling circle mechanism, yielding

concatemers which must be cleaved into monomers and packaged as linear dsDNA into capsids. After packaging of DNA into preassembled capsids, the nucleocapsid buds through the nuclear membrane and acquires tegument and envelope in intracellular sites by poorly elucidated mechanisms (115, 116). Common to most descriptions of the mechanisms(s) employed by the virus to exit the nucleus is a modification of the supporting lamin structures of the nuclear envelope, usually by phosphorylation of nuclear lamins and dissolution of the nuclear cytoskeleton (120). Budding through the inner and outer leaflet of the nuclear membranes has not been adequately investigated, but contemporary findings have suggested that interactions of newly formed capsids with at least two nuclear viral proteins results in the accumulation of viral capsids at the periphery of nucleus and eventually the envelopment and budding of capsid into the cytoplasm. Once in the cytoplasm the subviral particle acquires additional tegument protein and then undergoes a secondary envelopment with viral glycoproteins in the cytoplasm in proximity to the TGN or late endosomal compartment. Once enveloped, the particle most likely leaves the cell through exocytosis or as the result of virus lysis of the infected cell (Fig. 1).

HCMV clinical features

HCMV is an important human pathogen and is a major cause of disease in the immunocompromised host. In the normal, immunocompetent host infection with HCMV is rarely symptomatic and although over 60% of the US population has been infected with this virus, only a small number of people develop symptoms from acute infection with HCMV (17, 20). It has been estimated based on limited epidemiological information that up to 10% of primary HCMV infections in older children and adults will manifest as

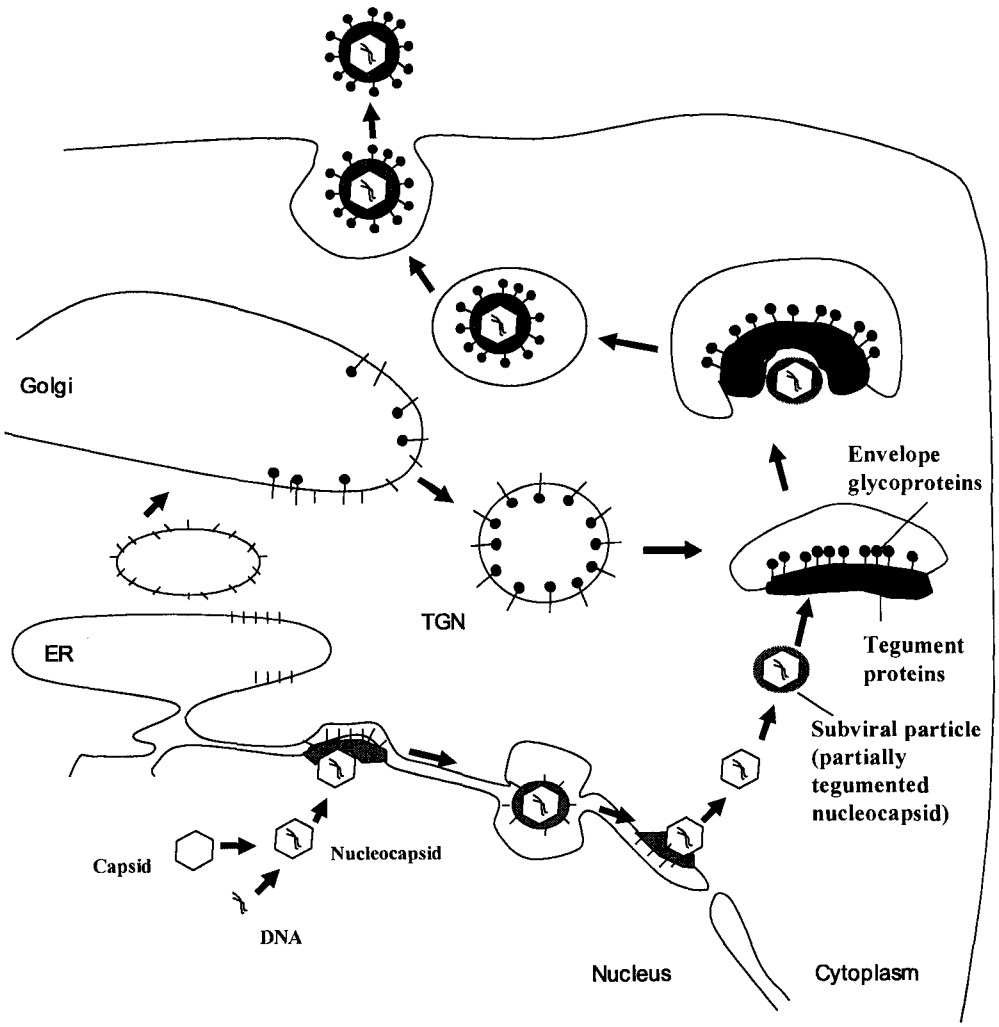


FIG. 1. Summary diagram of the proposed pathway of herpesvirus egress.

a mononucleosis syndrome that can be indistinguishable from that of EBV mononucleosis, a much more common entity (17, 20). These patients characteristically have fever, malaise, atypical lymphocytosis, pharyngitis and, rarely, cervical adenopathy or hepatitis. The disease is almost always self-limited and currently there is little evidence supporting the treatment of this syndrome with antiviral medications.

In the developmentally immunocompromised newborn infant, HCMV can cause very serious and not infrequently life threatening symptomatic infections (154). Congenital (present at birth) HCMV infection occurs in approximately 1% of all live births in the United States and represents the most common intrauterine infection in the US. Approximately 10% of congenitally infected children will have severe congenital infection that can result in significant end-organ disease and death in about 16% of infected babies (154). Moreover, about 10–17% infants with congenital CMV infections will develop hearing deficits ranging from mild to profound deafness and between 5-10% of infected infants will exhibit neurodevelopmental sequelae including microcephaly, encephalitis, seizures, deafness, psychomotor retardation, and, rarely, myopathy and choroidoretinitis (11, 43, 57, 154). It is important to note that this infection is transmitted from infected pregnant mother to her offspring during intrauterine life and that only very rarely is the infected woman symptomatic. Thus, the study of the maternal infection associated with congenital CMV infection has been difficult and most information comes from observational studies of populations of women during pregnancy.

HCMV also is a leading cause of post-transplantation infections in the pharmacologically immunosupressed host (17, 20). More recently, HCMV infections have been associated with allograft rejection and in cardiac allografts, restenosis of the engrafted coronary vasculature (52, 72, 82, 111). Allograft recipients at risk for severe

disease associated with HCMV include solid organ transplant recipients, particularly heart and heart/lung transplant recipients and bone marrow allograft recipients. In addition, recent findings have strongly implicated HCMV infections as a leading contributor to heart allograft rejection secondary to HCMV's unique interactions with the vasculature and cells of the inflammatory response.

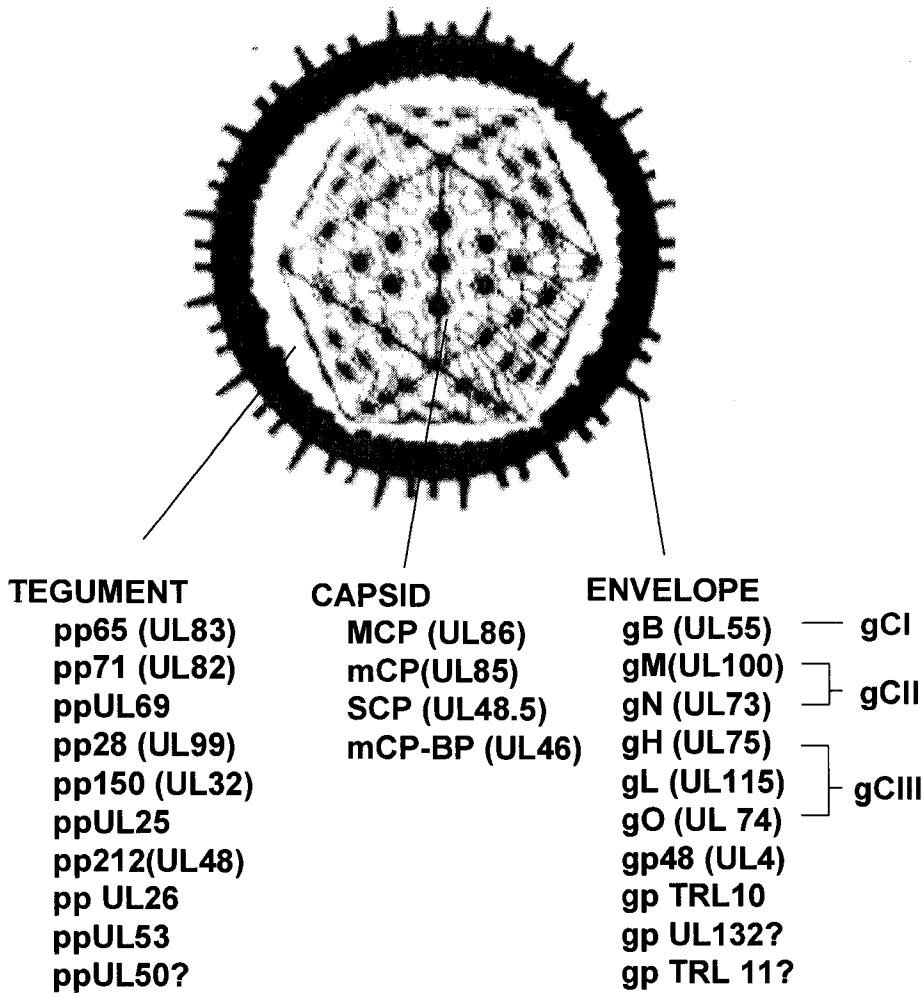
Individuals with human immunodeficiency virus (HIV) infection are also at higher risk for severe HCMV infection. The seroprevalence for HCMV is extremely high in this population exceeding 95% in communities of gay men (48). Infections with HCMV in these patients can result in severe acute disease or more chronic manifestations such as wasting disease. A particularly devasting manifestation of HCMV infection in this population is the development of retinitis that can rapidly lead to blindness. This disease represents a vasculitis with inflammatory cell infiltrates translocating into the retina followed by retinal edema, loss of retinal integrity and vasculature supply (59, 78, 84). Other clinical diseases associated with HCMV infection in HIV infected patient include gastrointestinal disease and more rarely, CNS disease. In the past, at one time, HCMV was the leading opportunistic infection in long-lived patients with HIV until the widespread utilization of ART (94, 122, 162). Although two licensed agents, foscarnet and ganciclovir, have shown clinical efficacy in immunocompromised transplant patients and HIV infected patients with HCMV disease, both exhibit dose related toxicity (33, 41, 49, 53, 101, 121, 137). Neither drug is licensed for use in children or in pregnant women. Safer and more efficacious therapeutic agents are needed for the treatment of invasive HCMV infections and with no prospects of an effective vaccine on the horizon, antiviral therapy may be the most promising approach for the prevention of sequelae from congenital infections. Development of these therapies is dependent on our understanding

of the various aspects of viral life cycle such as viral replication and assembly.

HCMV genome and structure

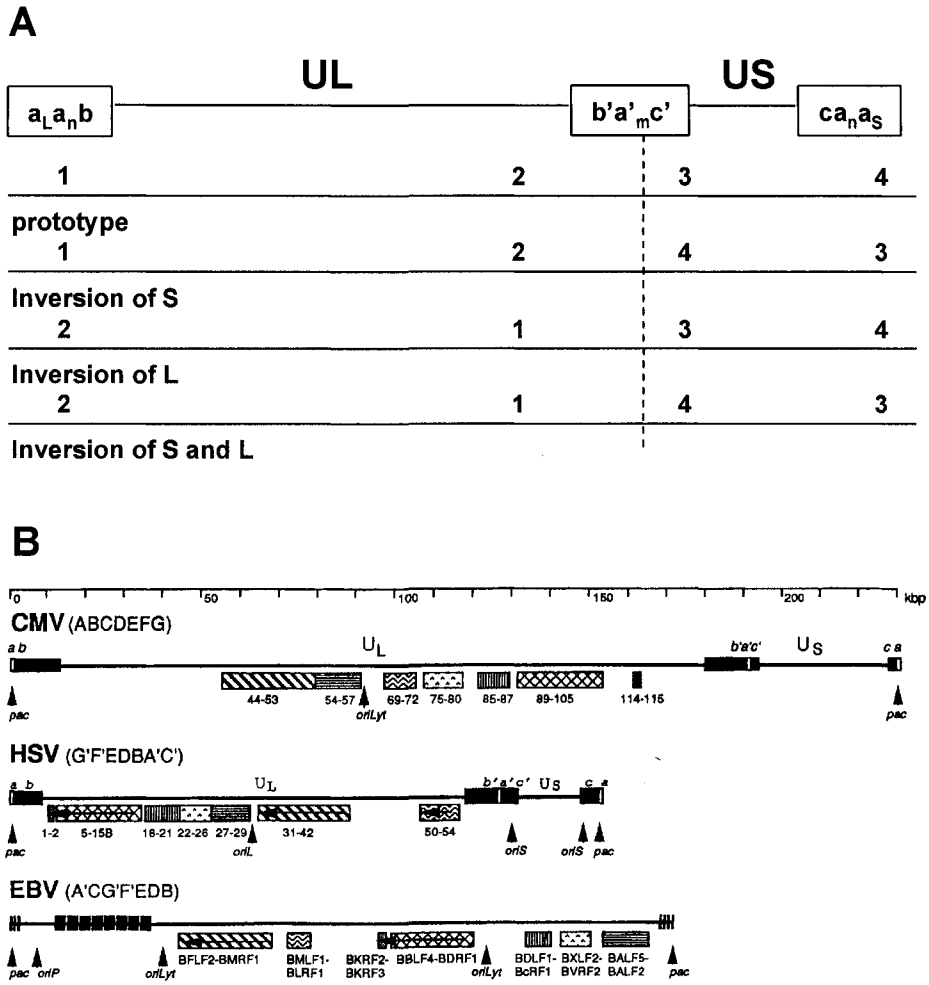
The virion of HCMV consists of a 100-nm diameter icosahedral nucleocapsid containing a 230-kbp, double-stranded linear DNA genome that may encode over 200 open reading frames (ORFs), surrounded first by a tegument layer and then by an envelope containing a large number of viral glycoproteins (Fig. 2) (98). The virion contains a minimum of 71 virus encoded proteins and large number of host proteins (164).

Virus genome. HCMV contains an arrangement of unique long (UL), unique short (US), and repeat regions. Since each long and short region can be oriented in either direction, four genome isomers are produced in viral progeny (Fig. 3). Inversion of UL and US regions is mediated by direct (a, b, c) and/or inverted (a', b', c') repeat sequences which contain the cis-acting pac (packaging) elements needed for DNA cleavage (Fig. 3) (117). The genome has been subdivided into seven conserved sequence blocks (A-G) relative to the genomes of the other herpesviruses (Fig. 3). These conserved blocks suggest that the functions encoded are probably conserved in all herpesviruses. Moreover, comparison of the amino acid (aa) sequences encoded by HCMV genome with those of other herpesviruses has revealed that the protein products of more than 40 ORFs share high similarity to proteins encoded by other herpesviruses (28, 92). However, many ORFs remains to be functionally characterized. Analysis of the phenotypes of virus-bearing deletions or inactivation at specific loci, has indicated that the products of more than 50 HCMV ORFs are dispensable for productive replication in tissue culture suggesting that many of these dispensable ORFs have important roles in in-vivo growth



Note: Adapted from "Herpesviridae" by Roizman, B. 1996. Fields Virology, Third Edition. p. 2222. Copyright 1996 by Lippincott-Raven. Adapted with permission.

FIG. 2. Schematic representation of HCMV virion structure. The virion consists of three structures: an icosahedral capsid containing a double-stranded linear DNA genome, an amorphous tegument, and a lipid envelope containing a large number of viral glycoproteins. Experimetally defined components of each structure are listed. gCI, gCII, and gCIII indicate three complexes of six major viral glycoproteins.



Note: Adapted from "Cytomegaloviruses and their replication" by Mocarski, E. S. 1996. Fields Virology, Third Edition. p.2454. Copyright 1996 by Lippincott-Raven. Adapted with permission.

FIG. 3. Schematic representation of the genomic sequences of HCMV. (A) Structure of the four HCMV genome isomers. The genome structure is shown on the top line with unique sequences (UL and US) flanked by inverted repeats (boxed areas). The four lines below the depiction of the HCMV genome show the four genome isomers. (B) Conserved blocks of sequence between HCMV, HSV-1, and EBV. The top line is a scale of kilobase pairs. The second line depicts the prototype arrangement of the CMV genome with conserved sequence blocks (A-G) shown below the UL genome segment. The third line depicts the prototype arrangement of the HSV-1 genome, and the fourth line depicts the EBV genome, each with conserved sequence blocks shown below. Numbers below the sequence blocks indicate the ORFs contained within each block. Leftward pointing arrows within blocks below the HSV-1 and EBV genome denote the inverted orientation of the blocks relative to CMV. A lytic origin of DNA replication (oriLyt) is indicated on the HCMV genome, and packaging signals (pac) are indicated near the genomic termini.

and pathogenesis.

The capsid. The capsid is constituted by four capsid proteins, the major capsid protein (MCP, pUL86), two minor capsid proteins (mCP, pUL85 and mC-BP, pUL46), and small capsid protein (SCP, pUL48.5 or pUL48/49). Three assembly proteins encoded by UL80 also associate with capsid and play roles in maturation. The capsid consists of a 162 capsomer shell which is composed of hexameric and pentameric units of pUL86 located at the vertices of a T = 16 icosahedral lattice, where adjacent capsomers are joined by surface structures produced by the association of pUL46 and pUL48.5. The mechanism by which this process occurs will be discussed in later sections.

The tegument. This amorphous layer contains approximately 30 virus-encoded proteins (1, 5, 31). Several of the major tegument proteins are phosphoproteins (pp), including pp150, pp71, pp65, and pp28 encoded by UL32, UL82, UL83, and UL99, respectively. The functions of most tegument proteins have not been completely elucidated. However, the tegument proteins may influence viral and cellular events in the early or late stages of infection. For example, the pp65 protein has been reported to block major histocompatibility complex (MHC) class I presentation of a viral immediate-early protein (68). The UL47 protein acts during disassembly of the newly infecting virus particle (7), and the pp71 and UL69 proteins are transactivators that help to activate the viral genes within infected cells, and dysregulate host cell cycle progression (4, 15, 76, 100, 105). Recently, it has been reported that pp71 relieves hDaxx-mediated repression of HCMV IE gene expression and replication (26, 27).

The envelope. The phospholipid envelope contains 6 virus-encoded glycoproteins: gB, gN, gO, gH, gM, and gL encoded by UL55, UL73, UL74, UL75, UL100, and UL115, respectively. These glycoproteins play essential roles in virus entry into host cells, cell-to-cell spread, and virion maturation (21). These six major viral glycoproteins associate to form three complexes that are highly conserved within herpesviruses and have been designated as gCI, gCII, and gCIII (73). The gCI is composed of homodimeric gB molecules. gB plays a role in virus binding, since it is the major cell surface, heparan sulfate proteoglycan-binding glycoprotein (160). It also participates in viral entry, cell-to-cell spread, and cell fusion. gCII results from the association of gM and gN (91). gCIII as a heteroligomeric complex is composed of gH, gL, and gO (160). gCIII is necessary for the final stage of virus entry via pH-independent fusion between the viral envelope and the cell membrane (Fig. 2) (83).

HCMV replicative cycle

Virus attachment and penetration. Viral entry results from a cascade of interactions between viral and cellular proteins that culminate in fusion of the virion envelope with the cellular plasma membrane. Like other herpesviruses, HCMV attaches to the cell surface by binding of gB to heparan sulfate proteoglycans at low-affinity (34). A more stable binding state is acquired by the subsequent interaction of gB with its nonheparin receptor. However, it is believed that the final fusion of the virus and cell membranes requires a further priming event mediated by the heteroligomeric gHgLgO complex with as yet unidentified receptors (160). After viral attachment and penetration, the deenveloped nucleocapsid and tegument proteins in the host cytoplasm are rapidly translocated into the nucleus where transcription and replication of the genome occurs.

Viral entry takes place in both permissive and nonpermissive cell types. However, a post-penetration block to viral gene expression restricts replication in nonpermissive cells (146).

Viral gene expression. The HCMV genome is expressed in a temporally and spatially regulated cascade of transcriptional events that lead to the synthesis of three categories of viral proteins, immediate-early (IE), early (E), and late (L) proteins (Table 1 and Fig. 4). HCMV genes are transcribed by RNA polymerase II and the associated basal transcription machinery with the intervention of host-encoded transcription factors which may be activated by viral transactivators (56, 119).

The immediate-early (IE) gene expression. The IE gene expression initiates from a restricted set of IE proteins without de novo protein synthesis. The IE genes include the major IE genes, UL122/123 (IE1 and IE2) and auxiliary genes such as UL36–UL38, UL115–UL119, IRS1/TRS1, and US3 (Table 1). The major IE region produces a set of exon 4 (UL123) and 5 (UL122). The IE1 and IE2 proteins are required for subsequent expression of viral genes and regulate the expression of a large number of host cell genes (56). The IE1 protein is able to positively autoregulate expression of the UL122/123 and US3 genes (118). IE1 also cooperates with IE2 to regulate the expression of subsequent viral genes, E (UL44 and UL54) as well as L (UL83) genes (56). In addition, IE1 stimulates the activity of several cellular promoters such as those of dihydrofolate reductase and thymidylate synthase genes (74, 165). The IE2 protein, a nuclear phosphorylated polypeptide, is critical for viral replication. It regulates the expression of E and L genes, and the transition from the IE to the E and L phases of HCMV gene

TABLE 1. HCMV genes discussed in the dissertation.

Gene	Kinetic class	Protein name(s)	Function(s)
UL36	IE	pUL36	Inhibition of apoptosis
UL37	IE	vMIA	Inhibition of apoptosis
UL122	IE	IE1	Regulation of viral and host gene expression
UL123	IE	IE2	Regulation of viral and host gene expression
IRS1	IE	pIRS1	Transactivator of viral gene expression
US3	IE	gpUS3	Down-modulation of MHC Class I expression
TRS1	IE	pTRS1	Transactivator of viral gene expression
UL44	E-L	ppUL44, p52	DNA processivity factor
UL46	E-L	mC-BP	Minor capsid-binding protein, capsid structure
UL54	E	DNA pol, pUL54	DNA polymerase
UL55	E	gB	Major envelope glycoprotein, constituent of gCI
UL57	E	pUL57, ssDNA BP	Single-stranded DNA-binding protein
UL69	E-L	ppUL69	Transactivator, dysregulation of host cell cycle
UL70	E-L	Helicase-primase	Subunit of the helicase-primase complex
UL84	E-L	ppUL84	Initiation of oriLyt-specific DNA replication
UL85	E-L	mCP	mCP, capsid structure
UL97	E-L	pUL97	phosphotransferase, GCV-activating enzyme
UL102	E	Helicase-primase	Subunit of the helicase-primase complex
UL105	E	Helicase-primase	Subunit of the helicase-primase complex
UL112	E	Early pp family	Organization of viral DNA replication centers
UL113	E	Early pp family	Organization of viral DNA replication centers
UL114	E	pUL114	Uracil DNA glycosylase
US2	E	gpUS2	Down modulation of MHC Class I expression
US11	E	gpUS11	Down modulation of MHC Class I expression
US27	E	pUS27	Similar to glucocorticoid receptors
US28	E	pUS28	C-C chemokine receptor, immune evasion
UL32	L	pp150, ppUL32	Major tegument component, basic phosphoprotein
UL48.5	L	SCP	Smallest capsid protein, capsid structure
UL73	L	gN	Envelope glycoprotein, constituent of gCII
UL74	L	gO	Envelope glycoprotein, constituent of gCIII
UL75	L	gH	Envelope glycoprotein, constituent of gCIII
UL80	L	Assemblin precursor	Assemblin protease (pUL80a), capsid assembly
UL80.5	L	AP	AP scaffolding protein, capsid assembly
UL82	L	pp71, ppUL82	Transactivator, dysregulation of host cell cycle
UL83	L	pp65, ppUL83	Major tegument component, lower matrix protein
UL86	L	MCP	MCP, capsid structure
UL94	L	pUL94	Virion protein?
UL99	L	pp28	Tegument protein
UL100	L	gM	Envelope glycoprotein, constituent of gCII
UL115	L	gL	Envelope glycoprotein, constituent of gCIII

expression. The IE2 protein functions as a strong transcriptional regulator by either stimulating or repressing both HCMV and cellular genes (56, 119, 151). It mediates

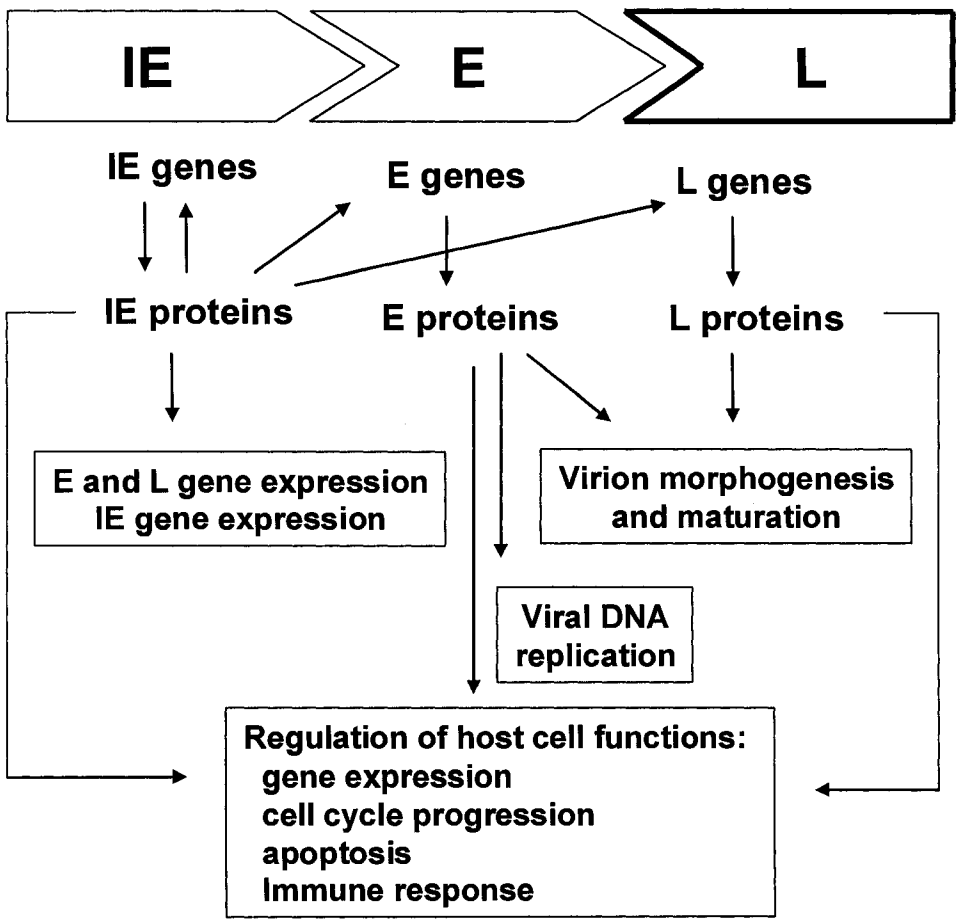


FIG. 4. HCMV gene expression and viral gene product functions during infection.

autoregulation of its own expression and reduces IE gene expression in the late stages of infection. IE2 protein also modulates several cell processes such as host cell cycle and apoptosis (90, 172). It is able to arrest cells and thus synchronize into the late G1/early S phase and inhibit host DNA synthesis (14, 47, 86, 104, 133). These modifications lead to a favorable environment for viral DNA replication, in which the precursors for DNA synthesis produced by the host are available for viral replication, but are not utilized for the host DNA replication (55, 90). The other IE genes are involved in heterogeneous functions. UL36 and UL37 encoded by UL36-38 genes are associated with antiapoptotic properties and play a role in the caspase cascade (69, 147). TRS1 and IRS cooperate with IE1 and IE2 in the transactivation of early gene promoters. The glycoproteins encoded by US3 are involved in the establishment of immune evasion in infected cells. The glycoproteins, which localize in endoplasmic reticulum (ER), prevent the transport of the assembled MHC class I from ER to the Golgi and thus, down-modulate MHC class I antigen presentation (89, 155). UL36, UL37, IRS1, and US3 are all dispensable for replication in cell culture.

The early (E) gene expression. Transcription of E genes is stimulated by IE2 alone or in cooperation with IE1 through transactivation and is unaffected by inhibitors of viral DNA replication (56). In addition, expression of several E genes is regulated by both transcriptional and post-transcriptional mechanisms (119). The E genes encode mostly non-structural proteins, including viral DNA replication factors, repair enzymes, and proteins involved in immune evasion (119). The UL54 encoded protein is the viral DNA polymerase and the UL44 gene product acts as a polymerase processivity factor (119). However, several other E proteins are involved in establishment of immune

evasion. The glycoproteins encoded by US2 and US11, bind the MHC Class I heavy chains and transport them from the ER into the cytosol where they are degraded by the proteasome (143, 157). The US27 and US28 ORFs have homology to the CC chemokine receptors (29). Moreover, US28 alone prevents elimination of HCMV infected cells by chemokine-activated immune cells, since it is a receptor for the CC chemokines RANTES and monocyte chemoattractant peptide-1 and thus, sequesters them from the extracellular environment by internalization (9, 62). Unlike the genes in the UL region, most US genes are E genes (Table 1).

The late (L) gene expression. Late gene expression leads to the synthesis of two subclasses of L proteins (leaky and true late) in accordance with their time of expression and sensitivity to viral DNA replication inhibitors. Leaky late transcription occurs 24–36 hrs post infection, and is reduced by replication inhibitors. True late transcription occurs 24–48 hrs post infection, and is strictly dependent on DNA replication. The transcriptional regulation of L genes is little known. The L proteins have mainly structural roles and primarily contribute to the assembly and morphogenesis of the virion (Table 1) (119).

Viral DNA replication. HCMV genome replication, inversion, and packaging take place in the nucleus. Viral DNA synthesis requires the activities of several viral and cellular proteins (119). Unlike other herpesviruses, the CMV genome does not encode deoxyribonucleotide biosynthetic enzymes. Thus, the virus has developed strategies to stimulate the expression of cellular enzymes for DNA precursor synthesis. Moreover, CMV-infected cells fail to undergo cellular DNA replication and division as a result of

blocks in cell cycle progression that prevent the host DNA replication machinery from competing with the virus for access to DNA precursors (55, 90). This favorable environment is crucial for its productive replication.

The HCMV genome has six herpesvirus-conserved ORFs encoding the core replication proteins for viral DNA replication (Fig. 5). The ppUL57 single-stranded DNA-binding protein prevents the reannealing of DNA strands following unwinding by the helicase-primase complex which is composed of three subunits encoded by UL70, UL102, and UL105, the DNA polymerase encoded by UL54 and the DNA polymerase processivity factor UL44 that prevents dissociation of UL54 from the template (119). Replication also requires other viral proteins for efficient DNA replication. A phosphoprotein encoded by UL84 stably interacts with IE2 and stimulates the viral origin-dependent DNA synthesis (136). The phosphoproteins encoded by UL112/113 recruit the core replication proteins to replication centers, subnuclear sites of HCMV DNA synthesis (2, 125). Finally, the protein encoded by UL114 expresses a functional uracil DNA glycosylase activity that is required for efficient viral DNA replication in post-mitotic cells (38).

HCMV DNA replication proceeds through initial circularization of the input genome followed by DNA synthesis via a bidirectional θ mechanism from a single origin of replication that undergoes a switch to a late-phase rolling circle form of DNA replication (119). During the late stages of infection, the rolling circle form is responsible for most of the viral DNA produced in the form of large concatemeric replicating units lacking terminal fragments that are subsequently cleaved into lengths that can be encapsulated. During the late stages of viral DNA replication, newly synthesized genomes mature through their inversion, cleavage, and packaging (113). Inversion occurs in concatemeric units and leads to the generation of progeny genomes as a pool of four

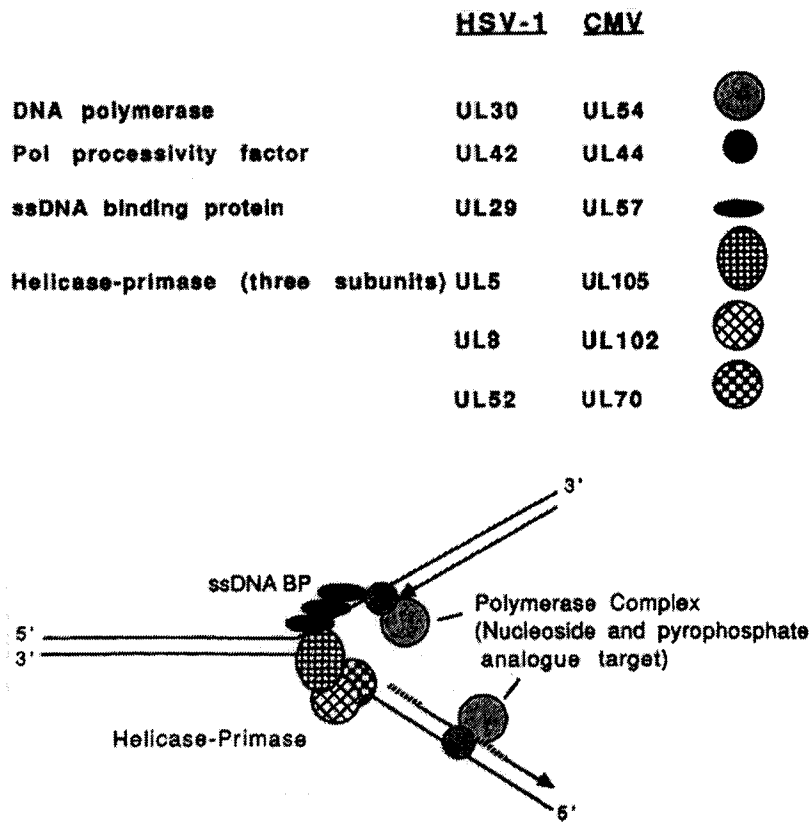


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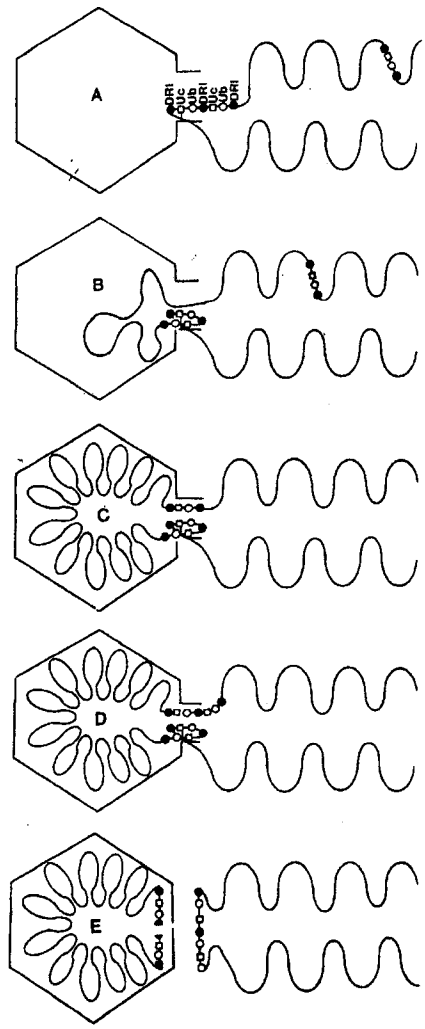
FIG. 5. DNA replication fork functions in HSV-1and HCMV. Top: List of polypeptides known to be a part of the HSV-1 replication fork and HCMV ORFs with functional or sequence similarity to these replication functions. All of these are contained in the set of viral trans-acting factors necessary to direct oriLyt replication. The predicted viral replication fork shows DNA strands as parallel lines. The two polypeptide polymerase complex (UL54 and UL44) is depicted in its expected roles carrying out leading (upper) strand and lagging (lower) strand synthesis and as an RNAseH in removing RNA primers (hatched lines) from the lagging strand. The three-polypeptide helicase-primase is depicted in its likely roles of unwinding DNA ahead of the polymerase and synthesizing RNA primers to prime lagging strand DNA synthesis.



Note: Adapted from "Herpes simplex viruses and their replication" by Roizman, B., and A. E. Sears. 1996. Fields Virology, Third Edition. p.2254. Copyright 1996 by Lippincott-Raven. Adapted with permission.

FIG. 6. Packaging of herpesvirus DNA. The model requires that proteins attach to components of the a sequence, probably Uc; empty capsids scan concatemeric DNA until contact is made in a specific orientation with the first protein-Uc sequence (capsid A); the DNA is than taken into the capsid B until a "head full" or contact is made with an a sequence whose nucleotide arrangement is in the same orientation (i.e., one genome equivalent in length away) is encountered (capsid C); the packaging signal requires nicking of both strands from signals on opposite sites of a DR1 sequence. In the absence of two adjacent a sequences (capsid D), the juxtaposition of the a sequences would results in duplication of the a sequence (capsid E).

isomers as described above (Fig. 3). Packaging of the genome into preformed B capsids then follows its cleavage at the essential cleavage/packaging signals, pac1 and pac2 near the repeated a sequence to release unit length DNA from its replicating form (Fig. 6) (119).

HCMV morphogenesis: assembly, maturation, and egress

HCMV infected cells produce infectious virions that consist of capsid, tegument, and envelope. In addition, the infected cells produce another two types of morphological particles, noninfectious enveloped particles and dense bodies (DB). Noninfectious enveloped particles are composed of enveloped immature capsids (B capsids) that lack DNA, but contain the viral scaffolding/assembly protein absent from mature nucleocapsids (C capsids). DB are enveloped particles that lack an assembled nucleocapsid and viral DNA, but contain several tegument proteins and capsid proteins.

The assembly of HCMV is a multistage and poorly understood process. The proposed models of capsid formation and envelopment are based on studies of alpha herpesviruses, HSV, VZV, and PRV (115). Although proposed models include steps of capsid assembly within the nucleus of an infected cell, the mechanism for acquisition of the final envelope remains contentious.

Nuclear phase. Formation of HCMV capsids and packaging of viral DNA occur in the nucleus. Nucleocapsid particles accumulate and form a nuclear inclusions that confer the typical ''owl's eye'' cytopathic effect of the infected cell nucleus. Initially, MCP (pUL86) interacts with a assembly protein precursor (pAP) which is generated from the carboxyl portion (UL80.5) of UL80 derived capsid protein by autocatalytic cleavage

events mediated by a serine-like maturational protease, assemblin (UL80a) in the cytoplasm. Assemblin is itself embedded in the amino-terminal portion of UL80. Subsequently, MCP-pAP complexes are translocated into the nucleus (Fig. 7) (67), where these complexes are oligomerized leading to the formation of hexons and pentons that interact with mCP-mCP-BP (pUL85-pUL46) complexes. The capsid intermediates are subsequently associated with SCP (pUL48.5) to form the B capsid precursor shell (preB-capsid). The maturational cleavages of internal precursor proteins (pAP and assemblin precursor) convert preB- to B-capsid (Fig. 7). The maturational cleavages sever the C-terminal tails from the pAP and assemblin precursors, thereby dissociate MCP from pAP, and separate the assemblin and nonproteolytic halves of the assemblin precursor (67). The cleaved pAP is eliminated from B-capsids and DNA is packaged to form the nucleocapsid (C-capsid) (Fig. 7). Although it has been known that B-capsids package viral DNA, it has been reported that in absence of DNA packaging, B-capsids accumulate (99, 144), compatible with them being assembly intermediates, suggesting that the processes of scaffold elimination and DNA packaging are coupled events. In addition, DNA that is not stably packaged appears to be lost from nascent nucleocapsids giving rise to A-capsids, which have no assembly protein (Fig. 7). It remains to be determined whether the assembly protein is eliminated from B-capsids prior to DNA packaging. Finally, after packaging DNA, the nucleocapsids mature into virions.

Cytoplasmic phase. Whereas capsid assembly is relatively well understood, the mechanism for acquisition of the final envelope has been controversial. Two alternative pathways have been proposed for herpesvirus maturation (115). In one model, the capsid buds through the inner nuclear membrane and then travels through the secretory pathway,

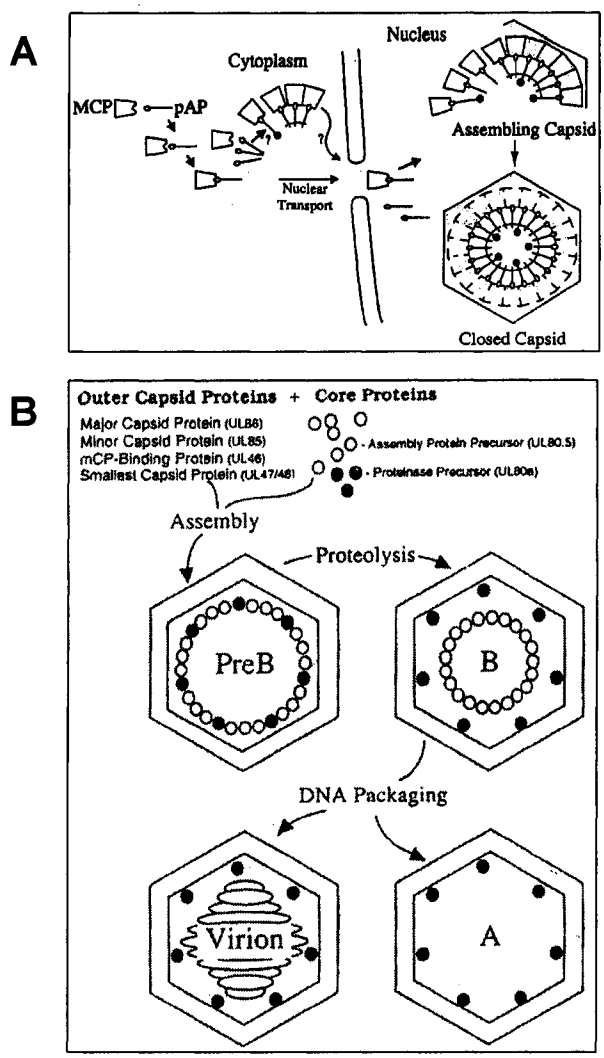


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FIG. 7. Herpesvirus capsid assembly pathway. (A) Cytoplasmic formation of pAP-MCP complexes. Shown here a hypothetical pathway in which MCP interacts with pAP to form nuclear transport complexes. MCP-pAP dimmers are shown as minimal transport complexes. An alternate pathway leading to the formation of larger MCP-pAP complexes (e.g., protocapsomer) is indicated by upper set of arrows. Open oval at end of pAP represents C-terminal tail; closed circle represents assemblin precursor. (B) Working model of capid assembly pathway. Six viral proteins (shown at top) associate to form the preB-capsid. Conversion of preB-capsids to B-capsids involves proteolytic cleavage of the pAP (open circles) and the proteinase (assemblin) precursor (closed circles), and results in conformational changes in the particle. The cleaved pAP is eliminated from B-capsids and DNA is packaged to form the nucleocapsid (C-capsid). A-capsids arise as probable abortive products of the DNA packaging step.

eventually exiting the cell with an envelope derived from the inner nuclear membrane. In the second model, nucleocapsids undergo a sequential envelopment and de-envelopment crossing the nuclear membrane to release the capsid into the cytoplasm (Fig. 1). Initially, nucleocapsids are enwrapped by budding at the nuclear membrane, where they acquire a primary envelope derived from its inner leaflet (67). The enveloped nucleocapsids then cross the lumen, fuse with the outer leaflet of the nuclear membrane or the ER membrane, lose their primary envelope, and move into the cytoplasm. Here, the de-enveloped nucleocapsids further mature by reenvelopment process in the cytoplasm (Fig. 1) (115, 119). They are associated with tegument proteins in the cytoplasm. The tegumented capsids then acquire their envelope by budding into a cytoplasmic compartment whose membrane is modified with viral envelope proteins (67, 140), which is a juxtanuclear structure proposed to be the final assembly site (134). Both tegumentation and reenvelopment are driven by multiple specific protein-protein interactions (115). These mature particles are retained within the assembly site, transported to the cell surface, and released into the extracellular compartment via an exocytotic-like pathway (Fig. 1). Recently, evidence from several sources have favored the envelopment/de-envelopment model. Electron microscopic studies of HSV morphogenesis have visualized the movement of its capsid from the nucleus to cytoplasm by sequential envelopment and de-envelopment as it passes through the inner and outer nuclear membranes (148). Extracellular particles were shown to contain tegument proteins, which were not present in particles found in the perinuclear space (156). Moreover, recent studies of HSV, PRV, and VZV morphogenesis using EM, inhibitors of protein transport in the secretory pathway, and analysis of carbohydrate modifications have provided evidence consistent with envelopment and de-envelopment at the nuclear envelope, followed by final

envelopment in a cytoplasmic compartment (22, 70, 71, 75, 87, 115, 148, 166, 173). Definitive findings suggesting a cytoplasmic site of envelopment were derived from the study of a triple deletion PRV mutant lacking gM, gE, and gI. This virus cannot assemble an enveloped virus particle, and non-enveloped capsids together with tegument proteins accumulated in the cytoplasm of infected cells (13). Finally, a substantial portion of the tegument proteins is acquired by the maturing virion in the cytoplasm. These and other findings suggested that the final envelopment of herpesviruses likely takes place in the cytoplasm.

Assembly compartment (AC)

Previous studies from our laboratory initially identified a juxtanuclear compartment in which virion structural proteins accumulated during infection and final envelopment appeared to take place (134). This juxtanuclear structure was termed the assembly compartment (AC). It is believed that this compartment is comprised of modified elements of the secretory apparatus. Initial studies indicated that the AC was in proximity to the TGN but excluded well defined markers of the TGN, did not contain lysosomal markers and was not an aggresome (134). Other reports have suggested that the AC is comprised of TGN derived vacuoles (81, 85). More recently, compelling data has indicated that enveloped HCMV can be found in (late) endosome derived multivesicular bodies (MVBs) (58). In agreement with this report, we have demonstrated that the luminal late endosome marker CD63 co-localized with HCMV tegument protein pp150 within the AC and that Rab7, a Rab found in late endosome following maturation from early endosomes, was also found in the AC (Fig. 8). These findings were consistent with an older study that demonstrated virion assembly in cellular compartments derived

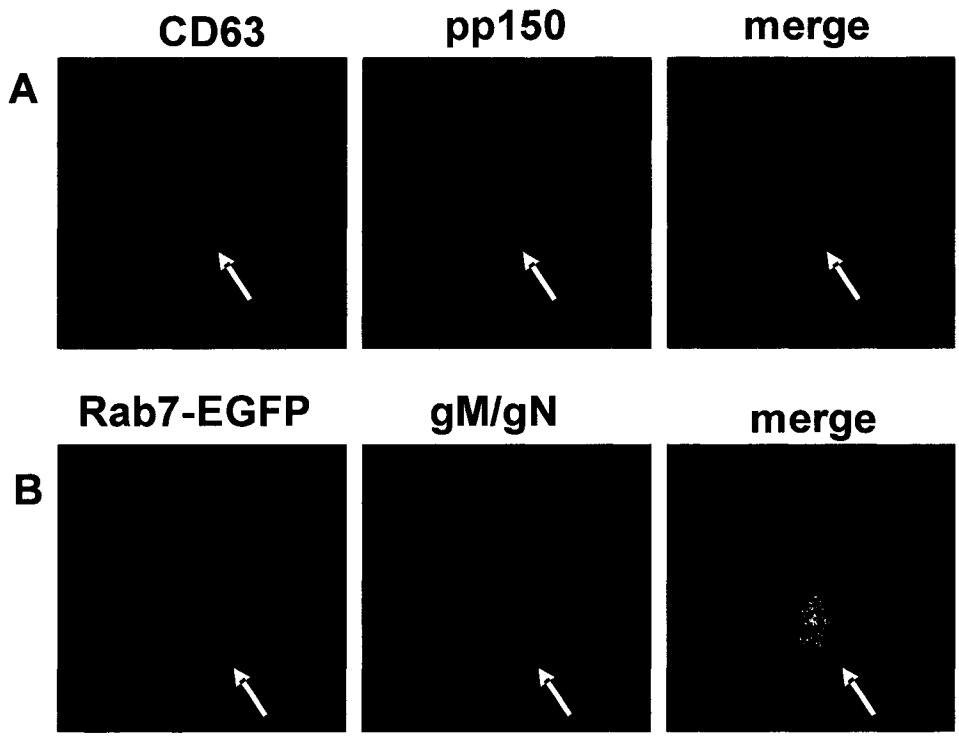

FIG. 8. Co-localization of markers of late endosome/MVBs with viral proteins in the AC virus infected cells. (A) Co-localization of CD63 with pp150 in the AC virus infected cells. Cells are stained with anti-CD63 mab and anti-pp150 mab followed by FITC- and TRITC-labeled anti-mouse IgG. (B) EGFP-Rab7 transiently expressed in infected cells and AC detected with anti-gM/gN mab and TRITC-labeled anti-mouse IgG (red). Arrows depict AC and demonstrate co-localization.

from endosomes (161). Thus, it appeared that the previously defined AC was derived from late endosomes localized to a juxtanuclear position in infected cells.

HCMV cytoplasmic assembly in terms of tegumentation

The cytoplasmic assembly to acquire the final envelope has been an area of intense investigation for many years. Immunofluorescence and electron microscopic studies have shown that viral tegument proteins and viral envelope glycoproteins accumulated in the AC late in infection (134), and tegument-coated particles bud into this AC (67, 140). Thus, it is possible that one or more virion tegument proteins participate in this intracellular budding event. Consistent with this, early ultrastructural studies of HSV and HCMV noted that nonenveloped cytoplasmic particles in HCMV-infected cells were coated with a thick tegument layer but that nonenveloped HSV particles often had the appearance of naked capsids (150). These and other findings suggested that tegument proteins play a key role in the cytoplasmic phase of HCMV assembly. Thus, understanding the assembly of the virion in terms of tegumentation of the particle will likely provide important clues about the assembly pathway.

The role of tegument proteins in the viral assembly. The structure of the tegument of HCMV remains undefined. Recent cryoelectron microscopic studies of HCMV have suggested that tegument formation requires an intact capsid to direct virion tegumentation (30, 112, 128). Some tegumentation is thought to take place in the nucleus because tegumented particles were seen in both the nucleus and cytoplasm (150). However, heavily tegumented capsids are found in the cytoplasm of HCMV infected cells (150). Thus, the tegument appears to be formed by the sequential addition of proteins, first in

the nucleus and then in the in cytoplasm. The role of individual tegument protein in the maintenance of the structure of the virion remains to be determined. However, the role of tegument proteins in HCMV assembly is proposed through that of tegument or matrix layers in the assembly of structurally less complex viruses, such as small RNA viruses whose assembly is more well understood.

Studies have shown that the retroviral matrix protein, gag protein alone is sufficient to organize the particle for budding, but envelope protein and gag protein interactions are required for infectious particle formation (35, 37, 66, 79, 127, 168). Structural domains for most retroviral gag proteins include; (i) a myristoylated domain at the amino terminus and a cluster of basic amino acids which may interact with the plasma membrane (membrane binding domain), (ii) an interaction domain proposed to mediate specific interactions between adjacent gag proteins leading to multimerization, and (iii) a late domain required for budding through interactions with cellular proteins (35, 64, 80). Trimerization of matrix proteins then leads to formation of a hexameric lattice which causes membrane deformation and eventual budding or pinching off of that region of the membrane (80). Multimerization of myristoylated HIV gag protein has been linked to conformational changes and exposure of gag subdomains, including the myristoylated amino terminus leading to membrane association (myrsitic acid switch) (45, 132, 159). Similarly, the M protein of VSV, paramyxoviruses, Ebola, and influenza virus are membrane associated and may undergo a multimerization to create lattice like structures at the host cell plasma membrane (32, 50, 60, 63, 65, 77, 93, 109, 138, 139, 171). Proline rich late domain has been identified in the matrix protein of both Rous sarcoma virus and HIV which appears to be essential for the late phases of budding and has been proposed

to facilitate the pinching off the matrix containing host membrane by interaction with host cell proteins (124, 167).

Although mechanisms of RNA virus assembly will only partially describe the HCMV assembly process, a working model for HCMV assembly is budding of subviral HCMV particles through an intracellular membrane utilizing interactions between viral proteins and host cell proteins, similar to pathways described for the budding of RNA viruses through cellular membranes. HCMV tegument proteins could directly participate in the deformation of the glycoprotein containing intracellular membranes, and/or facilitate interactions between cytoplasmic tails of envelope proteins and the tegumented particle. Therefore, understanding the role of tegument proteins in the process of envelopment will help to better define the process of assembly of the infectious particle.

Trafficking of tegument proteins for the cytoplasmic assembly. Studies of alpha herpesvirus tegument proteins have provided insight into the cytoplasmic assembly of all herpesviruses. Like HSV, PRV is believed to undergo nuclear tegumentation followed by detegumentation and entry into the cytoplasm essentially free of tegument (70, 71, 115). However, the PRV Vp22 homolog (UL49) appears to either traffic from the nucleus to the cytoplasm or a cytoplasmic form of the protein is incorporated into the virion during cytoplasmic tegumentation, suggesting another route of tegument protein trafficking to sites of virion assembly (44). In addition, the HSV tegument proteins, UL11 protein and VP16 appear to be incorporated into the virion in the cytoplasm, perhaps as a result of interactions with other viral proteins (16, 51, 103, 156). The HSV UL11 protein is required for wild type levels of viral replication, and UL11 null mutants have impaired nuclear virion budding (3, 108). Transiently expressed UL11 protein has been shown to

traffic in the cytoplasm of cells to the plasma membrane (PM) and is retrieved from the PM and to the TGN through interactions between an acidic cluster of aa in UL11 and the cellular protein, PACS-1 (102). The PRV UL11 is also localized to the PM and TGN in virus infected cells and deletion of UL11 results in a replication impaired virus (96). Electron microscopic studies of cells infected with this UL11 mutant virus revealed defects in secondary envelopment including aggregation of non-enveloped capsids, findings similar to those reported for PRV lacking glycoproteins E, I, and M (96). Deletion of UL11 in PRV in combination with UL100 (gM) resulted in a growth impaired virus and a nearly complete block in cytoplasmic envelopment (95). It has been argued that gM and UL11 have different roles in assembly and that UL11 is responsible for localizing other tegument proteins to the site of virus assembly; however, it is also interesting that deletion of PRV UL100 (gM) alone has little effect on virus replication (46, 95). These findings demonstrate that UL11 has critical role in alpha herpesvirus cytoplasmic envelopment. These studies suggested that the analysis of the trafficking of cytoplasmic tegument proteins together with the study of these components in isolation could provide a greater understanding of the cytoplasmic assembly.

HCMV cytoplasmic tegument protein, phosphoprotein 28 (UL99)

Several reports have described proteins comprising the HCMV tegument and have demonstrated that there are abundant HCMV tegument proteins that lack homology (sequence or positional) or cellular distribution with tegument proteins of HSV or other alpha herpesviruses (5, 134, 164). Several tegument proteins of HSV and PRV appear to be expressed in both the cytoplasm and nucleus of infected cells whereas at least three HCMV virion tegument proteins (ppUL25, pp150 and pp28) are expressed exclusively in

the cytoplasm throughout the replicative cycle of HCMV (Table 2) (6, 81, 97, 134). In addition, some HCMV tegument proteins such as ppUL53 and pp65 are expressed in the nucleus of cells early after infection but are localized predominantly in the cytoplasm late in infection (Table 2) (134). Moreover, and in contrast to alpha herpesviruses, heavily tegumented capsids are also found in the cytoplasm of HCMV infected cells (150). These studies suggest that tegumentation and envelopment of HCMV and alpha herpesviruses could differ significantly.

TABLE 2. Cellular Localization of a subset of HCMV tegument proteins in infection.

Gene	Protein name	Nucleus	Cytoplasm
UL25	ppUL25	-	+
UL26	ppUL26	+	-
UL32	pp150	-	+
UL48	pp212	+	-
UL50	pp35	+	-
UL53	ppUL53	+	+[1]
UL69	ppUL69	+	-
UL82	pp71	+	-
UL83	pp65	+	+[1]
UL94	pp36	+	-
UL99	pp28	-	+

[1]This set of tegument proteins is expressed in the nucleus of cells early after infection but is localized predominantly in the cytoplasm late in infection.

We and others have approached the problem of HCMV cytoplasmic assembly by attempting to understand the intracellular trafficking of a tegument protein, pp28 (UL99). Homologues of pp28 are found in all herpesviruses. The HCMV homolog of HSV UL11, pp28, is a cytoplasmic protein expressed very late in the replicative cycle and like UL11, pp28 is myristoylated (107, 135). The pp28 protein is 190 aa in length compared to HSV UL11 (96 aa) and PRV UL11 (63 aa). The primary sequence of HCMV pp28 has limited

homology with either HSV UL11 or PRV UL11; however, these three proteins share structural motifs in the amino terminus including a cluster of acidic aa and myristoylation and palmitoylation sites. It interesting to note that the HSV UL11 acidic cluster is 7 aa (DIESEEE) as compared to the 16 aa acidic cluster (DEGEDDDDGEDDDNEE) of pp28, suggesting a potentially different role in assembly. In contrast to HSV (PRV) UL11, pp28 is localized to the ER-Golgi-Intermediate Compartment (ERGIC) in the absence of other viral functions (135). pp28 has not been detected on the PM of cells following transient expression or in virus infected cells late in infection (18); however, ectopic expression of pp28 driven by a SV40 promoter was reported to result in PM expression early after virus infection (88). In transient expression assays, pp28 can be relocalized from the ERGIC to the Golgi following coexpression with HCMV glycoproteins suggesting different mechanisms for intracellular localization of pp28 compared to UL11 (135). These differences between pp28 and the HSV (PRV) UL11 protein suggest that these proteins may have related but virus specific functions during virus assembly. The function of the pp28 protein in the HCMV infectious cycle is also unknown, although recent evidence suggests that it has a role in envelopment (145). The true late protein, pp28 is localized in a cytoplasmic AC during infection, suggesting that the protein may be involved in late steps of viral morphogenesis such as final tegumentation or envelopment (134).

The focus of the dissertation

The main focus of this dissertation is to further characterize the assembly of HCMV, specifically the final tegumentation and envelopment of the virion in the cytoplasmic phase by defining the role of a tegument protein, pp28 in the process. Previous studies from our laboratory and others revealed that pp28 is essential for the

production of infectious virus and that myristoylation of this protein is essential for its authentic intracellular localization as well as the production of infectious virus (18, 145). The first part of this work was to produce recombinant viruses in which the pp28 gene has been deleted or mutated and to investigate sequence requirements for intracellular trafficking of pp28 to the AC and for production of infectious virus. This work suggested that authentic localization of pp28 within virus-infected cells required the first 50 aa as well as myristoylation of pp28, and is required for assembly of infectious virions. The second part of my work focused on the characterization of recombinant virus exhibiting a growth impaired phenotype. This work emphasized the importance of post localization function of pp28 within the AC for cytoplasmic envelopment process of HCMV. The third part of my work was to examine the role of multimerization of pp28 in HCMV assembly. This work suggested that like other matrix proteins of small RNA viruses, multimerization of pp28, as a post localization function could play a role in viral envelopment process such as budding or pinching off in the membrane of AC. Our findings have indicated that at least one essential HCMV tegument protein, pp28, has characteristics of prototypic viral matrix proteins; (i) pp28 is membrane associated, (ii) pp28 multimerizes, (iii) localization of pp28 to the AC is required for virus assembly, and (iv) viruses with mutant forms of pp28 are replication impaired and exhibit defects in production of enveloped particles. Therefore, pp28 could play a key role in the cytoplasmic phase of HCMV assembly, specifically pp28 plays an essential role in envelopment either acting directly in the process of budding into cytoplasmic vacuoles or as an adaptor that is required for essential interactions between envelope and other tegument proteins. Finally, we could suggest an assembly pathway in which structural proteins accumulate at a common site and subviral particles (partially tegumented

capsids) acquire additional coats of protein either directly through protein-protein interactions (tegument) or by budding through envelope glycoprotein modified membranes, possibly facilitated by tegument proteins (Fig. 9). Thus, understanding the trafficking of pp28 to the assembly site and the protein-protein interactions which promote envelopment will offer a more complete understanding of the process of assembly.

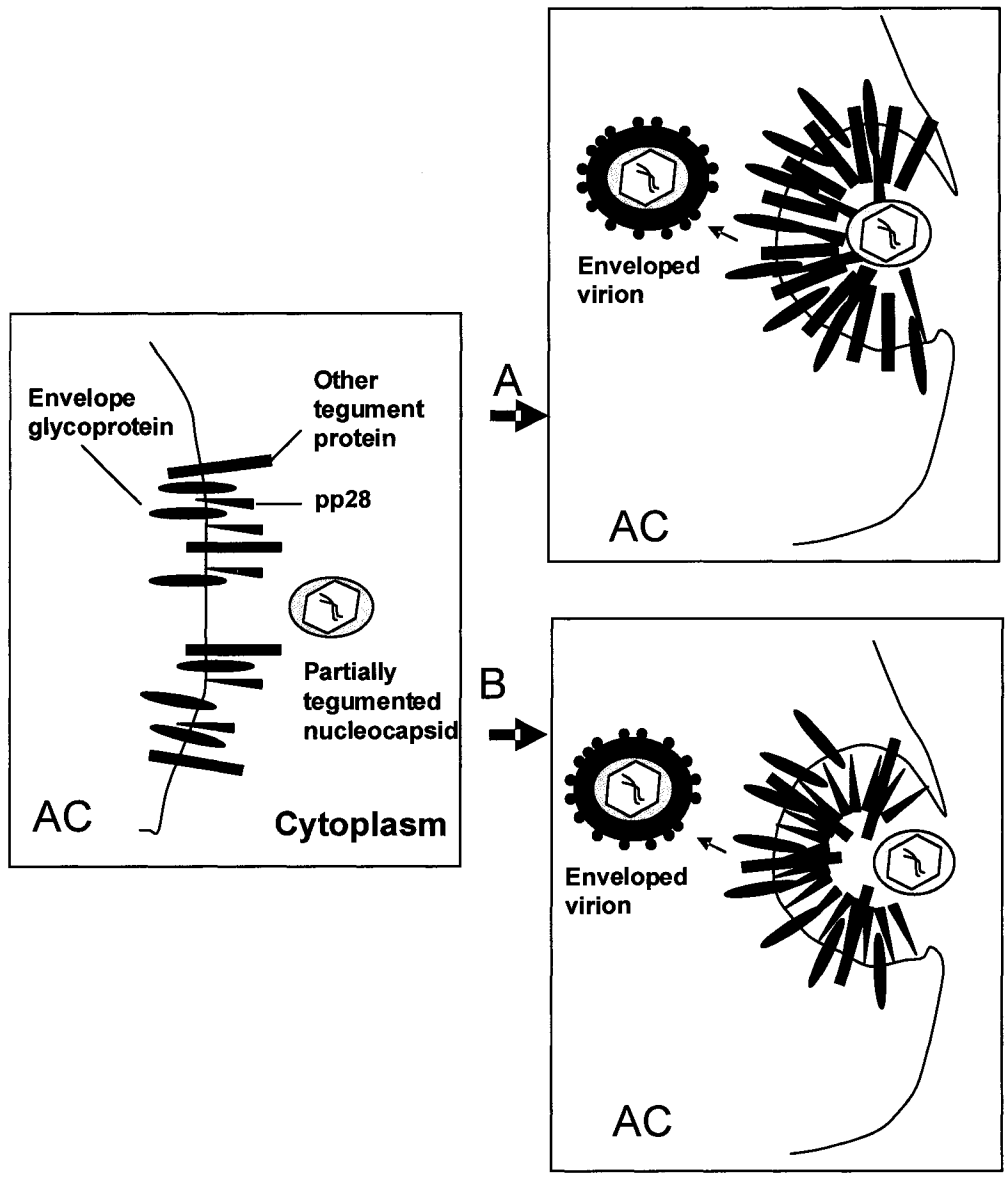

FIG. 9. Model of cytoplasmic assembly of HCMV in the AC virus infected cell. Viral structural proteins including viral tegument proteins and envelope glycoproteins accumulate at the AC and subviral particles (partially tegumented capsids) acquire additional coats of protein either (A) by through protein-protein interactions mediated by pp28 as an adaptor or (B) by budding through envelope glycoprotein modified membranes, possibly facilitated by a tegument protein, pp28. In box, an arrow depicts budding into the AC and result in production of enveloped virion.

SEQUENCE REQUIREMENTS FOR LOCALIZATION OF HUMAN CYTOMEGALOVIRUS TEGUMENT PROTEIN PP28 TO THE VIRUS ASSEMBLY COMPARTMENT AND FOR ASSEMBLY OF INFECTIOUS VIRUS

by

JUN-YOUNG SEO AND WILLIAM J. BRITT

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ABSTRACT

The human cytomegalovirus UL99 open reading frame encodes a 190 amino acid (aa) tegument protein, pp28 that is myristoylated and phosphorylated. pp28 is essential for assembly of infectious virus and non-enveloped virions accumulate in the cytoplasm of cells infected with recombinant viruses with a UL99 deletion. pp28 is localized to the ERGIC in transfected cells, while in infected cells it is localized together with other virion proteins in a juxtanuclear compartment termed the assembly compartment (AC). We investigated the sequence requirements for pp28 trafficking to the AC and assembly of infectious virus. Our studies indicated that the first 30-35 aa were required for localization of pp28 to ERGIC in transfected cells. Mutant forms of pp28 containing only the first 35 aa localized with other virion structural proteins to cytoplasmic compartments early in infection, but localization to the AC at late times required a minimum of 50 aa. In agreement with earlier reports, we demonstrated that deletion of a cluster of acidic aa (aa 44-59) prevented wild type trafficking of pp28 and recovery of infectious virus. A recombinant virus expressing only the first 50 aa was replication competent and this mutant pp28 localized to the AC in cells infected with this virus. These findings argued that localization of pp28 to the AC was essential for assembly of infectious virus and raised the possibility that aa in the amino terminus of pp28 have additional roles in the envelopment and assembly of the virion other than simply localizing pp28 to the AC.

INTRODUCTION

Human cytomegalovirus (HCMV) is the largest and most complex member of the family of human herpesviruses. The virion of HCMV consists of three distinct structures,

a nucleocapsid containing a double-stranded linear DNA genome, an envelope including an as yet undefined number of viral glycoproteins, and a tegument layer located between the capsid and envelope (27, 41, 45, 46). HCMV assembly is a multistage and poorly understood process. Although all proposed models include well studied mechanisms of capsid assembly within the nucleus of infected cells, final tegumentation and envelopment in the cytoplasm of infected cells remains poorly understood (25). The assembly pathway and protein interactions that are required for formation of the tegument layer are not defined. As an example, the tegument protein, ppUL69 is expressed only in the nucleus, whereas some tegument proteins such as pp150 (ppUL32) and pp28 (ppUL99) are expressed only in the cytoplasm during the replication of HCMV (34). Others tegument proteins such as ppUL53 and pp65 (ppUL83) are expressed in the nucleus of cells early in infection but are localized predominantly in the cytoplasm late in infection (34). Thus, it is unclear whether tegument proteins associate with the capsid in the nucleus or in the cytoplasmic assembly compartment at a later step (25, 34). Electron microscopic studies have revealed that both nuclear and cytoplasmic subviral particles have an additional electron dense layer consistent with a tegument layer, suggesting that tegumentation takes place partially in the nucleus and is presumably completed within the cytoplasm.

The role of individual tegument proteins in the replication and assembly of infectious HCMV have not been completely elucidated. However, it has been shown that many of the tegument proteins regulate viral gene expression or modify host cell responses to HCMV infection and likely have less important or non-essential roles in the assembly of the virion. As examples, pp71 (ppUL82) has been shown to transactivate immediate early viral promoters, target cellular Rb family members for degradation, and

inhibit degradation of incoming DNA; ppUL69 has been shown to restrict cell cycle progression, and pp65 has been shown to inhibit the expression of genes associated with induction of interferon responses (1, 10, 11 , 15, 18, 23). Deletion of viral genes encoding any of these tegument proteins results in various degrees of impaired replication but none exhibit comparable null phenotype of viruses with deletions in structural proteins such as the envelope glycoproteins gB, gM, or the tegument protein, pp28 (7, 8, 16, 17, 24, 37).

The HCMV pp28 is a 190 amino acid (aa) tegument protein that is encoded by the UL99 open reading frame (orf). It is a true late protein that is both myristoylated and phosphorylated (19, 26, 35). The pp28 protein is one of the most abundant constituents of the tegument layer and is highly immunogenic (26, 43). Our previous studies have approached the investigation of the envelopment and assembly of HCMV by studying the intracellular trafficking of this protein. We have determined that pp28 is expressed only in the cytoplasm and localized to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) in the absence of other viral proteins, suggesting that viral functions are required for its localization to the cytoplasmic assembly compartment (AC) late in infection (34, 35). Because pp28 is essential for the assembly of infectious virus, its localization in the AC suggested that pp28 may be involved in late steps of viral morphogenesis such as final tegumentation or envelopment (7, 17, 37). The findings that a mutant virus lacking pp28 failed to spread as cell-free infectious virus and the demonstration of non-enveloped cytoplasmic virions in cells infected with this pp28 deletion mutant virus were consistent with a key role of this tegument protein in the assembly of an enveloped virus (7, 37).

In this study, we investigated sequence requirements for intracellular trafficking

of pp28 as an initial attempt to define the function of pp28 in the infectious cycle of HCMV. Our previous studies as well as those of other investigators have determined that myristoylation at glycine 2 is required for localization of pp28 in the ERGIC in the absence of other viral proteins as well as for the production of infectious virus (7, 17, 35, 37). More recently, Jones and coworker have argued that an acidic cluster (aa 44-57) in the amino-terminus of pp28 was required for the cytoplasmic localization of pp28 in virus infected cells and for replication of infectious virus (17). They have also reported that the carboxyl-terminal two-thirds (aa 58-190) of pp28 were not essential for virus replication (17). To investigate sequence requirements for trafficking and function of pp28, we created a panel of C-terminal deletion mutants after each 30 aa of pp28 and produced recombinant viruses expressing this series of C-terminal deletions by the use of a lambda phage based linear recombination system. In addition, to define the possible functions of the acidic cluster domain for trafficking of pp28, we made two mutants, an acidic cluster (aa 44-59) deletion mutant and an acidic cluster deletion and insertion mutant in which the acidic cluster (aa 44-59) was transplanted to the C- terminus of pp28 . Our data indicated that in addition to myristoylation at glycine 2, the first 30-35 aa were required for localization of pp28 in the ERGIC in the absence of virus infection. Furthermore, the first 35 aa were sufficient for the cytoplasmic trafficking of pp28 with other virion structural proteins early in infection but this mutant expressing only the 1^{st} 35 aa of pp28 as well as other pp28 mutants that contained less than the first 50 aa of pp28 failed to accumulate mutant forms of pp28 in the AC late in infection. In addition, we also found that the first 50 aa were sufficient for production of infectious virus and for wild type trafficking of pp28 late in infection. Finally, our findings suggested that the sequences between aa 35-60 of pp28, especially, the acidic cluster (aa 44-59) may

function in a context dependent fashion in protein interactions required for the final envelopment and assembly of virus within the infected cell.

MATERIALS AND METHODS

Cells, viruses, plasmids, and antibodies.

Primary human foreskin fibroblasts (HF) were prepared, propagated, and infected as previously described (9). HCMV strain AD169 was used for all experiments. Infectious stock were prepared from supernatants of infected HF cells which exhibited 100% cytopathic effect and were titered as described previously (9).

For transient expression assay, a panel of carboxyl terminal deletions was generated by insertion of a stop codon into the gene encoding pp28 after each 30^{th} aa (cloning vector, pEF1; Invitrogen, San Diego, Calif.) (Fig. 1). To test the function of an acidic cluster domain in trafficking of pp28, we also made two mutants, an acidic cluster (aa 44-59) deletion mutant (pp28Δac) and an acidic cluster insertion mutant (pp28Δac-CtTR) in which the acidic cluster (aa 44-59) was deleted and then transplanted to the carboxyl terminus (between aa 189-190) (Fig. 1). In addition, these truncated forms of pp28 (expressing 10, 14, 20, 25, 30, 35, 40, 50, 61, 80, 110, or 145 aa from the N-terminus) were fused to EGFP (cloning vector, EGFP-N2; Clontech, Palo Alto, Calif.). All pp28 deletion mutants were constructed using standard cloning techniques and PCR-based mutagenesis and each was sequenced prior to use in experiments described in this report. Each truncated gene was transiently expressed in Cos 7 cells or 293T cells following calcium phosphate mediated transfection (35). For transient expression/ infection assay, pp28 mutants were transfected into HF cells followed by infection with HCMV. In some experiments, cellular proteins such as ERGIC53 (a recycling ERGIC

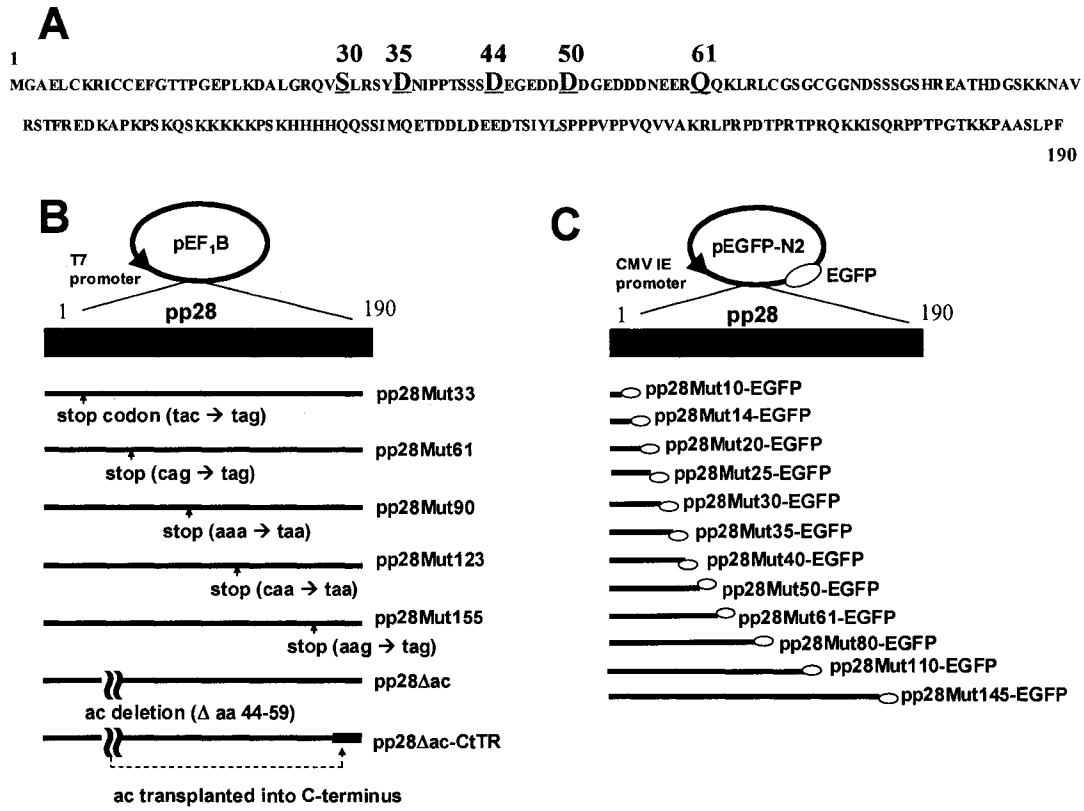

FIG. 1. Generation of pp28 mutants and pp28 EGFP fusion proteins. (A) Amino acid sequences of 190 aa pp28. Amino acids that are relevant to this study are identified by larger font and position listed above sequence. (B) pp28 deletion mutants. In pp28Mut33-Mut155 mutants, a stop codon was inserted into the nucleotide sequence following the codon designated in the mutant. In the pp28Δac mutant, the stretch of acidic amino acids (aa 44-59) was deleted internally, leaving the wild type reading frame of the remainder of the molecule intact. The pp28Δac-CtTR mutant was generated by transplanting the acidic cluster (aa 44-59) onto the C-terminus of the pp28Δac mutant. The mutants were cloned into pEF1B vector or pCDNA vector for transient expression assays. (C) Generation of the pp28 deletion mutants fused with EGFP. pp28 truncation mutants (Mut10-Mut145) were fused with EGPF by cloning into the pEGFP-N2 vector. The mutants are numbered such that the final aa of the wild type pp28 sequence that is expressed is designated in the mutant.

protein), mannosidase II (a Golgi protein,;Man II), and galactosyl transferase (a trans-Golgi protein; Gal T) were fused to EGFP and served as markers for compartments of the secretory system. The EGFP Gal T and Man II were kindly provided by Dr. Brian Storrie (Univ. of Arkansas Medical Center, Little Rock, AR) (40).

HCMV-encoded proteins were detected with monoclonal antibodies (MAbs) as previously described (33). MAbs used in this study included those specific for IE-1 (UL123, MAb P63-27), pp150 (UL32, MAb 36-14), pp28 (UL99, MAb 41-18), gB (UL55, MAb 7-17), and gM/gN complex (UL100/UL73, MAb 14-16A) (8, 24, 33). The antibodies reactive with cellular markers included a MAb specific for ERGIC53 (generously provided by Dr. Peter Hauri, University of Basel, Basel, Switzerland) and a MAb specific for p115 (purchased from Transduction Laboratories, Lexington, KY) as recycling ERGIC proteins, a rabbit antiserum specific for mannosidase II (kindly provided by Dr. Marilyn Farquhar, University of California, San Diego) and a rabbit antiserum against GM130 (purchased from Transduction Laboratories, Lexington, KY) as probes for Golgi proteins, a rabbit antiserum against TGN46 (purchased from Serotec Ltd, Oxford, UK) as a trans Golgi protein , and a rabbit antiserum specific for calreticulin as a ER-resident protein (purchased from Affinity BioReagents, Golden, Colo.). A rabbit antiserum against the cMyc epitope tag was purchased from Affinity BioReagents, Golden, Colo. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) subclass specific antibodies and FITC-conjugated goat anti-rabbit IgG antibodies, Texas red-conjugated goat anti-mouse immunoglobulin G (IgG) subclass specific antibodies and Texas red-conjugated goat anti-rabbit IgG antibodies, and Tritc-conjugated goat anti-mouse immunoglobulin G (IgG) subclass and Tritc-conjugated goat anti-rabbit IgG antibodies were purchased from Southern Biotechnology

Associates, Birmingham, Ala.

Generation of recombinant viruses.

Recombinant viruses were constructed utilizing a two-step strategy for introduction of point mutations into the HCMV genome maintained as an infectious BAC in E. coli as previously described (Fig. 7)(7). The BAC containing HCMV AD169 (HB-5) was provided by Drs. Martin Messerle and Ulrich Koszinowski (University of Munich, Germany) (6). In the first step, the UL99 ORF was deleted from a BAC-maintained HCMV genome by replacement with an Amp^r lacZ cassette. Growth in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) enabled identification of HCMV recombinants by visual inspection for blue bacterial colonies. The mutagenesis was performed with RED locus-mediated recombination to delete sequences between positions 145310 and 145795 of the AD169 genome. A primer set amplifying sequences from positions 145210 (forward primer) and 146025 (reverse primer) was used to produce a recombination cassette of approximately 2.0 kbp that carried the Amp^r lacZ cassette together with approximately 100 to 200 bp of viral sequence flanking the site of deletion within the HCMV genome. The cassette was recombined into the AD169 BAC by RED locus-mediated recombination using the protocol described by Lee et al., resulting in production of Amp^r blue colonies (7, 20). Insertion of the cassette resulted in the deletion of the entire pp28 ORF except for 100 bp of the 3' end (recombinant designated 99KO BAC). To ensure correct targeting of the recombination cassette into the desired genomic location, Southern blot analysis was performed. The recombinant BAC DNA was digested with *Hind*III, electrophoresed in agarose gels, transferred to nitrocellulose membranes, and

hybridized with a ^{32}P-labeled probe specific for the Amp^r lacZ cassette or the UL99 orf. Nucleotide sequence analysis of PCR products amplified from these recombinant BACs confirmed correct insertion of the Amp^r lacZ cassette and replacement of the pp28 ORF. In the second step, RED recombination was used to replace the pp28 deletion in 99KO BAC with either the wild-type pp28 ORF sequence or the pp28 sequence containing desired mutations by using a linear DNA fragment. Recombination removed the Amp^r lacZ cassette from the 99KO BAC, and repaired or mutagenized recombinants were identified by visual inspection for white colonies. The same primer set described above was used to prepare linear DNA fragments containing the desired mutations by PCR from a pp28 template as previously described (7). This methodology allowed insertion of single nucleotide changes in the native UL99 ORF to create stop codons and maintain the transcription program and the normal regulation of the expression of this region of the wild type genome.

In the two-step strategy above, we produced mutated BACs that contained mutations in the pp28 ORF that resulted in lethal mutations. Using a similar recombination strategy, the mutated BACs could also be repaired by using single-stranded oligonucleotides that contained a repaired stop codon and approximately 20 nucleotides flanking on each side of the repair codon. The repair oligonucleotides were electroporated into *E. coli* containing the mutated HCMV BAC, recombined and following expansion of this mixed population of *E.coli* (mutant and repaired BACs), BAC DNA was purified and electroporated together with a plasmid encoding pp71 into HF cells. Finally, 14 recombinant BAC DNAs (UL99KO; a series of pp28 deletion mutants expressing the first 33 (pp28STOP33), 50 (pp28STOP50), 61 (pp28STOP61), 90 (pp28STOP90), 123(pp28STOP123), or 155 aa (pp28STOP155) of pp28; an acidic

cluster deletion mutant in pp28 (pp28Δac); an acidic deletion mutant in which the acidic cluster was deleted and transplanted to the C-terminus (pp28Δac-CtTR); and a series of revertants BAC DNAs repaired from the pp28 mutants (pp28Rev33, 61, 90, 123 and 155) were generated for this study (Fig. 7).

To determine whether the recombinant pp28 gene was essential for virus infectivity in HF cells, DNA was purified from the BAC-containing *E. coli* cells and electroporated into HF cells. One ug of an expression plasmid encoding pp71 was included to enhance the recovery of infectious virus. Infectivity was monitored by observing the production of visible plaques. In assays for virus replication, virus titers were determined by fluorescent virus infectivity assay on HF cells infected with WT or recombinant viruses at the indicated times after infection (2) .

SDS-PAGE and immunoblotting.

SDS-polyacrylamide electrophoresis under reducing conditions and immunoblotting were carried out as described previously (4). Virus-infected cell proteins were extracted from wild type or recombinant virus-infected HF cells grown in 35 mm diameter tissue culture dishes. Following washing in phosphate-buffered isotonic saline (PBS; pH 7.4), the cells were lysed in sample buffer containing 5% 2 mercaptoethanol and 2% sodium dodecyl sulfate (SDS) and heated to 100°C. The solubilized proteins were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Murine MAbs or in some cases a 1:100 dilution of the IgG fraction of the rabbit anti-cMyc serum were used to detect specific proteins. Antibody binding was detected by ^{125}I-protein A followed by autoradiography.

Immunofluorescence microscopy.

HF cells were grown in 24-well tissue culture plates containing a 13-mm-diameter coverslip. After the cells were 90% confluent, the cells were infected with HCMV strain AD169 derived from the HB-5 BAC or from mutant viruses generated from the HB-5 BAC for 1 to 2 h, washed once and incubated for the indicated time. The coverslips were harvested by first washing the cells with PBS and then fixing for 45 min at room temperature in 3% paraformaldehyde (PFA) freshly prepared in PBS. The coverslips were washed in PBS and permeabilized with 0.05% Triton X-100 and 0.001% SDS in PBS for 7 min. The coverslips were then blocked with PBS containing 10% normal goat serum for 20 min at room temperature followed by the addition of primary antibody, and incubated for 60 min at 37°C. Following washing (x3) with 0.2% Tween in PBS (PBS-T), the coverslips were incubated with FITC-conjugated and/or Texas Red conjugated or Tritc secondary antibody diluted in PBS-T containing 2.5% normal goat serum for 45 min at 37°C. The coverslips were washed three times and then rinsed once in PBS, mounted with SlowFade antifade reagent (Molecular Probes, Eugene, Oreg.) and viewed on a Leitz Diavert fluorescence microscope or an Olympus confocal microscope. The images were captured with a digital camera (Photometrics, Tucson, Ariz.) using the Leitz epifluorescence microscope at a magnification of 60x. In some cases, images were processed with Image Pro software (Media Cybernetics, Silver Spring, Md.). Deconvolution was accomplished with Hazebuster (Vaytek, Fairfield, Iowa).

Cos7 cells grown on coverslips were transfected with an expression vector containing the truncated pp28 sequence or a vector encoding a pp28 EGFP fusion protein. Transfected cells were fixed 36 to 48 h posttransfection, and cells expressing pp28 were reacted with MAb 41-18 followed by Texas Red-conjugated goat anti-mouse IgG

antibody as described above. In transient expression/infection assay, HF cells were electroporated with approximately 5 ug of DNA from an expression vector containing the truncated pp28 sequence, a vector encoding a pp28 EGFP fusion protein, or a vector encoding EGFP fused to markers for cellular compartments. Forty-eight hours later, the cells were infected with HCMV and then washed once and incubated for the indicated time. The infected cells were fixed and stained as described above.

Subcellular fractionation.

A 75-cm^2 flask of HF cells was electroporated with 5 ug of an expression vector encoding pp28Mut40EGFP, pp28Mut50EGFP, or pp28ΔacEGFP. Twenty-four hours later, the cells were washed and infected with HCMV at an moi of 0.2. The HF cells were harvested on day 6 postinfection by trypsinization an the cell pellet washed twice with cold PBS and then resuspended in 1 ml of homogenization buffer (0.25 M sucrose, 10 mM Hepes, pH 7.4, 1 mM EDTA). The cell suspension was repeatedly passed through a 23-gauge needle until there were no intact cells in the suspension as determined by light microscopy and a post nuclear supernatant collected following centrifugation at 1000xg for 10 min. Subcellular fractionation was performed using a density gradient prepared from Iodixanol (Optiprep, Sigma, St. Louis, Mo) and ultracentrifugation as modified by a protocol as described (38, 44). A discontinuous gradient was prepared using 30%, 25%, 20%, 15%, and 10% (vol/vol) Optiprep solution. The gradient was allowed to equilibrate vertically for 30 min at room temperature. The post-nuclear supernatant was overlaid onto the discontinuous gradient and centrifuged at 100,000x g in an SW41 rotor for 3 h at 4 °C. Equal fractions were collected from the top of the gradient, and individual fractions were assayed for viral and host cell proteins by immunoblotting.

RESULTS

Sequence requirements for trafficking of transiently expressed pp28 to the ERGIC.

Initially we determined sequences within pp28 that were required for its localization in the ER- Golgi Intermediate-Compartment (ERGIC). Previously we have shown that pp28 is localized in the ERGIC and does not traffic to more distal compartments of the Golgi/Trans-Golgi (TGN) in cells transiently expressing pp28 in the absence of other viral proteins (35). To define specific domains required for the localization of this protein in the ERGIC, we transfected Cos 7 cells with plasmids encoding a series of pp28 C-terminal deletion mutants and determined their intracellular localization. This panel of pp28 mutants was generated by insertion of stop codons into the coding sequence of the wild type gene or generation of C-terminal truncations of pp28 followed by fusion of the remaining coding sequence with EGFP as described in figure 1. In addition, we also created two mutants in which either the acidic domain located between aa 44-59 was deleted or was excised and transplanted to the C-terminus of the molecule as detailed in figure 1. These latter two mutants were constructed because it has been reported that sequences within the first acidic domain of pp28 (aa 44-57) are required for its localization within infected cells (17). All mutations were confirmed by nucleotide sequencing and expression of the mutant form of pp28 was demonstrated by western blotting of pp28 mutants following transient expression in 293T cells (data not shown). The intracellular trafficking of pp28 deletion mutants was monitored by localizing of their intracellular expression using antibodies reactive with proteins specific for cellular compartments (Fig. 2). Surprisingly, the trafficking of most pp28 deletion mutants was indistinguishable from that of wild type pp28 and remained localized within the ERGIC (Fig. 2). Mutants expressing polypeptides of the first 33, 61, 90, 123, or 155

aa of pp28 all appeared to have similar intracellular localization as wild type pp28 (Table 1). Co-localization with a protein marker of the ERGIC, p115, was also observed following expression of C-terminal truncation pp28 mutants that encoded the first 30, 35, 40, 50, 61, 80, 110, or 145 aa of pp28 fused to EGFP (Table 1). The two acidic cluster mutants, pp28Δac (aa 44-59) and pp28Δac-CtTR also co-localized with markers of the ERGIC (Table 1). An example of this pattern of localization is shown by the distribution of pp28Mut30EGFP following transient expression in Cos 7 cells (Fig. 2). We noted that the vesicular distribution of this pp28 mutant protein closely resembles that of wild type pp28 and there was partial overlap with the protein marker of the ERGIC, p115 (Fig. 2). In contrast, pp28 mutants that contained less than the first 30 aa failed to co-localize with markers of the ERGIC and, their intracellular localization differed from that of wild type pp28. This is illustrated by the mutant pp28Mut10EGFP that was distributed in a pattern most consistent with ER resident proteins such as calreticulin when transiently expressed in Cos 7 cells Fig. 2). Each of these pp28 mutants contained a glycine codon at position 2 and therefore, was presumably myristoylated and thus, mislocalization cannot be ascribed to a lack of membrane association. These results indicated that the first 30 aa of pp28 together with the myristoylation modification at aa position 2 were required for the localization of this pp28 protein in the ERGIC when pp28 was expressed in the absence of virus infection.

Localization of pp28 to the assembly compartment of virus infected cells requires a late viral function.

In virus-infected cells, pp28 was localized to a membranous cytoplasmic compartment that we have designated as the virus assembly compartment (AC) based on

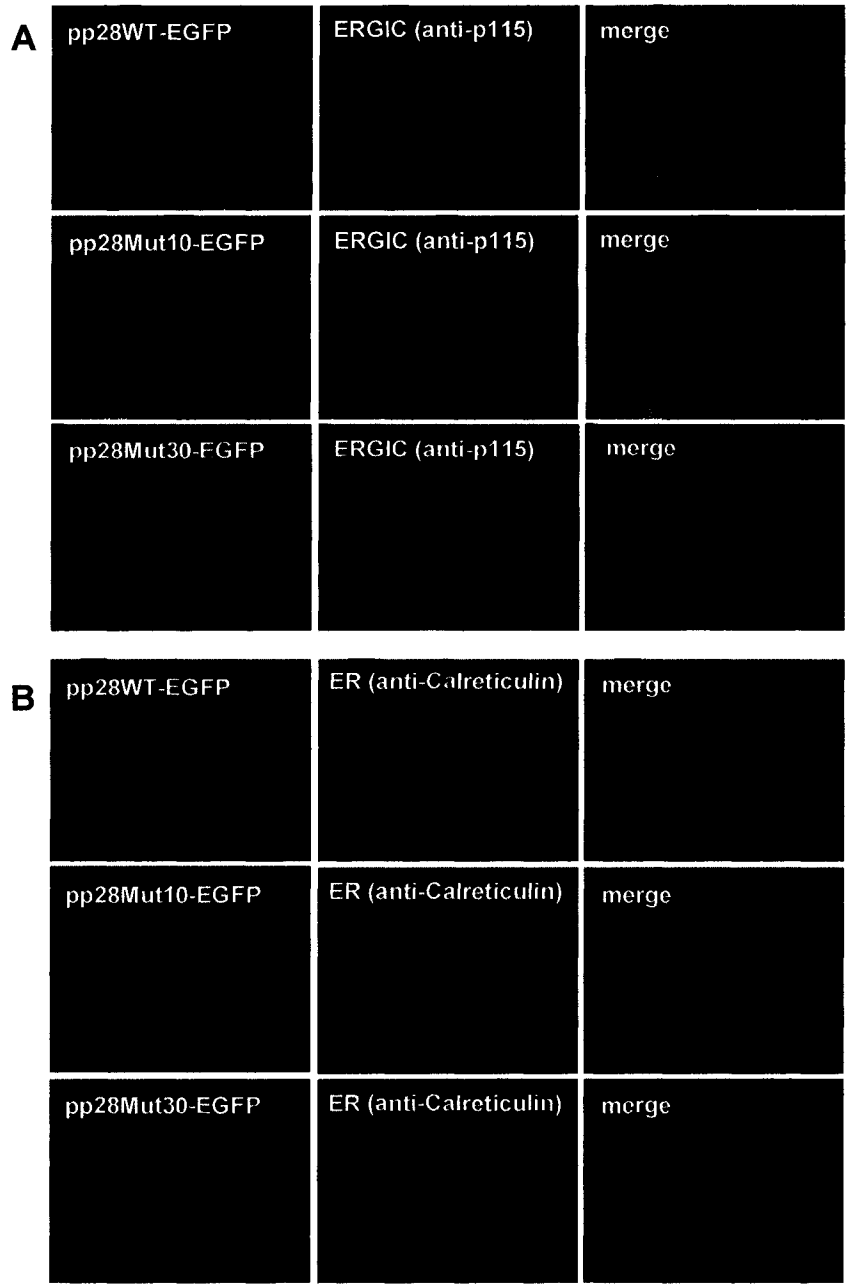

FIG. 2. Localization of transiently expressed pp28 deletion mutants in the ERGIC. pp28 deletion mutants were transfected into Cos-7 cells. At day 2 post transfection, the cells were fixed with 3% paraformaldehyde and examined by fluorescence microscopy as described in the Materials and Methods. (A) pp28WTEGFP or pp28MutEGFP fluorescence is green and organelle (p115, ERGIC marker) fluorescence is red (Texas-Red). (B) pp28WTEGFP or pp28MutEGFP fluorescence is green and organelle (calreticulin, ER marker) fluorescence is red (Texas-Red). Nuclei are pseudocolored blue following staining with Hoechst dye.

TABLE 1. pp28 sequence requirements for localization in the ERGIC.

Protein[2]	ERGIC[1]	ER[1]
pp28Mut33	++	
pp28Mut61	++	
pp28Mut90	++	
pp28Mut123	++	
pp28Mut155	++	
pp28ac	++	
pp28ac-CtTR	++	
pp28Mut10-EGFP	-	++
pp28Mut14-EGFP	-	++
pp28Mut20-EGFP	+	++
pp28Mut25-EGFP	+	++
pp28Mut30-EGFP	++	-
pp28Mut35-EGFP	++	-
pp28Mut40-EGFP	++	-
pp28Mut50-EGFP	++	-
pp28Mut61-EGFP	++	-
pp28Mut80-EGFP	++	-
pp28Mut110-EGFP	++	-
pp28Mut145-EGFP	++	-
pp28WT-EGFP	++	-

[1]Localization in the ERGIC was determined by colocalization of the pp28 mutant with ERGIC53 (ERGIC) or p115, and localization to the ER was determined by colocalization with calreticulin (ER). The intensity of the signal based on colocalization with the marker was scored as partially (+), primarily (+ +), or not (-) detected in the organelle.
[2]pp28 mutants were designated by the last remaining amino acid from the amino terminus of pp28.

its morphologic appearance, the isolation of this membrane bound compartment by cell fractionation, and localization of a number of structural tegument and envelope proteins in this compartment late in infection (34). Because pp28 remained in the ERGIC when transiently expressed in the absence of other viral proteins, the trafficking pathway of pp28 to the assembly compartment likely required expression of an undefined viral function. To formally demonstrate this possibility, we developed an assay based on transient expression of an EGFP or epitope tagged pp28 or mutant pp28 followed by

virus infection to supply the virus function in *trans* (transient expression /infection assay). In this assay, the EGFP tagged pp28 protein was expressed in HF cells following electroporation of a plasmid encoding the pp28 molecule fused at its C-terminus to EGFP. Electroportated HF cells were infected with HCMV 48 hrs later at a multiplicity of infection (moi) of 0.1. In uninfected cells, the pp28EGFP protein was expressed throughout the cytoplasm of the cell in a punctuate distribution that was similar to the distribution of ERGIC markers in uninfected HF cells (Fig. 3). Shortly after infection of electoporated cells, the pp28EGFP fusion protein was expressed in the cytoplasm of infected cells in a similar distribution as in uninfected cells (data not shown). However, late in infection the transiently expressed pp28EGFP could be co-localized with the gM/gN glycoprotein complex in the AC as illustrated by its expression in infected cells expressing IE-1 (pp72) in the nucleus and the gM/gN complex in the cytoplasm (Fig. 3). In contrast to these findings, when monolayers of pp28EGFP electroporated/infected cells were treated with ganciclovir to block expression of late viral proteins, the pp28EGFP fusion protein no longer localized to the AC at late times in infection but was expressed diffusely in the cytoplasm (Fig. 3). Transiently expressed pp28EGFP fusion protein was localized to the AC when these cultures of electroporated/infected cells were treated with the antiviral compound, BDCRB, a compound that inhibits late steps of virus assembly, cleavage and packaging of viral DNA, but not late protein synthesis (Fig. 3) (42). These results demonstrated that authentic localization of pp28 to the AC required expression of a late viral function and that localization of the pp28 to the AC was not inhibited by antiviral compounds that specifically blocked nuclear events of virus assembly but not late protein synthesis.

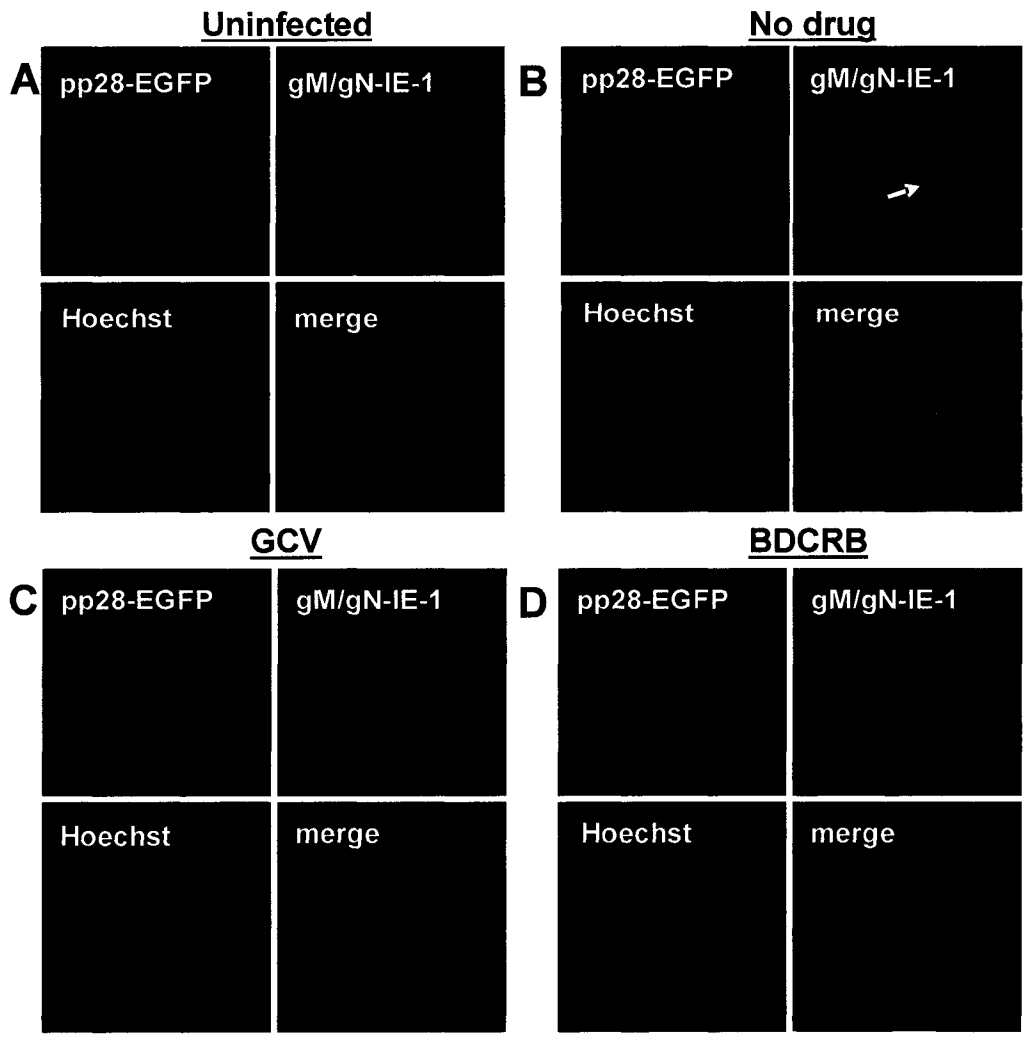

FIG. 3. pp28 localization in assembly compartment requires late gene expression. HF cells (3x 10^{6}) were electroporated with approximately 5ug of pp28EGFP and either left uninfected (A) or infected with HCMV 48 hr later at an moi of 0.2 (B-D). Individual wells were treated with media control (no drug, control) (B), ganciclovir (GCV) (C), or BDCRB (D). Cells fixed 120 hr post infection and stained with anti-gM/gN and IE-1 MAbs and developed with Tritc anti-mouse IgG secondary antibodies. Nuclei were stained (blue) with Hoechst dye and IE-1(red) in infected cells. Arrow in panel B demarcates assembly compartment as indicated by gM/gN staining. Note that pp28-EGFP was present in assembly compartment of >90% of cells in control wells as compared to <10% in GCV treated cultures.

Sequence requirements for localization of pp28 to the AC in virus infected cells.

Having demonstrated that wild type pp28EGFP localized to the AC in cells transfected with pp28EGFP and infected with HCMV, we utilized this assay to investigate the intracellular localization of transiently expressed mutant forms of pp28 to identify sequence requirements for localization of pp28 to the AC. The EGFP plasmids expressing the first 30, 35, 40, 50, 61 aa of pp28 (pp28Mut30EGFP, Mut35EGFP, Mut40EGFP, Mut50EGFP, Mut61EGFP, respectively) were used in this transient expression/ infection assay. In addition, the localization of an acidic cluster mutant, pp28Δac (aa 44-59) fused with EGFP (pp28ΔacEGFP) and the pp28 mutant in which the acidic cluster (aa 44-59) was deleted and transplanted to the carboxyl terminus and tagged with the Myc epitope (pp28ΔacCtTRMyc) were also studied. After electroporation of plasmids expressing pp28EGFP, pp28Mut30EGFP, Mut35EGFP, Mut40EGFP, Mut50EGFP, Mut61EGFP, pp28ΔacEGFP, and pp28ΔacCtTRMyc, we observed comparable intracellular distribution within 48 hours after electroporation (data not shown). The electroporated cells were then infected with HCMV at a moi of 0.2 and assayed the localization of the pp28EGFP or mutant forms of pp28 in infected cells (IE-1 expressing cells). Interestingly, all of the pp28 mutants with the exception of pp28Mut30EGFP, were expressed in a perinuclear compartment early after infection (day 3-4 post infection; data not shown). However, the efficiency of localization of wild type and mutant forms of pp28 in this compartment varied between mutants such that the relative efficiency of localization to the perinuclear compartment early infection depended on the length of the pp28 mutant. The shorter mutants such as pp28Mut35EGFP localized to this perinuclear compartment less efficiently than the longer mutants such as pp28Mut50EGFP. These results suggested that only the first 35

aa of pp28 was required for trafficking to a perinuclear compartment that appeared to form shortly after virus infection, although the efficiency of localization to the this compartment was decreased for the shorter pp28 mutants.

In marked contrast to findings early in infection, the localization of mutant forms of pp28 on day 7 pi revealed qualitative differences between several mutants and wild type pp28EGFP. The mutants pp28Mut40EGFP, pp28ΔacEGFP, and pp28ΔacCtTR-Myc failed to localized to the AC and were distributed peripherally to the AC as compared to the wild type pp28EGFP (Table 2; Fig. 4). In some panels of this figure, we co-stained with anti-IE-1 Mab and anti-GM130 Mab to allow detection of infected cell nuclei and the Golgi to help further define the intracellular localization of mutant pp28s. In the experiments in which we successfully visualized GM130, we noted that this Golgi marker was displaced peripherally from the AC (Fig. 4). This finding was further explored in a series of experiments (see following section). Interestingly, pp28Mut50EGFP was localized to the AC in only about 50% of EGFP positive cells at 7dpi (Table 2; Fig. 4). The pp28Mut61EGFP concentrated in the AC with comparable efficiency as the wild type pp28EGFP protein (Table 2). The differences in the localization of pp28 mutant forms in the AC at late times in infection suggested that sequences between aa 35-61 of pp28, particularly, the N-terminal acidic cluster (aa 44-59) might function through specific interactions with other viral proteins or cellular proteins for the final localization of pp28 to the AC. Interestingly, the pp28Mut50EGFP containing one-half of the acidic cluster of aa appeared to have an intermediate phenotype. These findings were consistent with the replication defective phenotype of the previously reported recombinant viruses expressing less than 57 aa of the amino-terminus of pp28 (17). Together these findings argued that a defect in the localization or

FIG. 4. Localization of pp28 deletion mutants to assembly compartment late in infection in HCMV infected HF cells. HF cells were electroporated with approximately 5 ug of expression plasmids encoding pp28WT-, pp28Mut40-, pp28Mut50-, pp28Mut61-, or pp28Δac -EGFP, or the pp28 ΔacCtTR-Myc mutant and infected 2 days later with HCMV at moi of 0.2. The cells were harvested at day 7 post infection, fixed with 3% paraformaldehyde, stained with anti- IE-1 Mabs combined with anti-GM130 Mabs (pp28WTEGFP, pp28Mut40EGFP, pp28Mut50EGFP, pp28Mut61EGFP, and pp28ΔacEGFP) to localize AC to a secretory compartment or anti-IE-1 Mabs only (pp28 ΔacCtTR-Myc) followed by Tritc labeled anti-mouse IgG to identify infected cells (red nuclei), and examined by confocal microscopy. The expression of the pp28ΔacCtTR-Myc tagged mutant was detected with an anti-Myc Mab followed by FITC anti-mouse IgG (green fluorescence). The AC can be seen in the juxtanuclear position in cells expressing the pp28WTEGFP, pp28Mut61EGFP, and pp28Mut50EGFP. Note the weak staining from the GM130 Mab staining of the Golgi that is surrounding the AC in cells expressing pp28WTEGFP, pp28Mut40EGFP, pp28Mut50EGFP, and pp28Mut61EGFP. The GM130 reactivity in infected cells expressing pp28ΔacEGFP cannot be appreciated in this photograph.

TABLE 2. pp28 sequence requirements for localization to the AC following transient expression and infection of HF cells

Protein[2]	AC[1]
pp28Mut10-EGFP	NT
pp28Mut14-EGFP	NT
pp28Mut20-EGFP	NT
pp28Mut25-EGFP	NT
pp28Mut30-EGFP	-
pp28Mut35-EGFP	-
pp28Mut40-EGFP	-
pp28ac-EGFP (aa 44–59)	-
pp28ac-CtTR-Myc	-
pp28Mut50-EGFP	+
pp28Mut61-EGFP	+ +
pp28Mut80-EGFP	+ +
pp28Mut110-EGFP	+ +
pp28Mut145-EGFP	+ +
pp28WT-EGFP	+ +

[1]Localization to the AC was assayed by imaging of cells 6 to 7 days postinfection. Localization was scored as - if there was no localization in the compact AC, + if 50% of cells demonstrated localization to the AC, and + + if _70% of cells exhibited localization of mutant pp28 or wild-type pp28 in the compact AC. NT, not tested in this experiment.
[2]pp28 mutants were designated by the last remaining amino acid of the amino terminus of pp28.

concentration of pp28 mutant protein to the mature AC late, but not early in infection, was limiting in the process of the envelopment and assembly of infectious virus.

The striking differences between the localization of wild type pp28 in the AC and the distribution of pp28 mutants such as pp28Mut40 on the periphery of the AC suggested that pp28 mutants such as pp28Mut40 were restricted to more proximal compartments of the secretory pathway that were reorganized during HCMV infection.

This possibility was consistent with previous imaging findings that demonstrated exclusion of protein markers of the Golgi and TGN from the AC (34). To explore the possibility that morphological reorganization of the host cell secretory compartment took

place during virus infection, we investigated the distribution of markers of the secretory compartment including, ERGIC53-EGFP, mannosidase II (Man II)-EGFP, and galactosyl transferase (Gal T)-EGFP in transient expression/infection assays. These markers of the secretory system redistributed early in infection (<4d) into perinuclear spherical structures, that also contained viral proteins such as gB, and pp28 (data not shown). We then utilized the same transfection/infection assays to determine the distribution resident proteins of compartments of the secretory system late in infection. The analysis was carried at late times in infections for several reasons including that characteristics of the AC have been defined in cells late in infection, pp28 is expressed with true late kinetics and virus production and presumably, virus assembly is maximal late in infection. By day 7 pi, signals from these protein markers of the secretory compartment revealed a dramatic morphological reorganization of the host cell secretory system and, demonstrated that the secretory compartment had been reorganized into layers that ringed the pp28 containing, juxtanuclear AC (Fig. 5). Moreover, we observed that late in infection, the AC was a compact structure as compared to a more diffuse and spherical perinuclear structure early in infection and in contrast to the co-localization with markers of the secretory pathway early in infection, the AC containing pp28 was devoid of markers of the Golgi or TGN (Fig. 5). These findings were consistent with a the maturation of the AC during the course of infection in an individual cell, a mechanism consistent with results from earlier studies (34). Previously, we demonstrated that several viral proteins were localized in the AC late in infection, but interestingly in some experiments utilizing these markers of the host secretory pathway, gB could also be detected in both the center of the AC and partially overlapped with the signal from the Golgi (data not shown). Thus, it appeared that the AC represented a site of viral protein

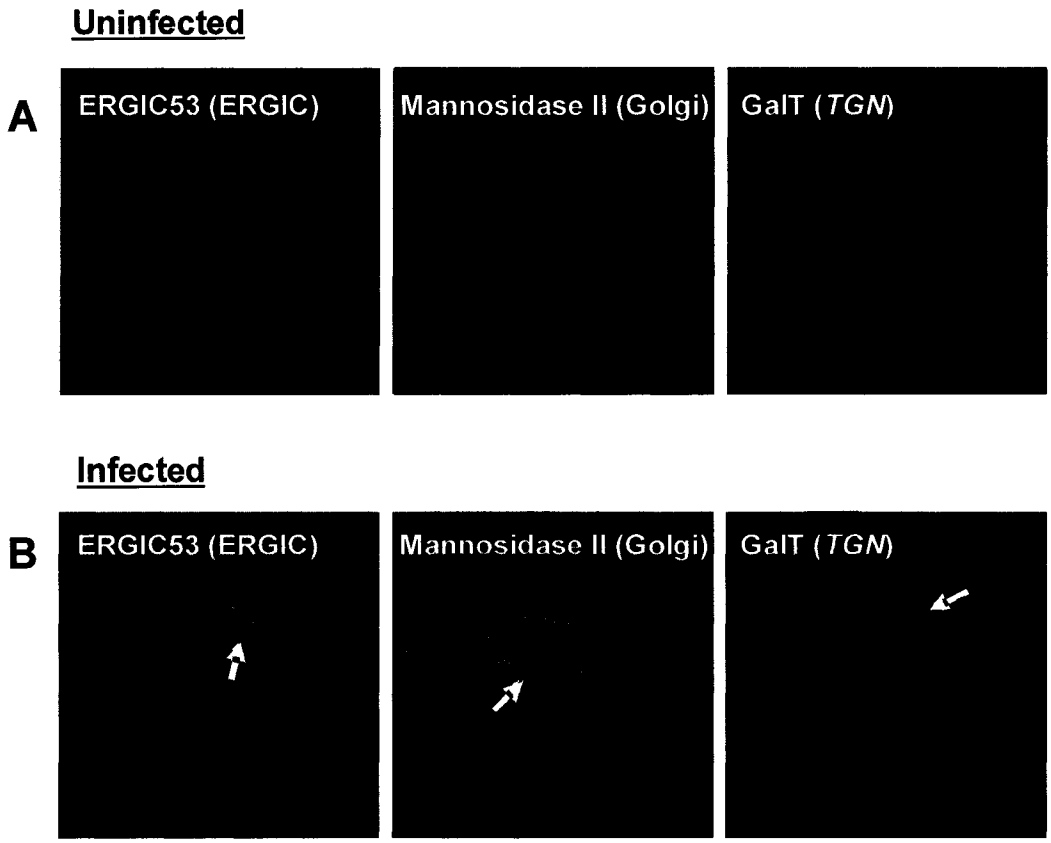

FIG. 5. Altered morphology of secretory compartment following HCMV infection. HF cells were electroportated with approximately 5ug of plasmids encoding ERGIC 53 (ERGIC marker), mannosidase II (Golgi marker), and galactosyl transferase (Gal T, TGN marker) proteins fused to EGFP and either (A) left uninfected or (B) infected with HCMV at a moi of 0.2 36 hr after electroporation. The infected cells were incubated for 7 days prior to fixation as described in Materials and Methods. Cells that were fixed 7 days post infection were reacted with anti-pp28 MAb followed by TRITC anti-mouse IgG to demonstrate assembly compartment. Nuclei were stained (blue) with Hoechst dye. Arrow demarcates assembly compartment.

accumulation within a morphologically altered secretory compartment displaced to the periphery of the AC. Some viral glycoproteins such as gB could be co-localized with the Golgi and TGN as well in the more compacted AC late in infection suggesting the possibility that this molecule and other viral proteins trafficked from the Golgi/TGN into the AC during virus assembly.

Subcellular localization of wild type pp28 and pp28 mutants.

To further investigate the nature of the AC containing the pp28 WT protein and to compare the intracellular distribution of wild type pp28 with mutant forms of pp28 late in infection, we used centrifugation through density gradients to analyze the distribution of transiently expressed viral proteins in cells transfected with pp28Mut40EGFP, pp28Mut50EGFP, or pp28)acEGFP and then infected with HCMV. Post nuclear supernatants were prepared from cells harvested late in infection (> 6d) and subjected to centrifugation through discontinuous iodixanol (Optiprep) density gradients and individual fractions analyzed by western blotting for the presence of the virus encoded wild type pp28, the mutant pp28EGFP proteins, and host cell proteins GM130 (Golgi) and CD63 (late endosomes). The amount of protein detected in each fraction was quantified by densitometry and presented as a fraction of the total amount of the specific protein recovered from the entire gradient. The wild type pp28 viral protein was distributed in two broad peaks, with one being associated with the first 3 fractions and a second broad peak migrating further into the gradient (fractions 6-9; Fig. 6). The mutant pp28, pp28Mut40EGFP, exhibited a very different distribution in the gradient with the majority of this protein migrating in the first 3 fractions of the gradient (Fig. 6). Similarly, the pp28 mutant, pp28)acEGFP also partitioned primarily in the top fractions

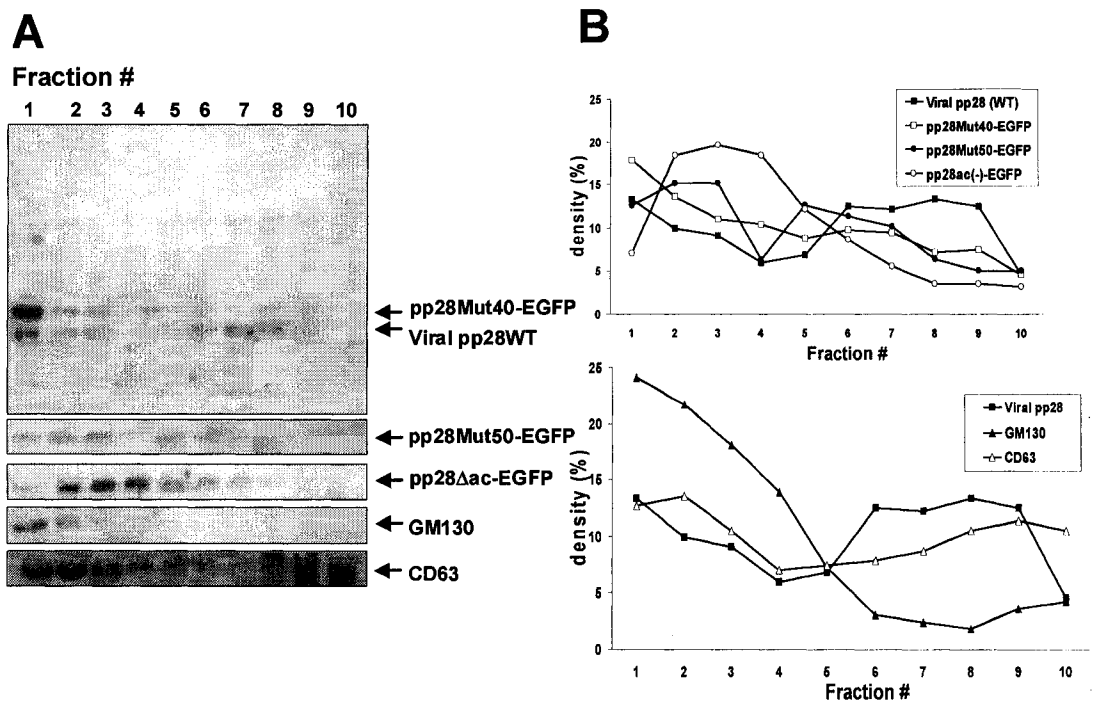

FIG. 6. Iodixanol density gradient fractionation of HCMV infected HF cells transfected with pp28 mutants. HF cells electroporated with approximately 5 ug of the designated pp28MutEGFP expression plasmids and infected 2 days later with HCMV at moi of 0.2. The cells were harvested at day 7 post infection and fractionated by centrifugation through iodixanol (Optiprep, Sigma Co, St. Louis, Mo) gradients as described in Materials and Methods. The gradient was fractionated by removing 1 ml fractions from the top, thus fraction 1 represents the top of the gradient, and fraction 10 the bottom of the gradient. (A) Gradient fractions were analyzed by western blot. Proteins were detected with specific antibodies, anti-pp28 MAb for pp28 WT and pp28Mut- 40, 50, and pp28Δac- EGFP and developed with ^{125}I-protein A. Mabs reactive with cellular proteins specific for compartments of the secretory pathway, anti-GM130, and anti-CD63, were used to localize pp28 and pp28 mutants to different intracellular compartments. (B) Results of panel A were analyzed by densitometry (density (%) = peak density of each fraction/ total density of signal from protein in all gradient fractions x 100). The top of panel B is graphic comparison of pattern of fractionation of virus encoded pp28 to pp28Mut40, 50 and Δac -EGFP. Viral pp28 (WT; ■); pp28Mut40EGFP (□); pp28Mut50EGFP (●); pp28ΔacEGFP (○). The bottom of panel B represents comparison of pattern of fractionation from viral pp28 to subcellular organelles (GM130, Golgi; CD63, late endosome). Viral pp28 (WT; ■); GM130(▲); CD63(Δ).

of the gradient as single broad peak (Fig. 6). Interestingly, the pp28 mutant, pp28Mut50EGFP, was found in two broad peaks in a distribution that was most similar to that of the wild type pp28 (Fig. 6).

To determine the distribution of the wild type pp28 and mutant forms of pp28 in different compartments of the cellular secretory pathway, we assayed the distribution of two host cell proteins within these same gradient fractions. Our results were similar to those reported previously by Sims, et al. (38). The Golgi specific protein GM130 could be localized to fractions 1-3 whereas the late endosomal marker CD63 was distributed over the gradient in two broad peaks, one of which coincided with the distribution of viral pp28 in fractions 6-9 (Fig. 6). This broad migration of endosomes has been observed by other investigators using similar conditions for density gradient separation of cellular organelles and is thought to be secondary to the heterogeneity of the density of endosomes as a result of differences in cargos (39). The results were also consistent with our imaging findings that suggested that mutant forms of pp28 that localized outside of the AC and could be co-localized with proteins such as mannosidase II, a marker of the Golgi and not with the late endosomal marker CD63 (data not shown). Furthermore, these data also demonstrated that wild type pp28 and the mutant pp28Mut50EGFP partitioned similarly in this gradient, and perhaps most importantly, could be found in fractions containing Golgi protein as well as fractions containing the late endosomal marker protein, CD63. In contrast, the distribution of both pp28Mut40EGFP and pp28ΔacEGFP mutants that were found most abundantly in fractions from the top of the gradient along with a cellular marker protein of the Golgi. Together with data from our imaging studies, these findings provided additional evidence that mutant forms of pp28 that failed to localize to the AC late in infection were distributed in different cellular

compartments as compared to either wild type pp28 or a mutant form of pp28, pp28Mut50. Lastly and most importantly, these data were also consistent with the capacity of the mutant pp28Mut50 to support virus replication when recombined into the viral genome in that the pp28Mut50 protein partitioned similarly to the wild type pp28 protein in these gradients and from image analysis, localized to the AC (see following section).

Mislocalization of pp28 results prevents assembly of infectious progeny virus.

To examine the effect of mislocalization of pp28 mutants on the assembly of infectious virus, we generated a panel of recombinant viruses with stop codons inserted into the pp28 coding sequence using a linear recombination system previously described (7). Comparison of restriction fragment pattern of recombinant BAC DNAs containing mutations in the UL99 were identical to that of the wild type parent virus and differed from the UL99 KO BAC utilized for the replacement mutagenesis (data not shown). Southern blot analysis using a ^{32}P-labelled probe generated by nick translation of the UL99 orf revealed the presence of the 6 kilobase pair (kbp) *Hind III* R fragment in the wild type and mutant pp28 BAC DNA and the approximate 8 kbp *Hind III* R fragment that contained the 2 kbp Amp/LacZ cassette in the UL99 KO BAC (data not shown). Nucleotide sequencing of BAC DNAs that were mutated by introduction of point mutations encoding translational stop codons verified the introduction of a stop codon into the predicted location and the insertion of a translational stop at the predicted location in the aa sequence. After electroporation of HF cells with DNAs from these recombinant BACs, infectious virus was recovered from pp28 deletion mutants expressing the first 50, 61, 90, 123, or 155 aa (STOP50, 61, 90, 123, or 155, respectively)

of pp28 (Fig. 7). As reported previously, infectious virus was not recovered following electroporation of HCMV BAC containing a gly→ala mutation at codon 2 and insertion of a stop codon at codon 4 (7). Infectious virus was also not recovered from a mutant BAC in which a stop codon had been inserted at codon 34 (STOP33), nor was infectious virus recovered from a recombinant BAC in which amino acids 45-59 were deleted (pp28Δac) (Fig. 7). Furthermore, we also failed to recover infectious virus from a recombinant BAC in which sequences encoding aa 45-59 were deleted and then transplanted to the C-terminus of pp28 (pp28Δac-CtTR) (Fig. 7). Revertants of some of the BACs that failed to produce infectious virus were generated and infectious virus could be recovered from these mutant HCMV BACs (Fig. 7). In the case of the pp28STOP33 mutant, the revertant was generated by linear recombination using a single defined oligonucleotide (Rev33) to repair the stop codon at position 33. Thus, the loss of infectivity in pp28STOP33 mutant was secondary to the engineered mutation and not by additional mutations introduced by the mutagenesis procedure. In the case of the acidic cluster deletion mutant in pp28 (pp28Δac), small plaques were observed shortly after electroporation of HF cells with the BAC DNA but these failed to increase in size and infectious virus was not recovered from the supernatant of these cultures (data not shown). To confirm expression of the truncated pp28s from the replication competent recombinant viruses, we performed western blot analysis of recombinant virus-infected HF cells at 5 dpi (Fig. 8). As expected, truncated pp28 was expressed from each recombinant virus. Because we repaired mutations in the coding sequence of pp28 by linear recombination and electroporation of a mixture of both repaired and mutant BAC DNA, revertants including Rev33 generated by recombination with a repair

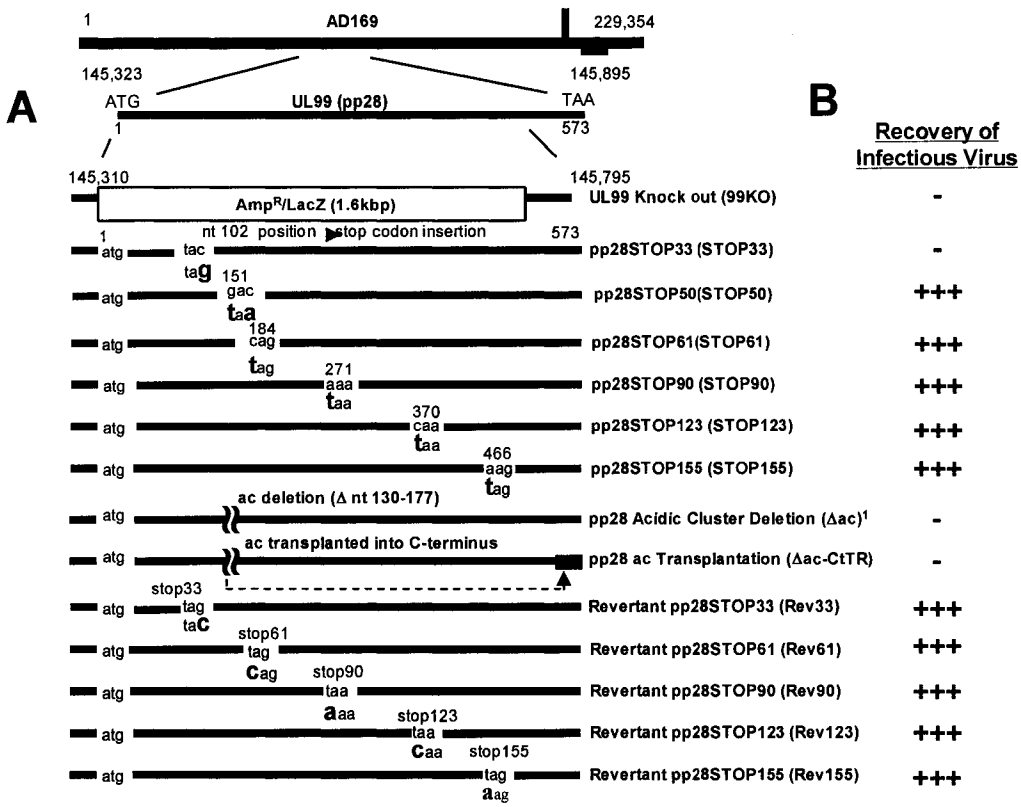

FIG. 7. Construction of UL99 recombinant BACs containing mutations in pp28. (A) Recombinant BACs containing pp28 deletion mutants were generated by insertion of single nucleotide (nt) changes in UL99 coding sequence that altered wild type codons to translational stops. Mutations were confirmed by sequence analysis. Revertant or repaired BACs were made by oligonucleotide directed repair of the single nt mutations. Recombinant viruses were recovered by electroporation of BAC DNA into HF cells. (B) Successful recovery of infectious viruses is listed on the right. In all cases, at least 3 independent attempts were made to recover infectious virus. The acidic cluster deletion BAC[1] was made by deletion of nt 130-177 in UL99 coding sequence. A virus was recovered from this pp28Δac BAC but it formed small and very slowly expanding plaques. We could not isolate cell-free infectious virus from these cultures nor did the plaques expand through the monolayer.

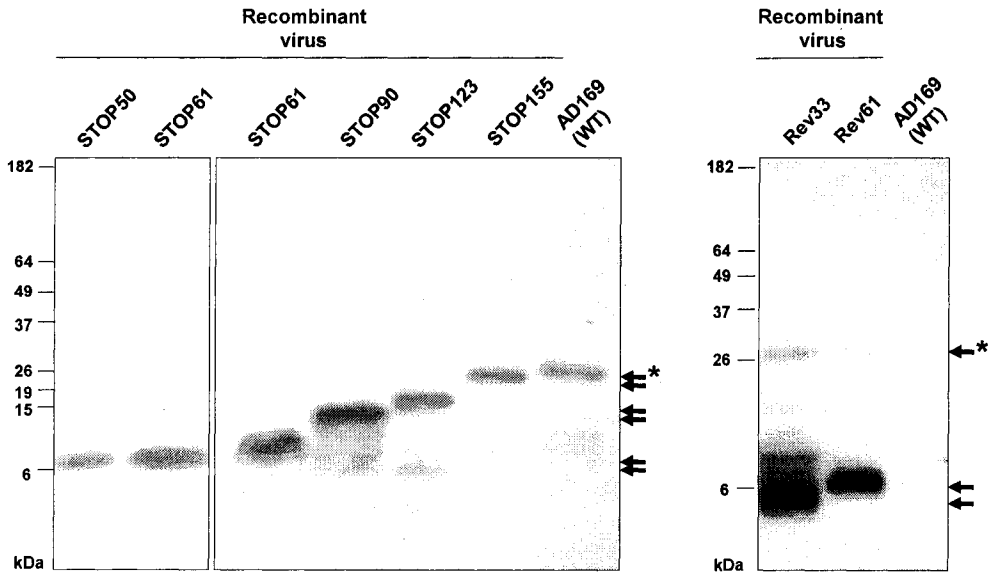

FIG. 8. Expression of truncated pp28 in HF cells infected with UL99 recombinant virus. HF cells infected with recombinant virus at an moi of 0.1. The cells were harvested at day 5 post infection, lysed, and detected with anti-pp28 MAb in a western blot analysis as described in Materials and Methods. Arrows indicate the mass predicted in kDa from the aa sequence of truncated pp28. Asterisk (*) indicates the migration of the wild type pp28.

oligonucleotide expressed both wild type pp28 and truncated pp28. This result suggested that at least in the case of pp28STOP mutants, a replication defective genome was maintained in these stocks of replicating virus that had been passaged through at least 3 serial passages. The phenotypes of the recombinant viruses generated from this panel of mutant HCMV BACs were characterized. As an example, image analysis showed that intracellular localizations of the mutant pp28 from the recombinant virus containing the pp28Mut50 mutant and other viral proteins including gB, gM/gN, and pp150 in infected cells were similar to that of wild type parental virus (data not shown). This result indicated that a pp28 deletion mutant virus expressing only the first 50 aa of pp28 contained sufficient targeting information for authentic intracellular localizations of this mutant tegument protein with other viral proteins required for virus replication. To determine the kinetics of replication for viruses recovered from the mutant BACs, the replication kinetics of each mutant virus and revertants of mutants that failed to support virus replication were compared to wild type HCMV in single-step growth assays. The growth curves for all recombinant viruses with the exception of pp28STOP50 were similar to that of the wild type parental virus (Fig. 9). The replication kinetics of the pp28STOP50 virus was delayed in comparison to wild type and the pp28STOP61 virus and the virus yield after 7 days in culture was approximately 10 fold less than wild type (Fig. 9). The decreased yield was even more obvious at lower moi (data not shown). These results indicated that the first 50 aa were sufficient for production of infectious virus and for normal trafficking of pp28 in the infected cell but that wild type virus replication kinetics required the first 61 aa.

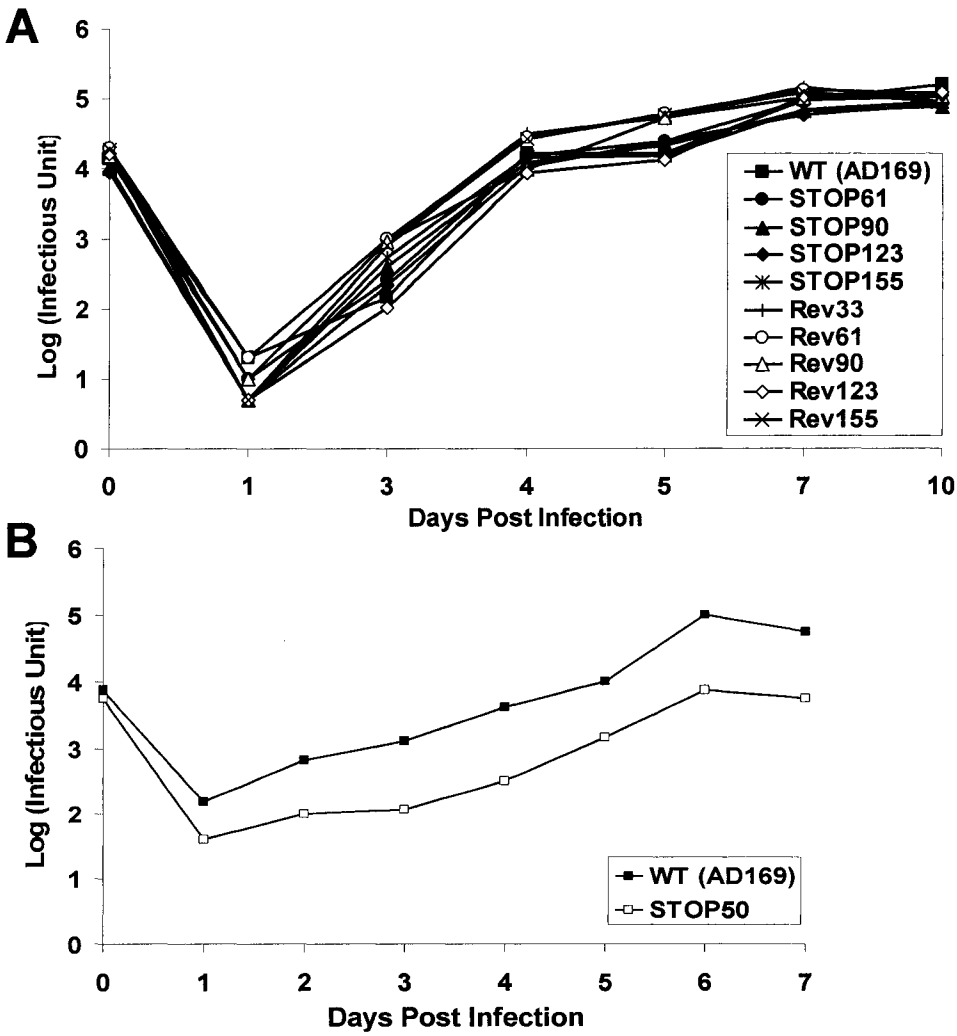

FIG. 9. Replication kinetics of wild-type and recombinant viruses. Virus titer was quantified by fluorescent based virus infectivity assay on HF cells infected with WT or recombinant viruses at the indicated times after an initial infection using an moi 0.1. Results are expressed as $\log_{10}$/ml (A) wild-type (■) versus STOP61 (●), STOP90 (▲), STOP123 (♦), STOP155 (*), Rev33 (+), Rev61 (○), Rev90 (Δ), Rev123 (◊), or Rev155 (x) virus. (B) wild-type (■) versus STOP50 (□) virus.

DISCUSSION

Although there is a consensus that HCMV virion assembly, more specifically virion envelopment, takes place in the cytoplasm of infected cells, the trafficking of protein constituents of the virion tegument and envelope to cytoplasmic sites of assembly is not well understood. Because localization of tegumented capsids, tegument proteins, and envelopment proteins to the site of virion envelopment are a prerequisite for the assembly of the infectious particle, characterization of intracellular trafficking of essential virion structural proteins should offer insights into the assembly of this large virus. In this study we investigated the sequence requirements for localization of the essential tegument protein, pp28, to a cytoplasmic site of virus assembly in HF cells, the AC. Our findings indicated that the first 50 amino acids of pp28 were required for localization to the AC and more importantly, a mutant virus encoding only the first 50 aa was replication competent. This finding was similar to the results of previous studies from Jones, et.al. that described a virus mutant expressing only the first 57 aa of pp28 that was replication competent(17) . Carboxyl-terminal deletions of the pp28 sequence at aa 44 resulted in a null phenotype of a recombinant virus leading these investigators to postulate that acid amino acids between positions 44-57 were responsible for localization of pp28 to sites of assembly, perhaps through interactions with cellular adaptor proteins (17). We also found that deletion of the acidic cluster (aa 44-59) in pp28 resulted in a pp28 mutant virus that was defective in production of infectious virus; however, our mutant virus was constructed differently than the C-terminal deletion mutants (aa 57 and aa 43) described in this earlier study in that this stretch of amino acids were deleted by excision of this sequence from the full length protein. Truncation mutants of pp28 retaining only the first 30 or 40 aa also exhibited a similar phenotype as the pp28 mutant

containing the internal deletion of aa 44-59 in that, they failed to localize to the AC and support virus replication. Thus, our studies utilizing both image and cell fractionation assays indicated that localization of pp28 to the AC late in infection was required for the assembly infectious virus and that the minimal sequence requirements for localization of pp28 to the AC were contained within the first 50 aa of the molecule. It is unclear whether domains or sequences within these 50aa are also responsible for other as yet undefined functions of pp28 in virion assembly after its localization to the AC.

Previously we have shown that pp28 localizes in the ERGIC in the absence of other viral functions. This initial observation was confirmed by the finding that pp28 transiently expressed in virus infected cells failed to localize in the assembly compartment in transfected/infected cells treated with ganciclovir, an inhibitor of late gene expression, but by a drug (BDCRB) that has been shown to inhibit DNA packaging but not late protein synthesis. Thus, intracellular trafficking of pp28 differs from the ∀-herpesvirus homologue, UL11, a virion tegument protein that has been reported to traffic to the Golgi and TGN when expressed in the absence of virus infection (22). We also determined that localization of pp28 to the ERGIC required expression of the first 30 aa based on findings that truncated forms of pp28 containing less than the first 30 aa co-localized with the ER resident proteins such as calreticulin. In contrast, deletion mutants expressing only the first 35 aa of pp28 trafficked similarly to wild type pp28 and co-localized with ERGIC localized proteins, suggesting that sequences within the first 35aa were required for ERGIC localization. It is unclear if specific sequences within this stretch of aa target pp28 to the ERGIC. Although amino acid sequences that target specific proteins to the ERGIC have not been well defined, both a carboxyl terminal dilysine ER retrevial signal and a phenylalanine ER export signal have been associated

with localization to this compartment presumably through export from and retrieval to the ER (14, 21, 29, 30). Because this compartment is dynamic, localization of proteins within this compartment likely requires interactions with host cell proteins that partition within this compartment such as ERGIC 53 (3). Several well studied ERGIC localized proteins are either integral membrane protein or membrane associated secondary to myristoylation and/or palymitoylation modifications. Interestingly, without the myristoylation modification, pp28 traffics throughout the cytoplasm and even enters the nucleus suggesting that membrane association of pp28 is necessary but not sufficient for its localization in the ERGIC (35). Whether other sequences within the first 35 aa of pp28 contribute to membrane association is not known, although preliminary studies have suggested that the protein is palmitoylated and a consensus sequence for this post-translational modification can be identified in the NH_2 terminus of pp28 (data not shown). Even though our results are consistent with the interpretation that specific aa residues within the amino terminal 35 aa of pp28 are required for ERGIC localization, other explanations include a loss of protein structure following deletions of these aa that results in the disruption of interactions with host proteins necessary for ERGIC localization.

The mechanism(s) that lead to pp28 trafficking from the ERGIC to the AC in virus infected cells is a second and perhaps more interesting question. At least two possibilities can be proposed based on our findings. The first is that HCMV infection and late gene expression remodel the cellular secretory compartments resulting in the approximation of the proximal compartments such as the ERGIC with more distal compartments including the TGN. Thus, viral protein trafficking through the secretory pathway could be less compartmentalized and could occur following mixing of closely

approximated viral protein containing vesicles. Alternatively, the dependence of pp28 localization to the AC on expression of late gene products raises the possibility that interactions between pp28 and other viral proteins results in the redistribution of pp28 from the ERIGIC to more distal sites in the secretory pathway. This mechanism has been observed in assembly pathways of other viruses, including vaccinia and Mason-Pfizer monkey virus (32, 36). Preliminary findings have suggested that pp28 localizes to the distal compartments of the secretory pathway when transiently expressed with HCMV virion glycoproteins (data not shown) suggesting that pp28 could traffic with viral glycoproteins in virus infected cells. This protein interaction could provide coordinated transport of this essential tegument protein with virion glycoproteins to the AC.

The defect in AC localization by the pp28 deletion mutants pp28Mut35, 40 and the internal deletion mutant, pp28Δac (deletion of aa 44-59) was readily observed in transfected/infected cells on day 7 post infection. The distribution of these pp28 mutants in the infected cell were clearly different than that of wild type pp28 or mutant ppMut50 or 61 in that deletion mutants at aa positions 35, 40 or 44-59 remained localized outside of the AC in a distribution that was consistent with proteins of the Golgi or ERGIC. Imaging studies of cells co-expressing Myc tagged pp28Mut35 and EGFP ManII suggested that this mutant form of pp28 co-localizes with this Golgi protein late in infection, suggesting that this mutant form of pp28 entered the secretory pathway but was not transported to the AC (data not shown). The correlation between pp28 localization to the AC and the assembly of infectious virions strongly argued that virus replication and production of infectious virus was dependent on pp28 localization within the AC. The finding that only the first 50 aa of this 191 aa protein were essential for the localization of pp28 to the AC and for the replication of infectious virus and prompted questions about

the function(s) of the remaining C-terminal 141 aa of pp28 that were non-essential for the replication of this virus in HF cells. Several possibilities could be considered such as protein interactions with virion proteins that are not essential for infectivity in HF cells, or alternatively, interactions between these sequences and host cell proteins that are destined for incorporation into the virion. It is also of interest that mutant pp28STOP50 that expressed only the first 50 aa of pp28 could be localized to the AC in infected cells and was incorporated into the virion even though the pp28STOP50 recombinant virus replicated less efficiently than the wild type virus. This finding suggested that the replication defect in pp28STOP50 virus was unrelated to authentic intracellular trafficking of pp28 but perhaps secondary to a loss or decrease in another function of pp28 such as interactions with virus-encoded or host cell proteins that contribute to the efficiency of virus assembly. Alternatively, the impairment in replication of pp28STOP50 virus could be secondary to a defect in the kinetics of localization of the mutant pp28 protein to the AC such that late in infection, a smaller fraction of pp28Mut50 localized to the AC and was available for assembly of infectious virions.

The role of the cluster of acidic aa 44-59 in pp28 in the assembly of infectious virus has not been defined but it has been suggested to be critical for localization to the AC through its interactions with cellular adaptor proteins such as PACS-1 (17). This potential function of the NH_2 terminal acid cluster of pp28 has not been formally demonstrated and is based on the extrapolation of findings of studies of the ∀-herpesvirus homolog, UL11 (22). As noted previously, the UL11 protein of HSV is localized to the Golgi and TGN in virus infected cells and in cells transfected with an expression plasmid encoding UL11 (22). The deletion of the acidic cluster in the amino terminus of UL11 results in a protein that is expressed on the plasma membrane of transfected cells and also

in the Golgi and TGN (22). This finding together with the observation that the function of the acidic cluster of aa in the trafficking of UL11 in transient expression assays could be replaced with the PACS-1 binding consensus aa sequence from furin suggested a potential role of PACS-1 in the intracellular trafficking of UL11 (22). These authors also noted that targeting of HSV UL11 to the Golgi appeared independent of the acidic cluster but required membrane association through fatty acid modifications in the extreme amino-terminus of the molecule (22). Thus, the role of the cluster of acidic aa in the amino terminus of UL11 in trafficking of this molecule remains unclear.

Although HSV UL11 and HCMV pp28 are presumed to be functional homologues and share some structural similarities, including a positionally conserved acid cluster of aa, several characteristics of the intracellular trafficking of HCMV pp28 appear to differ fundamentally from that of UL11. The first is that HCMV pp28 localizes in the ERGIC in the absence of other viral proteins and not in Golgi or TGN as has been reported for UL11 (35). In addition, unlike UL11 we have not detected the expression of pp28 nor pp28 mutants on the plasma membrane in transfected cells. It should be noted however, that we have detected expression on the plasma membrane in an occasional cell in transfection/infection assays following overexpression of pp28 very early in infection (data not shown), a finding that was reported by Jones, et.al using ectopic expression of pp28 from a strong, constitutive promoter (17). Furthermore, pp28 deletion m utants that fail to localize in the AC are not expressed on the plasma membrane in transfected/infected cells (data not shown). Thus, our findings suggest that the cluster of acidic aa in pp28 does not play an obvious role in retrieval from the plasma membrane as was observed in experiments with HSV UL11 (22) . Secondly, it has been reported that inhibition of PACS-1 expression with siRNA resulted in only a 2-3 fold decrease in virus

production whereas mutations in the UL99 orf that alter intracellular trafficking this viral protein result in the loss of infectious virus production (12). Thus, it could be argued that if pp28 interacts with PACS-1, then this interaction likely has only a limited role in localization of pp28 to the AC. Alternatively, the acidic aa cluster between aa 44-59 could interact with cellular adaptor proteins other than PACS-1; however, this motif lacks other requisite signals such as adjacent dileucines that have been shown to function as signals recognized by cellular adaptor proteins responsible for trafficking between compartments of the secretory pathway (5, 31). Lastly, it was of interest that transplantation of the NH_2 terminal acid cluster of pp28 (aa 44-59) to the carboxyl terminus of the molecule failed to direct trafficking of this molecule to the AC and when this UL99 mutant was incorporated into the viral genome, this recombinant molecule also failed to support virus replication. This result indicated that the role of the acid cluster in pp28 (aa 44-59) in localization to the AC and virus assembly was context dependent, a finding that is in contrast to the context independence of PACS-1 binding sequences and other trafficking motifs used to generate chimeric molecules for studies of intracellular trafficking. Thus, even though two independent studies have shown that the NH_2 terminal acid cluster of pp28 plays a critical role in the localization of pp28 to the AC late in infection, the mechanism through which this sequence of aa functions to direct this molecule to the AC remains to be determined.

In an earlier report we noted that the AC was localized in a cellular compartment that failed to co-localize with markers of the Golgi or TGN and exhibited only minimal overlap with lysosomal markers such as LAMP-1 (34). In the current study we further characterized the AC in transfection/infection assays in which EGFP tagged markers of the Golgi and trans-Golgi were used to mark these secretory compartments. These

experiments were carried out to further define the trafficking of pp28 and pp28 mutants to the AC, but unexpectedly we found that the secretory pathway was morphologically remodeled during virus infection resulting in the loss of ribbon-like stacks of the Golgi and TGN and formation of a juxtanuclear, more spherically shaped organelle. The remodeling of the secretory pathway began within 72 hr post infection and host cell markers such as mannosidase II and TGN 38 (gal T) co-localized with cytoplasmic structural virion proteins, including gB and gM/gN, as well as pp28 that was expressed transiently from an HCMV immediate early promoter in cells transfected with this plasmid and infected with wild type virus. However, at this relatively early time in infection, the characteristic compact AC containing virion structural proteins that has been described late in infection was not present. Only late in infection (>120 hr in these experiments) was the compact structure containing tegument proteins pp28 and pp150 localized to the center of the AC. These results suggested that the formation of the AC was associated with remodeling of the secretory compartment with the eventual displacement of host cell proteins of the secretory compartment to the periphery of this structure, a finding consistent with earlier studies (34). Furthermore, the dynamics of the changes in the morphology of the secretory compartment provided an explanation for results from experiments using transiently expressed pp28 mutants that demonstrated that pp28 mutants of greater than 35 aa of the amino-terminus of pp28 could localize in to the ERGIC in transfected cells and also traffic to a perinuclear compartment early in infection in transfected/infected cells. Although , this finding that was initially interpreted as evidence that even the shortest pp28 truncation mutant, including several mutants that failed to support virus replication when recombined into the viral genome could localize to a juxtanuclear compartment, we subsequently demonstrated that only

those pp28 mutants that localized to the compact AC that formed late in infection supported virus replication. These observations were consistent with a morphologic maturation of the AC during infection that can be described as a compaction of this virion protein containing structure during maximal virus production in infected HF cells. Thus, it is possible that the characteristic compact appearance of the AC represents the concentration of virion structural proteins within this membranous compartment during the assembly of infectious particles and that the rate of production of infectious virus is governed by the concentration of structural proteins in this compartment. This pathway could in turn be dependent on viral protein trafficking to this compartment. Previous studies have demonstrated that virions lacking pp28 fail to become enveloped and can be found as individual non-enveloped particles in the cytoplasm of infected cells (37). Together with findings presented in the current study, these results suggested that the failure of pp28 mutants to localize in the AC at late times in infection could be expected to result in a loss of infectious virus production. An extension of this proposed function of pp28 during virus assembly would be that once pp28 is localized to the AC by targeting signals located between aa 44-50, other post-localization functions such as interactions with other viral proteins and/or host cell proteins are required for virion envelopment and virus assembly.

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REFERENCES

1. **Abate, D. A., S. Watanabe, and E. S. Mocarski.** 2005. Major human cytomegalovirus structural protein pp65 (ppUL83) prevents interferon response factor 3 activation in the interferon response. Journal of Virology **78:**10995-1006.

2. **Andreoni, M., M. Faircloth, L. Vugler, and W. J. Britt.** 1989. A rapid microneutralization assay for the measurement of neutralizing antibody reactive with human cytomegalovirus. Journal of Virological Methods **23:**157-67.

3. **Appenzeller, C., H. Andersson, F. Kappeler, and H. P. Hauri.** 1999. The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. Nature Cell Biology **1:**330-4.

4. **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.

5. **Bonifacino, J. S., and L. M. Traub.** 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annual Review of Biochemistry **72:**395-447.

6. **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.

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

8. **Britt, W. J., M. A. Jarvis, D. D. Drummond, and M. Mach.** 2005. Antigenic domain 1 is required for oligomerization of human cytomegalovirus glycoprotein B. Journal of Virology **79:**4066-79.

9. **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.

10. **Browne, E. P., and T. Shenk.** 2003. Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. Proceedings of the National Academy of Sciences of the United States of America. **100:**11439-44.

11. **Cantrell, S. R., and W. A. Bresnahan.** 2005. Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early

gene expression and viral replication. Journal of Virology **79:**7792-802.

12. **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. Journal of Virology. **77:**11105-13.

13. **Crump, C. M., Y. Xiang, L. Thomas, F. Gu, C. Austin, S. A. Tooze, and G. Thomas.** 2001. PACS-1 binding to adaptors is required for acidic cluster motif-mediated protein traffic. EMBO Journal. **20:**2191-201.

14. **Dogic, D., A. Dubois, B. de Chassey, Y. Lefkir, and F. Letourneur.** 2001. ERGIC-53 KKAA signal mediates endoplasmic reticulum retrieval in yeast. European Journal of Cell Biology **80:**151-5.

15. **Hayashi, M. L., C. Blankenship, and T. Shenk.** 2000. Human cytomegalovirus UL69 protein is required for efficient accumulation of infected cells in the G1 phase of the cell cycle. Proceedings of the National Academy of Sciences of the United States of America. **97:**2692-6.

16. **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. Journal of Virology **74:**7720-9.

17. **Jones, T. R., and S. W. Lee.** 2004. An acidic cluster of human cytomegalovirus UL99 tegument protein is required for trafficking and function. Journal of Virology **78:**1488-502.

18. **Kalejta, R. F., and T. Shenk.** 2003. The human cytomegalovirus UL82 gene product (pp71) accelerates progression through the G1 phase of the cell cycle. Journal of Virology. **77:**3451-9.

19. **Kerry, J. A., M. A. Priddy, C. P. Kohler, T. L. Staley, D. Weber, T. R. Jones, and R. M. Stenberg.** 1997. Translational regulation of the human cytomegalovirus pp28 (UL99) late gene. J Virol **71:**981-987.

20. **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.

21. **Lontok, E., E. Corse, and C. E. Machamer.** 2004. Intracellular targeting signals contribute to localization of coronavirus spike proteins near the virus assembly site. Journal of Virology **78:**5913-22.

22. **Loomis, J. S., J. B. Bowzard, R. J. Courtney, and J. W. Wills.** 2001. Intracellular trafficking of the UL11 tegument protein of herpes simplex virus

type 1. Journal of Virology **75:**12209-19.

23. **Lu, M., and T. Shenk.** 1999. Human cytomegalovirus UL69 protein induces cells to accumulate in G1 phase of the cell cycle. J Virol **73:**676-683.

24. **Mach, M., B. Kropff, M. Kryzaniak, and W. Britt.** 2005. Complex formation by glycoproteins M and N of human cytomegalovirus: Structural and functional aspects. J. Virol. **79:**2160-2170.

25. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. Journal of Virology. **76:**1537-47.

26. **Meyer, H., A. Bankier, M. P. Landini, and R. Ruger.** 1988. Identification and procaryotic expression of the gene coding for the highly immunogenic 28-kilodalton structural phosphoprotein (pp28) of human cytomegalovirus. J Virol **62:**2243-2250.

27. **Mocarski, E. S., Tan Courcelle, C.** 2001. Cytomegaloviruses and their replication, p. 2629-2673. *In* D. M. K. a. P. M. Howley (ed.), Fields Virology, 4th ed, vol. 2. Lippincott Williams and Wilkins, Philadelphia.

28. **Molloy, S. S., E. D. Anderson, F. Jean, and G. Thomas.** 1999. Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis. Trend Cell Biol **9:**28-35.

29. **Nufer, O., S. Guldbrandsen, M. Degen, F. Kappeler, J. P. Paccaud, K. Tani, and H. P. Hauri.** 2002. Role of cytoplasmic C-terminal amino acids of membrane proteins in ER export. Journal of Cell Science **115:**619-28.

30. **Nufer, O., F. Kappeler, S. Guldbrandsen, and H. P. Hauri.** 2003. ER export of ERGIC-53 is controlled by cooperation of targeting determinants in all three of its domains. Journal of Cell Science **116:**4429-40.

31. **Ohno, H., R. C. Aguilar, D. Yeh, D. Taura, T. Saito, and J. S. Bonifacino.** 1998. The medium subunits of adaptor complexes recognize distinct but overlapping sets of tyrosine-based sorting signals. Journal of Biological Chemistry **273:**25915-21.

32. **Roper, R. L., and B. Moss.** 1999. Envelope formation is blocked by mutation of a sequence related to the HKD phospholipid metabolism motif in the vaccinia virus F13L protein. Journal of Virology **73:**1108-17.

33. **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.

34. **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. Journal of Virology **74:**975-86.

35. **Sanchez, V., E. Sztul, and W. J. Britt.** 2000. Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. Journal of Virology **74:**3842-51.

36. **Sfakianos, J. N., and E. Hunter.** 2003. M-PMV capsid transport is mediated by Env/Gag interactions at the pericentriolar recycling endosome. Traffic **4:**671-80.

37. **Silva, M. C., Q. C. Yu, L. Enquist, and T. Shenk.** 2003. Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids. Journal of Virology. **77:**10594-605.

38. **Sims, A. C., J. Ostermann, and M. R. Denison.** 2000. Mouse hepatitis virus replicase proteins associate with two distinct populations of intracellular membranes. Journal of Virology **74:**5647-54.

39. **Stockli, J., and J. Rohrer.** 2004. The palmitoyltransferase of the cation-dependent mannose 6-phosphate receptor cycles between the plasma membrane and endosomes. Molecular Biology of the Cell **15:**2617-26.

40. **Storrie, B., J. White, S. Rottger, E. H. Stelzer, T. Suganuma, and T. Nilsson.** 1998. Recycling of golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. Journal of Cell Biology **143:**1505-21.

41. **Trus, B. L., W. Gibson, N. Cheng, and A. C. Steven.** 1999. Capsid structure of simian cytomegalovirus from cryoelectron microscopy: evidence for tegument attachment sites.[erratum appears in J Virol 1999 May;73(5):4530]. Journal of Virology. **73:**2181-92.

42. **Underwood, M. R., R. J. Harvey, S. C. Stanat, M. L. Hemphill, T. Miller, J. C. Drach, L. B. Townsend, and K. K. Biron.** 1998. Inhibition of human cytomegalovirus DNA maturation by a benzimidazole ribonucleoside is mediated through the UL89 gene product. Journal of Virology. **72:**717-25.

43. **Varnum, S. M., D. N. Streblow, M. E. Monroe, P. Smith, K. J. Auberry, L. Pasa-Tolic, D. Wang, D. G. Camp, K. Rodland, S. Wiley, W. Britt, T. Shenk, R. D. Smith, and J. A. Nelson.** 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. Journal of Virology **78:**10960-10966.

44. **Wentz-Hunter, K., R. Kubota, X. Shen, and B. Y. Yue.** 2004. Extracellular myocilin affects activity of human trabecular meshwork cells. Journal of Cellular

Physiology **200:**45-52.

45. **Yu, X., S. Shah, I. Atanasov, P. Lo, F. Liu, W. J. Britt, and Z. H. Zhou.** 2005. Three-dimensional localization of the smallest capsid protein in the human cytomegalovirus capsid. Journal of Virology **79:**1327-32.

46. **Zhou, Z. H., M. Dougherty, J. Jakana, J. He, F. J. Rixon, and W. Chiu.** 2000. Seeing the herpesvirus capsid at 8.5 A. Science. **288:**877-80.

CYTOPLASMIC ENVELOPMENT OF HUMAN CYTOMEGALOVIRUS REQUIRES A POSTLOCALIZATION FUNCTION OF TEGUMENT PROTEIN PP28 WITHIN THE ASSEMBLY COMPARTMENT

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ABSTRACT

The assembly of herpesvirus remains incompletely defined secondary to the structural complexity of these viruses. Although the assembly of the capsid of these large DNA viruses is well studied and reasonably well conserved for all members of this diverse family of viruses, the cytoplasmic processes of tegumentation and envelopment are not well understood. The virion of the largest human herpesvirus, human cytomegalovirus (HCMV), contains over 70 virus encoded proteins that are incorporated during a nuclear and cytoplasmic phase of assembly. Envelopment of this virus requires the function of at least one tegument protein, pp28, the product of the UL99 open reading frame. However, the role of pp28 in envelopment of HCMV remains undefined. We have generated a pp28 mutant virus that encodes only the first 50 amino acids (aa) of this 190 aa virion protein. This virus is replication impaired and is defective in virus assembly. Characterization of both intracellular and extracellular virions from cells infected with this viral mutant indicated that the decrease in production of infectious virus was secondary to a defect in envelopment and the accumulation of tegumented, non-infectious intracellular particles. Image analysis using fluorescence recovery after photobleaching (FRAP) indicated that the mutant pp28 protein encoded by this virus failed to efficiently accumulate in the virus assembly compartment. Our results suggest that pp28 must accumulate in the AC for efficient envelopment of the particle and provide evidence for a direct role of this tegument protein in late stages of assembly such as envelopment.

INTRODUCTION

Infection with human cytomegalovirus (HCMV) has been associated with acute and chronic disease in both normal and immunocompromised populations (7, 21, 28).

Although a considerable body of knowledge about its replication has been gathered, particularly the regulation of viral gene expression and interactions with cellular transcription programs during infection, a similarly detailed description of its assembly has not been accomplished. Like more well described α-herpesviruses such as herpes simplex virus (HSV) and pseudorabies virus (PRV), DNA replication, encapsidation and capsid assembly takes place in the nucleus of infected cells (19). The process of nuclear egress, cytoplasmic envelopment, and virion release from the infected cell have not been completely defined for HSV or PRV and these processes remains even less well defined for HCMV because of the increased structural complexity of this prototypic member of the β-herpesvirus family (2, 11-13, 16, 18, 19, 30). Moreover, the infectious particle of HCMV contains proteins that have no homologous proteins in HSV or PRV and based on the eclipse period in permissive cells, is assembled with significantly delayed kinetics as compared to either HSV or PRV (8, 19). Additional findings also highlight the potential differences in the assembly pathway of HCMV as compared to HSV or PRV, including the presence of tegumented particles in the nucleus of HCMV infected cells (27). Furthermore, several tegument and envelope proteins that have been shown to be dispensable for assembly of infectious HSV or PRV particles have homologous proteins in HCMV that are essential for the assembly of infectious virus (5, 15, 17, 18, 24, 26, 27). More recent studies have also argued that HCMV could be enveloped in a different cellular compartment than has been proposed for HSV or PRV (10, 18). These observations have lead investigators to postulate that the cytoplasmic assembly of HCMV could share some features of pathways proposed for HSV but also differ fundamentally in many aspects of virus assembly.

Several approaches for the study of HCMV have been reported but the most

informative have been characterization of viral mutants that exhibit phenotypic defects in assembly. Utilizing genetic systems introduced by Messerle and Koszinowski, several laboratories have described phenotypes of viral mutants with defined mutations in single open reading frames (orfs) (4, 5, 24, 26). Deletion of essential envelope glycoproteins such as gB, gH, or gM resulted in the loss of virus infectivity (14, 17). Similarly, the deletion of capsid proteins also prevented recovery of infectious virus (14). In contrast to these findings, viral mutants with deletions of tegument proteins have exhibited more variable phenotypes in terms of virus replication (9, 32). One such tegument protein that has been studied by several laboratories is pp28, the product of the UL99 orf. This protein has been shown to be essential for the production of infectious virus and deletion of this orf resulted in a viral mutant that could not assemble enveloped particles (5, 15, 26). A more recent report has described the intercellular spread of pp28 negative virions, although the relevance of this finding to the in-vivo behavior of HCMV is unclear at this time (25). pp28 is a true late protein that is membrane linked by myristoylation and by palmitoylation (Britt, unpublished data) and is localized to the cytoplasmic compartment designated the virus assembly compartment (AC) (22, 23). This compartment is thought to be a modified compartment of the cellular secretory system and distal to the tran-Golgi network, possibly a compartment derived from late endosomes (22, 24). Localization of pp28 to the AC is required for virus assembly and mutations that impaired its localization in this compartment also limited the production of infectious virus (24). Two different studies have shown that only 1st 60 amino acids (aa) of this 190 aa phosphoprotein are required for the generation of viruses that exhibit a phenotype of wild type virus replication (15, 24). Furthermore, these same studies demonstrated that infectious virus was not recovered following deletion of a 16 aa cluster of acidic aa between aa 44-59,

suggesting that this stretch of aa was essential for assembly of infectious virus (15, 24). The role of this acidic cluster in the function of pp28 in the assembly of infectious HCMV is unknown, although potential roles in the interaction with cellular adaptor proteins have been proposed (15). Regardless of the contribution of this domain of pp28 to its function in virus assembly, pp28 is an essential structural protein and thus far, available data points to its critical role in envelopment of the infectious particle.

To further define the role of pp28 in the assembly of HCMV, we have characterized the phenotype of a replication impaired virus mutant generated by insertion of a translational stop codon at nucleotide position 151 resulting in a virus encoding only the first 50aa of pp28. The phenotype of this mutant virus, pp28STOP50, was compared to a previously described viral mutant (pp28STOP61) that has been shown to exhibit a wild type phenotype in terms of virus replication, localization of the mutant pp28 protein to the assembly compartment, and assembly of infectious enveloped particles (24). The phenotype of the pp28STOP50 viral mutant was particularly informative because although replication impaired, it produced infectious particles that could be characterized during assembly without the necessity of complementation, such as the expression of pp28 in *trans*. Our results indicated that cells infected with the pp28STOP50 mutant virus assembled intracellular particles that contained similar quantities of viral DNA, the virus major capsid protein, and at least one viral tegument protein, yet were significantly less infectious than particles produced by cells infected with the pp28STOP61 virus. Additional studies including electron microscopy and quantitation of a major envelope glycoprotein, gB, in particles produced by the pp28STOP50 virus infected cells indicated that the majority of intracellular particles produced by this mutant virus were non-enveloped and thus,presumably non-infectious. The mutant pp28 protein encoded by this

virus appeared to contribute to this loss of envelopment by failing to accumulate in the AC of infected cells, arguing that the role of pp28 in envelopment is expressed after its localization in the AC. Finally, our results also suggested that incorporation of a threshold amount of pp28 during assembly is sufficient for envelopment of an infectious particle and in turn for the release into the extracellular supernatant.

MATERIALS AND METHODS

Cells and viruses.

Human primary fibroblasts (HF) were prepared and maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 5% newborn calf serum (NCS) and penicillin/streptomycin antibiotics as described previously (24). Wild type virus (strain AD169) was obtained by electroporation of the infectious clone HB-5 maintained in E.coli, into HF cells. This BAC was obtained from Drs. Martin Messerle and Ulrich Koszinowski (University of Munich, Germany) and was used to generate all recombinant viruses in this study (4). The recombinant viruses, pp28STOP61 and pp28STOP50 have been described previously and were constructed by insertion of a translational stop codon at nucleotide positions 184 and 151 respectively (24).

Virus infections, infectivity titrations, and real time PCR assays.

Monolayers of HF cells in 35 mm dishes were infected with designated multiplicity of infection (moi) for 1 hour with gentle rocking, washed and then incubated at 37°C, 5% CO_2. Dishes were harvested daily and either separated into cells and supernatant or frozen at -80°C until titrated for infectivity. Results are expressed as infectious units/ml. Titrations were carried as described (1). Briefly, 0.2ml of 10 fold

dilutions was seeded in replicates (2-3) in wells of a 96 well flat bottom containing HF monolayers. After an 1 hour incubation, the wells were washed, and the plate incubated for 16 hrs. The plate was then washed with phosphate buffered salt solution (PBS, pH 7.4) and then fixed in absolute ethanol for 20 min. The plate was then washed, a monoclonal antibody (mab) reactive with the IE-1 protein was added and reactivity developed with FITC anti-mouse IgG antibodies (1). The number of fluorescent nuclei were counted and results expressed as infectious units per ml of input virus. Quantiation of viral genome copy number was carried out using real time PCR as we have described previously using the UL55 as the target template (24).

Electron microscopic (EM) analysis.

HF cells were infected with wild type, pp28STOP61, or pp28STOP50 virus at a moi of 0.1 and processed for EM on day 5 post infection. Cells were collected from the culture plate in 1 ml of PBS, and centrifuged into pellets. The resulting pellets were then rinsed with 0.1M PBS and fixed with 2.5% glutaraldehyde in PBS for 1hr at room temperature. After a rinse in 0.1M PBS three times, pellets were postfixed in 1% osmium tetroxide for 1 hr at 4°C in the dark. These pellets were rinsed with 0.1M PBS three times and with water once more. The pellets were then dehydrated in a graded series of ethanol solutions beginning with 25% and ending with 100% and embedded on a copper grid. Approximately 90-nm sections of the pellets were stained with 1% uranyl acetate and lead citrate, and examined with a Hitachi 7000 series electron microscope at an acceleration voltage of 75 kV.

Gradient separation of extracellular and intracellular virions.

Extracellular and intracellular virions were purified by centrifugation through sorbitol gradients as previously described (6). Briefly, supernatant virus was isolated by initially clarifying the supernatant by centrifugation at 3,000 g for 15 minutes. Virions were pelleted by centrifugation of the clarified supernatant through a 20% sucrose cushion at 25, 000 rpm in a SW28 rotor for 1 hr. The pellet was then resuspended in 1 ml of tris buffered saline (pH 7.4) and applied to a preformed 20-70% sorbitol gradient (10 ml) and centrifuged at 27,000 rpm in a SW41 rotor for 1 hr at 16° C. Intracellular particles were prepared from post-nuclear supernatants of the same cultures of virus infected cells by passing cells repeatedly through a 25 gauge needle followed by clarification of the cell lysate at 6,000 rpm for 10 minutes. This material was loaded directly onto preformed 20-70% sorbitol gradients and centrifuged as described above. The gradients were fractionated into 1 ml fractions from the bottom by pumping mineral oil onto the top of the gradient. The fractions were tittered for infectivity and analyzed for viral DNA by real time PCR. Subsequently, individual fractions were analyzed for viral proteins by western blotting as described below.

Antibodies and western blotting.

Mabs used in this study include, anti-major capsid protein (MCP), mab 28-4; anti-pp28, mab 41-18; anti-gB, mab 7-17; anti-gM, mab IMP anti-pp65, mab 28-19 (22). Samples were analyzed by western blotting as described in previous publications and developed using enhanced chemiluminesence (Pierce Biotechnology, Rockford, Ill).

Imaging and fluorescence recovery after photobleaching (FRAP).

Immunofluorescence assays of viral protein expression in virus infected cells was carried out as previously described (24). FRAP analysis was performed at day 6 post infection in HF live cells in which EGFP tagged pp28 protein was expressed following electroporation of a plasmid encoding the first 35aa, 50 aa or 61 aa of pp28 or the full length of pp28 molecule fused at the C-terminus to EGFP. Electroportated HF cells were infected with HCMV 48 hrs later at a multiplicity of infection (moi) of 0.1. The infected HF cells were grown on a 13mm glass coverslip at 37°C in DMEM containing 10 % fetal calf serum. At day 6 post infection, the infected live HF cells on a coverslip were washed with 25mM Hepes-buffered phenol red-free DMEM, the coverslip was mounted on slides. A drop of this HEPES-buffered medium directly was added in the well created by rubber gasket (Molecular Probes) on a slide. The coverslip with live cells onto slide was sealed. Photobleaching was performed on a confocal laser scanning microscope (Leica SP2, NJ). Cells expressing EGFP fusion proteins in moderate amounts, i.e. sufficiently high fluorescence above background levels, were monitored using the 488 laser line of the argon laser at 25% power and bleached at 100% laser power. For assembly compartment bleach or cytoplasm (except nucleus and AC) bleach, the regions of interest were bleached with three subsequent bleach frames. The images were taken 5 frames at 20-second intervals for pre-bleaching, 3 frames at 2.9-second intervals for bleaching 40 frames at 30-second intervals for post-bleaching. The recovered fluorescence intensity in the region of interest was measured and normalized. The intensity in the pre-bleach image was set to 100%, and the first post-bleach image was set as time point 0. The average fluorescence intensity was plotted over time. The recovery curves shown are mean values and standard errors of the recovery curves of at least five cells (or three cells

for cytoplasm bleach experiments) and are representative of two independent experiments.

RESULTS

A replication competent HCMV expressing only the first 50 aa of pp28 has delayed replication kinetics.

Previous studies from our laboratory and other laboratories have reported that a stretch of acidic amino acids (aa) between amino acids 44-59 of pp28 were required for the function of pp28 in the assembly of infectious virus (Fig 1) (15, 24). In addition, the function of this cluster of acidic aa was context dependent in that deletion of this cluster of acidic aa and transplantation to the C-terminus of pp28 failed to restore the function of the protein (24). To further define the function of this acidic domain in pp28 we generated three recombinant viruses containing translational stop codons at aa position 44, 51, and 62 (Fig 1). As reported previously, infectious virus could be recovered from the pp28STOP61 and the pp28STOP50 HCMV BAC but not from the pp28STOP43 BAC (24). Although infectious virus could be recovered from the pp28STOP50 BAC, the replication kinetics of this recombinant virus was delayed as compared to both wild type virus and the pp28STOP61 virus, a mutant virus that replicates with similar kinetics as the wild type parent virus (24). Comparison of the replication kinetics of the pp28STOP50 and pp28STOP61 viruses at low multiplicity of infection (moi) revealed that the pp28STOP50 virus infected cells produced about 2 logs less virus than that produced by cells infected with the pp28STOP61 virus (Fig 2). Similar levels of viral genome replication were observed in cells infected with the pp28STOP50 and pp28STOP61 viruses indicating that the decrease in virus yield was not secondary to a

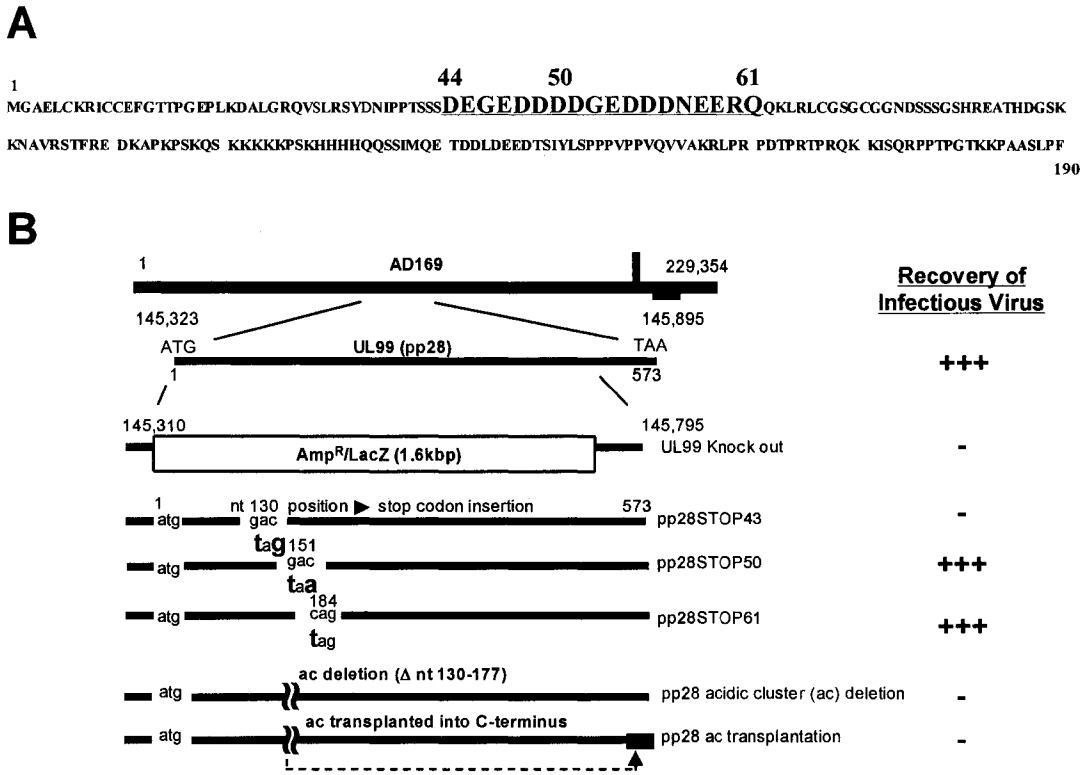

FIG. 1. Amino acid sequence of pp28 and diagrammatic representation of recombinant HCMV BACs constructed for generation of mutant viruses. (A) Amino acid sequence of pp28 with aa 44-61 bolded. The 16 aa acidic cluster is contained within this sequence. (B) The structure of the HCMV genome with the UL99 orf (nt 145,310-145,795) shown in greater detail. The two viral mutants characterized in this report, pp28STOP61 and pp28STOP50 have been previously described with the location of the translational stop codons indicated (24).

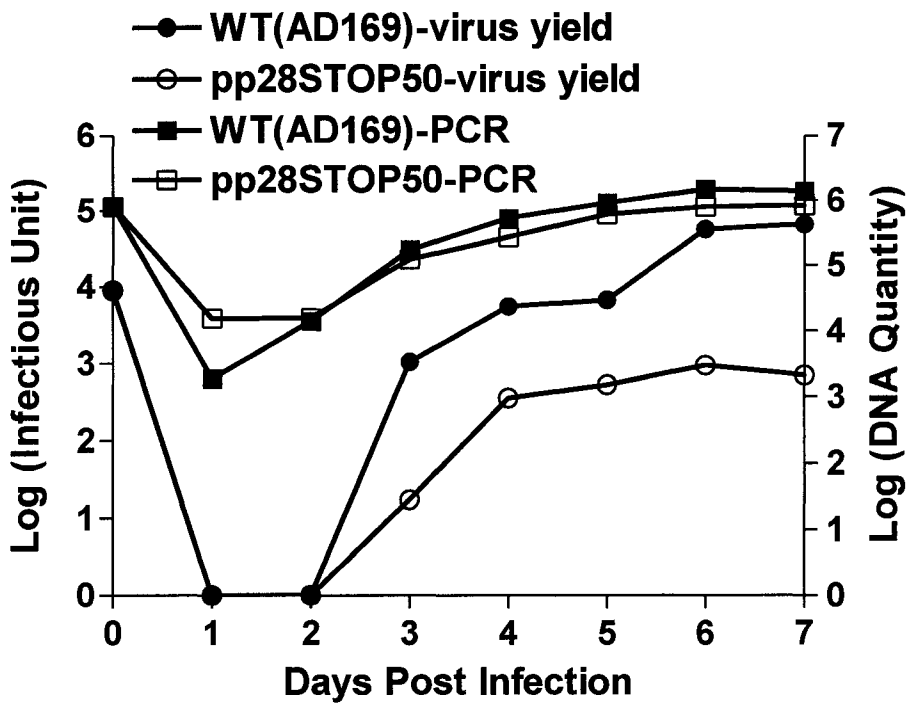

FIG. 2. Virus yield and viral DNA replication of HF cells infected with wild type and pp28STOP50 viruses (moi 0.01). Cells and supernatants from 35mm dishes infected with wild type and pp28STOP50 virus were combined and tittered as described in the Materials and Methods. Viral genome copy number was determined by real time PCR as previously described and reported as log of copy number (24).

defect in genome replication (Fig. 2). At higher moi (3-5), the differences in virus yields between the pp28STOP50 and pp28STOP61 viruses remained but were less apparent than that previously reported for infections using low moi (24).

The defect in the replication of the pp28STOP50 virus is not related to virus entry or spread.

To further investigate the role of pp28 in the replication and assembly of HCMV, we characterized the replication phenotype of the pp28STOP50 virus. Initially, we determined if the pp28STOP50 virus had a deficit in virus entry and/or spread within in a monolayer permissive human fibroblast (HF) cells. An assay of virus entry revealed that the pp28STOP50 virus entered HF cells as efficiently as the wild type and the pp28STOP61 virus, suggesting that the replication defect in the pp28STOP50 virus was not secondary to a defect in incorporation of a protein(s) essential for virus attachment and entry (Fig. 3). Furthermore, we also analyzed the capacity of the pp28STOP50 virus to spread between cells in a monolayer of HF cells maintained under an agarose overlay. This assay allowed us to measure the expansion of an infectious center as a function of time and thus provided quantitation of the capacity of these viruses to spread within a monolayer, independently of extracellular virus yield. We found no difference in the rate of spread between the pp28STOP50 and wild type virus again suggesting that the defect in virus production associated with the pp28STOP50 virus was not directly related to virus entry or spread between cells (Fig. 4). Our previous findings indicated that the pp28STOP50 virus replicated similar levels of viral genome as the wild type parental virus, thus it was unlikely that the decrease in virus yield from cultures infected with the pp28STOP50 virus was secondary to a defect in viral DNA replication (Fig. 2). Together

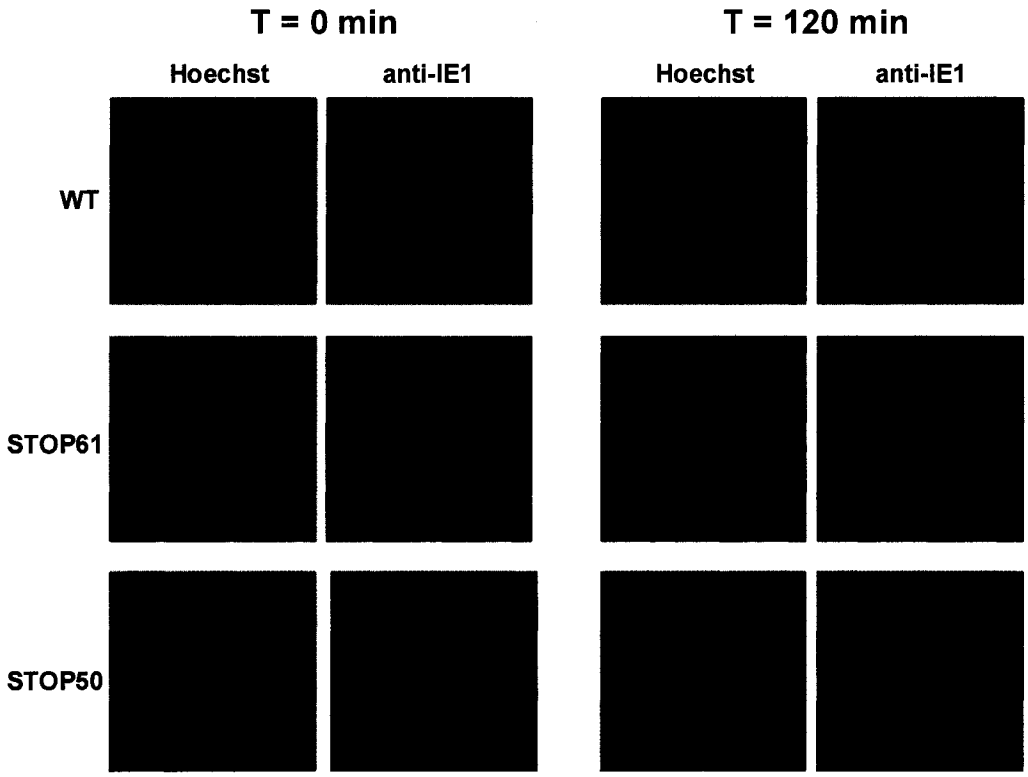

FIG.3. Virus entry of wild type, pp28STOP61, and pp28STOP50 viruses in HF cells. Monolayers of HF cells on 13mm coverslips were infected at 4° C with wild type, pp28STOP61 and pp28STOP50 viruses at a moi of approximately 5. Following an incubation at 4° C for 90 min, the cultures were shifted to 37° C for 2 hr. Coverslips were fixed at 0 min and 2 hr and then assayed for expression of IE-1 antigen using mab p63-27 developed with a Texas Red conjugated anti-mouse IgG (red). Nuclei were stained with Hoechst dye (blue).

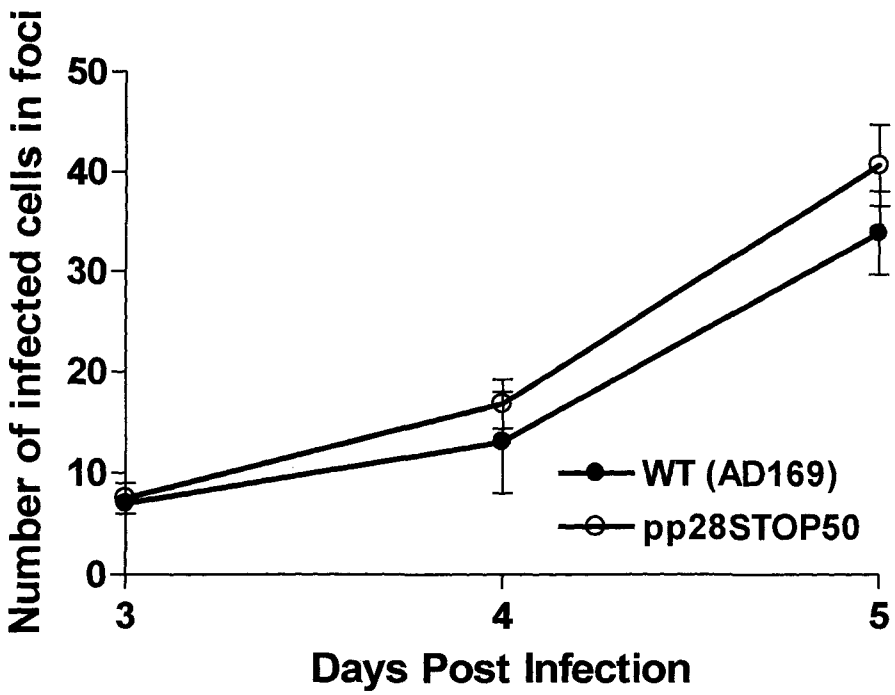

FIG. 4. Intercellular spread of wild type and pp28STOP50 virus. Monolayers of HF cells on 13mm coverslips were infected with wild type or pp28STOP50 virus at a moi of 0.1 and overlaid with agarose to prevent extracellular virus spread. The monolayers were fixed on day 3, 4, and 6 post infection and then stained with mab p63-27 to detect IE-1 expression. The number of nuclei per individual foci were counted. Approximately 20 foci per time point (and virus) were counted and the mean and standard error are shown at the various time points.

the results of these experiments suggested that the deficit(s) in infectious virus production in cells infected with the pp28STOP50 virus were likely related to a defect(s) in virus assembly and/or virus release.

The defect in the replication of the pp28STOP50 virus is associated with a decrease in the production of enveloped virions.

The assembly of the pp28STOP50, pp28STOP61, and wild type virus was investigated utilizing several assays. Initially, the assembly of enveloped virions was studied by electron microscopy. HF cells were infected with wild type, pp28STOP61, and pp28STOP50 virus at similar moi and fixed on day 5 post infection. The number of cytoplasmic capsids, tegumented capsids, and enveloped particles was counted in multiple frames and multiple cells within each frame (Fig. 5A). The ratio of enveloped particles to the total number of viral particles indicated that about 45% of wild type and pp28STOP61 viral cytoplasmic particles appeared to have an envelope (Fig. 5B). In contrast, using the same criteria that was used to identify enveloped particles in micrographs of the wild type and pp28STOP61 virus infected cells we determined that <15% of particles in cells infected with the pp28STOP50 virus were enveloped (Fig. 5B). In all cases, a similar number of particles could be observed in the nucleus of infected cells. This result suggested that the defect in the pp28STOP50 virus was associated with a decrease in the production of enveloped particles in the cytoplasm of infected cells.

These results were confirmed using a second methodology. Virions were gradient purified from HF cells that had been infected with similar moi of the pp28STOP61 and pp28STOP50 virus. In these experiments we used the pp28STOP61 virus instead of wild type virus for several reasons including; (i) the finding that the pp28STOP61 virus

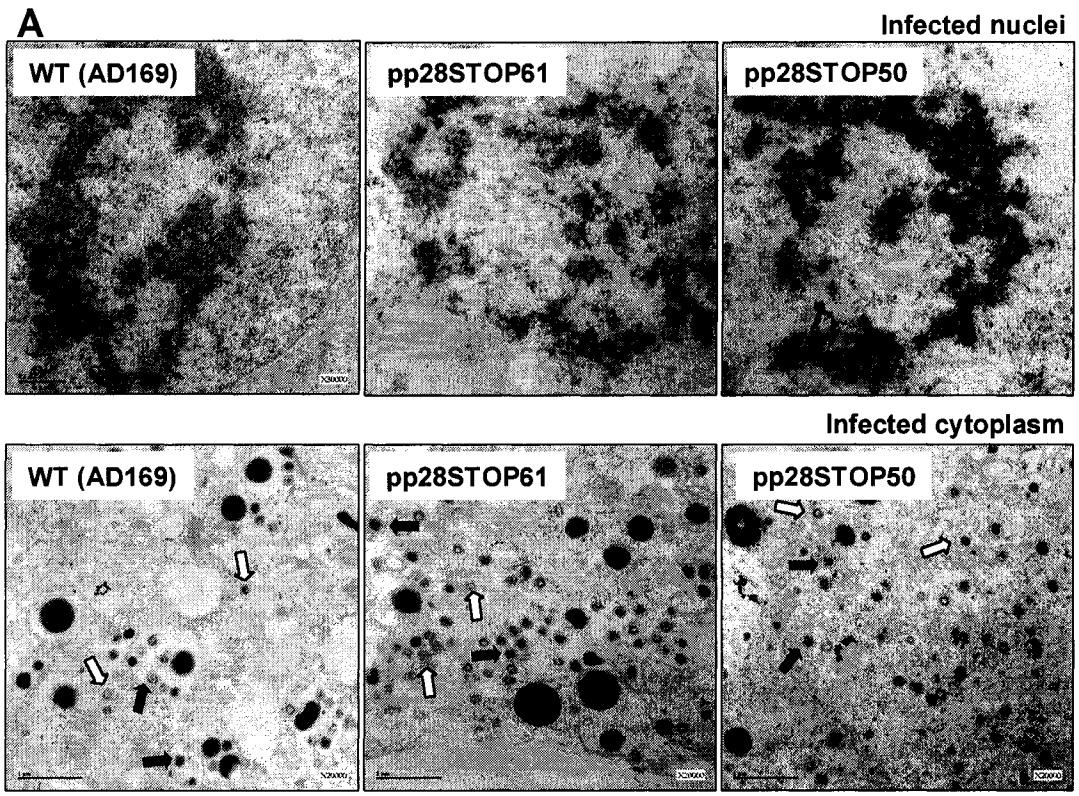

FIG. 5. Electron microscopic analysis of virus infected cells and estimation of frequency of enveloped particles in cytoplasm of infected cells. Monolayers of HF cells were infected with wild type, pp28STOP61 and pp28STOP50 viruses at a moi of 0.1 and processed for electron microscopy at 5 days post infection. (A) Multiple frames from each sample were imaged and photographed. Nuclear (top) and cytoplasmic particles (bottom) from a representative cell are shown at a final magnification of 30,000x or 20,000x, respectively. Open and filled arrows indicate non-enveloped particles and envelope particles respectively.

B

Cell	Ratios (enveloped virions / total viral particles)		
	WT	STOP61	STOP50
1	13 / 22	14 / 34	0 / 17
2	6 / 11	7 / 10	9 / 31
3	7 / 13	5 / 14	1 / 12
4	13 / 24	5 / 13	0 / 9
5	14 / 37	6 / 14	2 / 12
6	2 / 11	11 / 18	3 / 14
7	9 / 23	2 / 27	1 / 33
8	4 / 7	14 / 25	6 / 14
9	5 / 19	0 / 16	4 / 32
10	13 / 25	28 / 49	3 / 33
Total	86 / 192	100 / 237	29 / 207

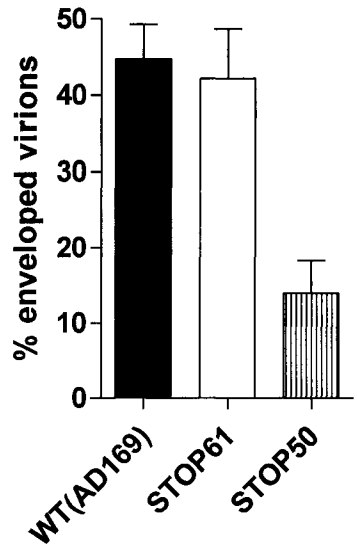

FIG. 5. (Continued) (B) The number of enveloped and non enveloped particles were counted in each frame and tabulated as a ratio of enveloped particles/total particles in the cytoplasm of infected cells (n=10). There was no significant difference between the ratio of enveloped particles/total particles for the wild type and pp28STOP61 mutant virus ($p > 0.5$) whereas the pp28STOP50 virus infected cells contained a smaller ratio of enveloped/total particles than either wild type ($p<0.001$) or pp28STOP61 ($p<0.001$) virus infected cells. Paired, one-tailed *t* tests were used to analyze the ratios of enveloped particles/total particles for the wild type, pp28STOP61, and pp28STOP50 viruses.

replicated to similar levels as wild type virus, (ii) the in-vitro replication phenotype of the pp28STOP61 virus was indistinguishable from wild type virus, and (iii) the pp28STOP61 virus allowed us to study the role of aa 44-59 of pp28 in virion assembly without introduction of the additional structural complexity of aa 62-190 in the wild type virus. Fractionation of the gradients revealed that infectious extracellular virus from both pp28STOP61 and pp28STOP50 virus infected cells exhibited similar densities and that peak infectivity could be found midway down the gradient, within one fraction of one another (Fig. 6A). The differences in the infectivity in these fractions was approximately 1.5 logs (Table 1), a value that was within the range of differences in viral DNA copy number determined for these fractions (Fig. 6B and Table 1). Thus, in this experiment it appeared that the pp28STOP50 virus infected HF cells produced infectious virions that banded with similar density following ultracentrifugation but the quantity of extracellular infectious particles and viral DNA containing particles was reduced in cells infected with the pp28STOP50 virus as compared to cells infected with the pp28STOP61 virus (Fig. 6A and B). Consistent with these results, total extracellular viral particles derived from HF cells that had been infected with the pp28STOP61 and pp28STOP50 virus prior to application to the gradient had similar differences in the infectivity (1.3 logs) and viral DNA copy number (1 log) (Table 1). These results suggested that the decrease in infectious virus production by cells infected with the pp28STOP50 virus could be explained by a decreased production of infectious extracellular particles and not secondary to the production of similar number of particles but with a decreased infectivity of individual particles.

Somewhat different results were noted when intracellular viruses were analyzed following purification by ultracentrifugation through density gradients. The results of

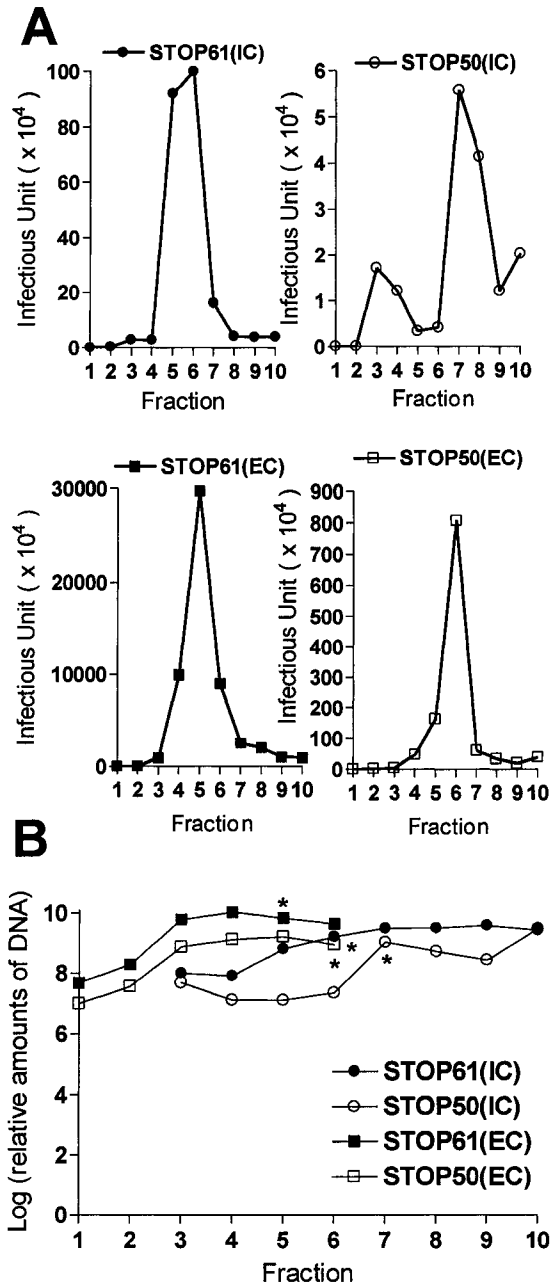

FIG. 6. Gradient separation and analysis of extracellular and intracellular viruses from pp28STOP61 and pp28STOP50 infected cells. (A) Intracellular (IC) and extracellular (EC) particles were prepared as described in Materials and Methods and fractionated on 20-70% sorbitol gradients as described (6). Gradient fractions (1ml) collected from bottom of density gradients were tittered for infectivity as described in Materials and Methods. (B) Real time PCR of viral genome copy number in fractions from gradients. 50ul of each fraction was extracted using column technology (Qiagen Blood PCR Kit, Qiagen, Valencia, Ca) and assayed by real time PCR as described in Materials and Methods. Results are expressed as genome copy number per fraction.

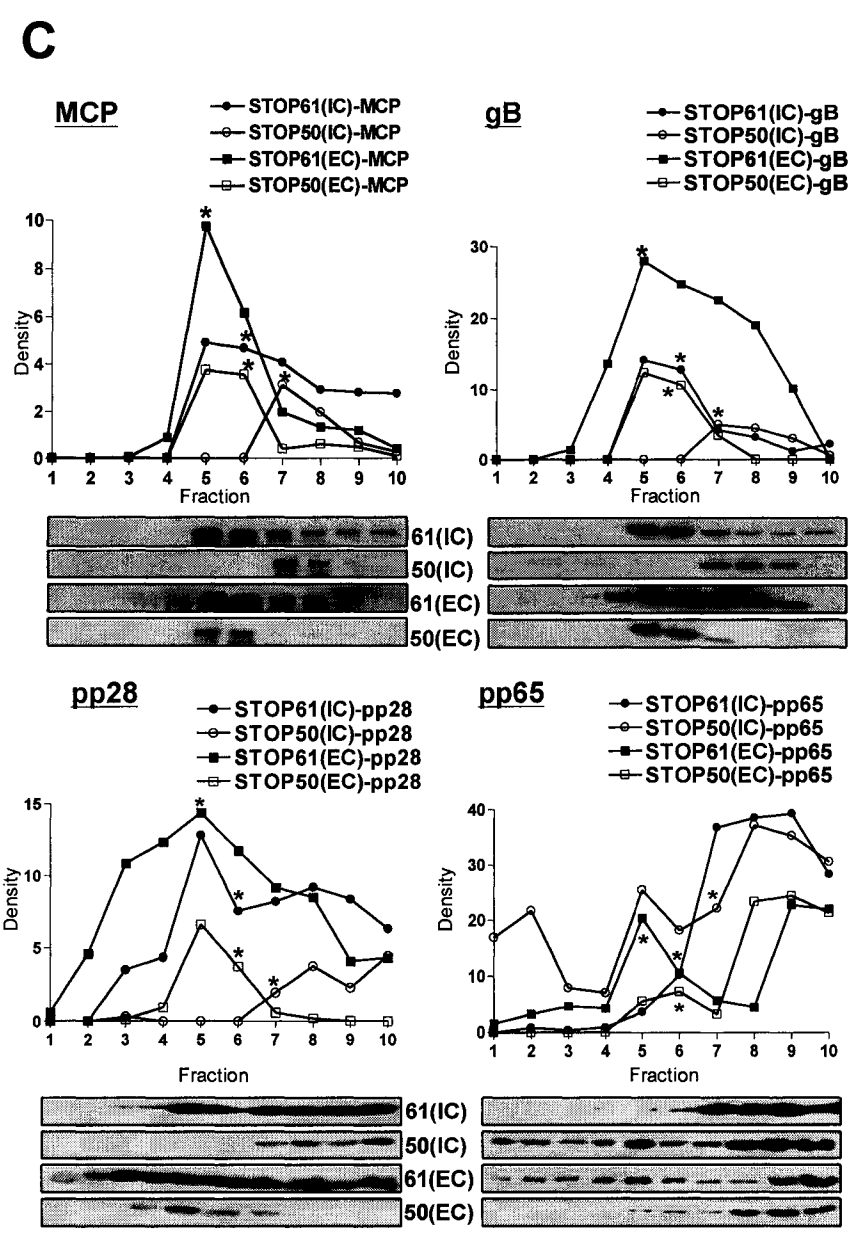

FIG. 6. (Continued) (C) Relative expression of major capsid protein (MCP), glycoprotein B (gB), pp28, and pp65 in each fraction as detected by western blotting with specific mabs. 50 ul of each fraction was loaded on 12% polyacrylamide gels and subjected to SDS-PAGE and western blotting as described in Materials and Methods. Following developing with ECL, densitometry was carried out and relative signal for each lane determined. Note that densitometry was only done on fluorograms acquired during the same experiment and developed for the same time interval. The fluorograms shown for pp28 and pp65 were processed in Adobe photoshop to remove non-specific background signals assumed to be secondary to non-specific binding of primary antibodies; however, densitometry was performed prior to image processing to remove background. The pp65 and pp28 specific bands were not processed. (*) indicates gradient fraction with peak infectivity.

TABLE 1. Estimation of amounts of viral proteins, viral DNA and infectivity in extracellular and intracellular virion particles produced in cells infected with pp28STOP61 or pp28STOP50 virus.

Virus[1]	MCP[2]	gB	pp28	pp65	gB/MCP[3]	pp28/MCP	pp65/MCP	DNA copy[4]	Infectivity
STOP61									
IC (6)	4.67	12.75	7.56	10.27	2.7 (0.05)	1.6 (0.02)	2.2 (0.10)	1.7×10^9 (9.22)	6.00
EC (5)	9.74	27.96	14.40	20.36	3.1 (0.10)	1.58 (0.08)	2.14 (0.14)	6.9×10^9 (9.84)	8.47
STOP50									
IC (7)	3.13	5.00	1.95	22.10	1.64 (0.07)	0.65 (0.06)	6.92 (0.23)	1.1×10^9 (9.04)	4.75
EC (6)	3.56	10.59	3.74	7.23	3.06 (0.07)	1.04 (0.01)	1.93 (0.05)	8.9×10^8 (8.95)	6.91

[1]Number in parentheses following IC (intracellular) and EC (extracellular) represents fraction containing peak infectivity.
[2]Density of respective protein in peak fraction from Figure 6 C is shown.
[3]Ratios of envelope (gB), tegument (pp28), and tegument (pp65) proteins to major capsid protein (MCP) were calculated at the peak fraction of infectivity. The ratios (gB/MCP, pp28/MCP, pp65/MCP) represent mean values with standard errors in parentheses from 3-4 independent experiments. A single representative experiment is shown in Figure 6 C.
[4]The viral DNA copy number in parentheses and infectivity are represented in log scale at the peak fraction of infectivity. Note that prior to application to the gradients the total extracellular viral particles from HF cells (as analyzed in clarified supernatant) that had been infected with the pp28STOP61 and pp28STOP50 virus differed by comparable levels of infectivity (1.3 logs) and viral DNA copy number (1 log).

these experiments demonstrated a greater disparity between the generation of infectious particles as compared to the replication of viral DNA in HF cells infected with the pp28STOP50 mutant virus and the pp28STOP61 virus (Fig. 6A and B, and Table 1). This result raised the possibility that a similar number of DNA containing particles were produced in pp28STOP50 virus and pp28STOP61 virus infected HF cells, but a fewer number of infectious particles were assembled in pp28STOP50 virus infected cells. To address the possibility that pp28STOP50 virus infected HF cells failed to assemble similar amounts of infectious virions as cells infected with the pp28STOP61 virus, we analyzed the quantity of several viral proteins together with DNA copy number in gradient fractions from both extracellular and intracellular STOP61 or STOP50 virus.

The quantity of viral proteins was measured by western blot analysis using specific mabs followed by densitometry (Fig. 6C). When the DNA copy number present in particles was determined from the fractions containing infectious virus, approximately 55% of the total (intracellular + extracellular) DNA containing particles from pp28STOP50 virus infected cells remained intracellularly as compared to only 20% of particles from pp28STOP61 virus infected cells (Table 1). In agreement with previous findings, this result suggested that the pp28STOP50 virus had a defect in virus assembly/release into the extracellular supernatant when compared to pp28STOP61 virus (Table 1). The amount of the UL86 (MCP) present in the peak fraction of intracellular particles from both viruses was similar (MCP pp28STOP61 / pp28STOP50 = 1.5) as was the copy number of viral DNA (DNA pp28STOP61 / pp28STOP50 = 1.52) suggesting that the defect in the production of extracellular particles by pp28STOP50 virus infected cells could not be explained by differences in the synthesis of MCP or assembly of DNA containing particles within the infected cell (Table 1). However, the infectivity of pp28STOP50 (4.75 logs) intracellular particles was about 1.3 logs less than the infectivity of the pp28STOP61 (6.00 logs) particles indicating that a significant number of MCP and DNA containing intracellular particles produced in pp28STOP50 virus infected HF cells were not infectious perhaps as a result of being non-enveloped as suggested by the EM findings (Table 1). This interpretation was also consistent with the observation that intracellular particles from the pp28STOP50 virus infected cells had a gB/MCP ratio (1.64) of about 50% of the gB/MCP ratio of either intracellular or extracellular particles isolated from pp28STOP61 infected HF cells (2.7 and 3.10 respectively) or more interestingly, extracellular particles from pp28STOP50 infected HF cells (3.06; Table 1). Interestingly, similar calculations from only one experiment in which the ratio of gM

complex to MCP was measured revealed results that were consistent with results from the more extensive analysis of gB. In these experiments, the gM/ MCP ratio for intracellular and extracellular particles from STOP61 virus infected cells was 2.99 and 3.02 respectively and 1.89 and 2.95 intracellular and extracellular particles from STOP50 virus infected cells. These results suggested that only a fraction of intracellular pp28STOP50 viral particles were enveloped (gB and gM containing) and therefore, infectious.

The deficit in envelopment of the pp28STOP50 mutant virus could be explained by a defect in tegumentation leading to a defect in envelopment. This possibility was examined initially by assaying the quantity of a major tegument protein, pp65, in both extracellular and intracellular particles. The amount of pp65 in intracellular particles purified from the pp28STOP50 virus infected cells was greater than the amount detected in intracellular or extracellular particles purified from the pp28STOP61 infected cells (Fig. 6C and Table 1). Decreased amounts of pp65 were noted in extracellular pp28STOP50 virions as compared to pp28STOP61 virions consistent with the conclusion that fewer particles were released by cells infected with the pp28STOP50 virus (Fig. 6C and Table 1). Thus, it was unlikely that the deficit in envelopment of the pp28STOP50 mutant virus was initially secondary to a defect in tegumentation. In contrast to these findings, the ratio of pp28/MCP from pp28STOP61 intracellular or extracellular particles was similar. However, intracellular particles from pp28STOP50 virus infected HF cells had a pp28/MCP ratio of about 50% of that of pp28STOP50 extracellular particles indicating that lesser amounts of pp28Mut50 protein were present in the population of intracellular particles (Table 1). This result also suggested that only a fraction of intracellular particles from pp28STOP50 virus infected HF cells contained the mutant

form of pp28 and therefore, were enveloped during assembly. Alternatively, the possibility that only a threshold amount of pp28 was required for assembly of an enveloped particle and that each particle derived from pp28STOP50 infected cells contained a decreased amount of the mutant pp28 could not be ruled out. Consistent with the latter possibility was the finding that extracellular particles from pp28STOP50 virus infected cells had a ratio of pp28/MCP that was less than that of pp28STOP61 extracellular particles arguing the possibility that only a threshold amount of pp28 was required for successful envelopment of an infectious particle and that each particle contained limiting amounts of the mutant pp28 protein (Table 1).

The mutant pp28 protein encoded by the pp28STOP50 virus fails to concentrate in the assembly compartment.

The pp28 protein is localized and increases in amount in the AC late in infection (22). To investigate the possibility that the deficit in the production of infectious virus by the pp28STOP50 virus infected HF cells was secondary to altered localization of the mutant pp28STOP50 to the AC or a lack of the accumulation of pp28 in the AC, we analyzed the kinetics of pp28 localization to the AC by fluorescence recovery after photobleaching (FRAP) experiments. An EGFP tagged pp28 protein was expressed in HF cells following electroporation of a plasmid encoding the first 35aa, 50 aa or 61 aa of pp28 or the full length of pp28 molecule, each of which was fused at its C-terminus to EGFP (pp28Mut35-EGFP, pp28Mut50-EGFP, pp28Mut61-EGFP, or pp28WT-EGFP). HF cells were electroporated with the indicated plasmid and then infected with HCMV 48 hrs later at a moi of 0.1. A FRAP assay of live infected HF cells was carried out 6 days post infection. The fluorescence of pp28Mut35-EGFP, pp28Mut50-EGFP,

pp28Mut61-EGFP, or pp28WT-EGFP in the prebleached compartment was saturated late in infection (Fig. 7A). We selectively bleached the entire AC and monitored the fluorescence recovery in this region (Fig. 7A). The fluorescence in the bleached region dropped to about 20% immediately after bleaching. Thus for these FRAP experiments, the fluorescence in the bleached region was normalized to 20% at time 0 postbleaching and the recovered fluorescence was monitored for 20 minutes postbleaching. For pp28WT-EGPF, the fluorescence in the bleached AC recovered to 32% (Fig. 7A). Similar values were noted for both the pp28Mut50-EGFP and pp28Mut61-EGFP proteins (45% and 41%, respectively) (Fig. 7A). These values indicated that transport of these pp28 mutants was similar to wild type and that differences between the rates of localization of pp28Mut50 and wild type pp28 (and pp28Mut61) to the AC could not explain the defect observed in the replication of the pp28Mut50 expressing recombinant virus (pp28STOP50). In contrast to these findings, the recovery of the pp28Mut35 protein in the AC reached only 25% (5% above post bleach levels) and then appeared to decline suggesting that the AC was less efficiently refilled by this mutant form of pp28 as compared to the wild type or pp28Mut61 or pp28Mut50 (Fig. 7A). This finding was consistent with our previous results using static imaging (24).

In the next series of experiments, the accumulation of pp28, pp28Mut61, pp28Mut50, and pp28Mut35 in the AC was assayed using transfected/infected cells and FRAP as described above. In these experiments, the entire cytoplasm (except AC and nucleus) was bleached and the recovery of the fluorescence signal in a region of cytoplasm surrounding the AC was measured over time (Fig. 7B). The fluorescence in the measured region was normalized to 20% at time 0 postbleaching. We noted significant differences in the recovery rates. The fluorescence signal in wild type pp28-

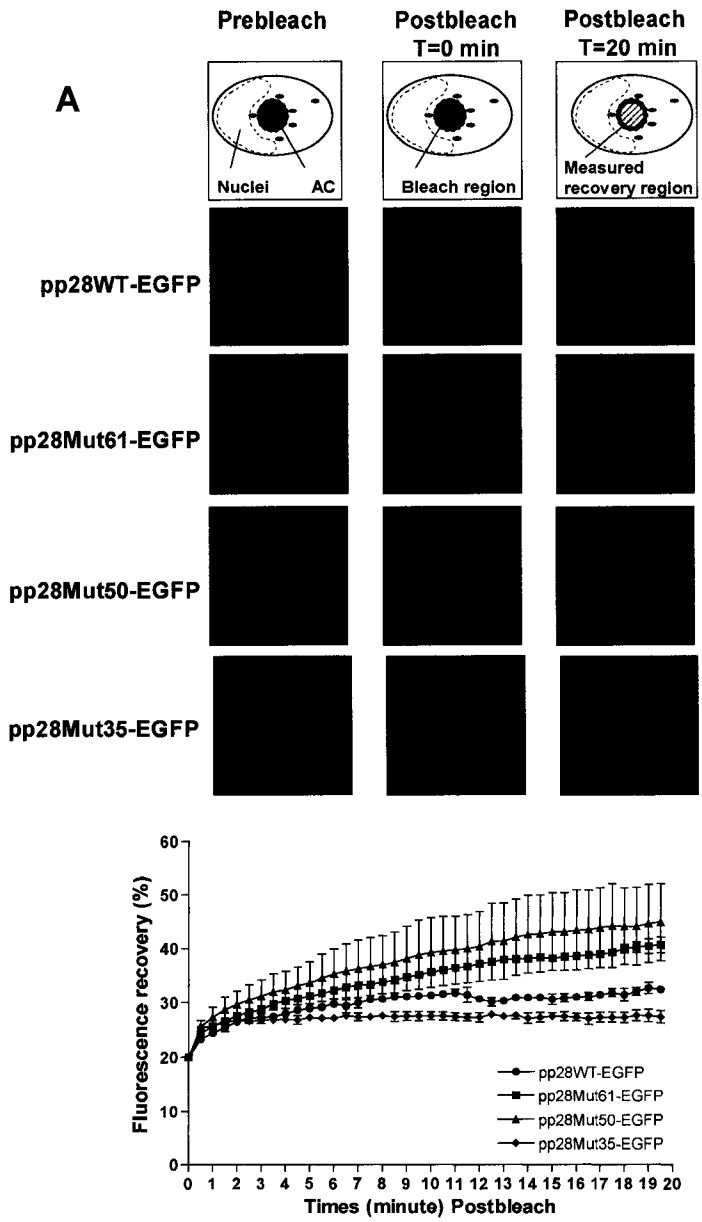

FIG. 7. FRAP analysis of accumulation of pp28 wild type, pp28Mut61, pp28Mut50, and pp28Mut35-EGFP in the AC. HF cells were transfected with pp28WT-EGFP, pp28Mut61-EGFP, pp28Mut50-EGFP, or pp28Mut35-EGFP and followed by infection with HCMV at a moi of 0.1 48 hrs later. At day 6 post infection, the infected live HF cells were subjected to FRAP analysis as described in Materials and Methods. Illustrations on the top of images show the areas of FRAP, a cell prior to bleaching (left), a region selectively bleached (middle, black colored), and a measured recovery region over time (right, green dashed). (A) Localization of pp28WT-EGFP, pp28Mut61-EGFP, pp28Mut50-EGFP and pp28Mut35-EGFP to the AC. FRAP was performed at the entire AC of infected cells (n=5). At time 0 postbleaching, the fluorescence in the bleached region was normalized to 20% of the prebleaching fluorescence. Fluorescence recovery was monitored and plotted over time.

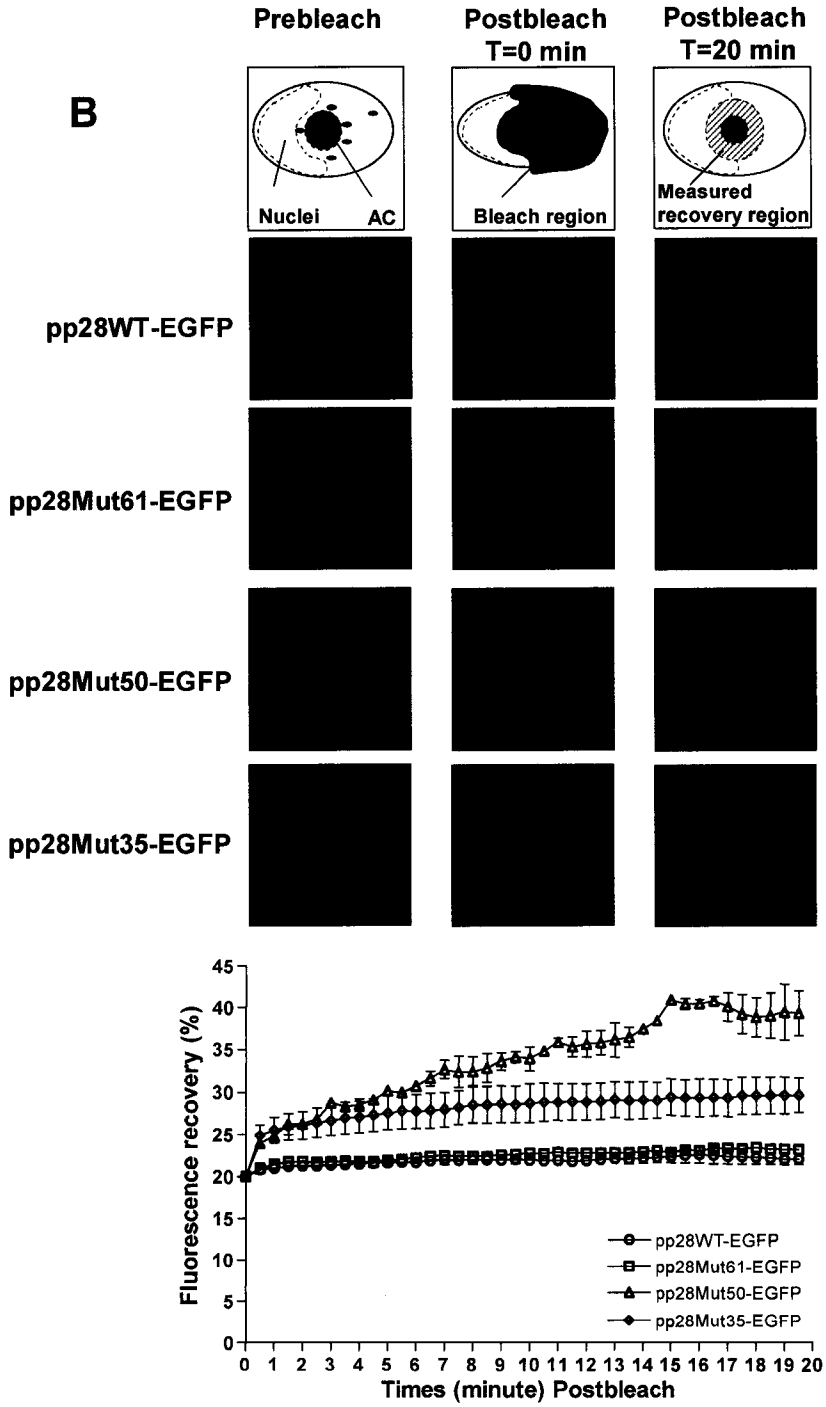

FIG. 7. (Continued) (B) Movement of pp28WT-EGFP, pp28Mut61-EGFP, pp28Mut50-EGFP, and pp28Mut35-EGFP out of the AC. FRAP was performed at the cytoplasm of infected cells (n=3). Fluorescence recovery was monitored and plotted over time as described above. Error bars represented standard errors of the means.

EGFP transfected/infected cells recovered to about 23% following photobleaching of the cytoplasm (Fig. 7 B). Similarly, the recovery of the fluorescence signal from the pp28Mut61 transfected/infected cells was 24% during the measurement interval (Fig. 7 B). The analysis of the pp28Mut35 protein suggested similarly inefficient refilling of this area of the cell, presumably because there was a minimal amount of the pp28Mut35 protein in the AC available to refill the cytoplasm of these previously bleached cells (Fig. 7B). In contrast to these findings, the recovery of fluorescence in the photobleached cytoplasm of pp28Mut50 transfected/infected cells was almost 40% indicating that the trafficking of pp28Mut50 out of the AC was significantly increased as compared to either the pp28Mut61 mutant or wild type pp28 (Fig. 7B). When the rate of movement of these proteins into and out the AC is expressed as a ratio of recovery of fluorescence at 20 minutes following photobleaching to a value of 20% at time 0, these differences are more apparent. The ratio of in/out for wild type pp28 and pp28Mut61 were 1.5 and 1.7 respectively, indicating that at steady state kinetics, localization of these proteins in the AC was favored (Table 2).

TABLE 2. Recovery kinetics of pp28 and pp28Mut in FRAP assays.

	pp28WT	pp28Mut61	pp28Mut50	pp28Mut35
Recovery rate in AC	1.6	2.0	2.2	1.4
Recovery rate in cytoplasm	1.1	1.2	2.0	1.5
Accumulation rate in AC	1.5	1.7	1.1	0.9

Ratios of fluorescence recovery were generated by ratio of fluorescence at T=20 min/ fluorescence at T=0 min. For kinetics of accumulation in AC, ie localization in the AC, the ratio of fluorescence recovery in the AC/fluorescence recovery in cytoplasm was calculated. Note that accumulation rate of pp28Mut35 in AC was < 1.0 indicating that at equilibrium the majority was localized outside of the AC as compared to pp28WT with accumulation rate of 1.5.

In contrast, the ratio of in/out for pp28Mut50 was 1.1 indicating that at late times following infection, nearly equivalent amounts of pp28Mut50 were exiting the AC as were trafficking into this compartment in HF cells (Table 2). Moreover, the recovery pattern on the plot showed that in contrast to wild type and pp28Mut61, the recovered fluorescence of pp28Mut50 in the cytoplasm was not saturated during the measured time (Fig. 7 B). When the same ratios were calculated for the pp28Mut35 protein, the ratio of in/out of the AC was 0.9 confirming our findings with static imaging carried out at equilibrium that the majority of the pp28Mut35 protein is localized outside of the AC (Table 2). Together these results were consistent with the favored kinetics for trafficking of the pp28Mut50 out of the AC and suggested that the failure of this mutant form of pp28 to accumulate in the AC could limit its concentration in the AC and therefore, its role in the envelopment of the virion.

DISCUSSION

Although pp28 has been shown to be an essential tegument protein for assembly of an infectious HCMV particle, its precise role in assembly has not been elucidated. Cells infected with recombinant viruses in which pp28 was deleted failed to produce enveloped particles and analysis of cells infected with this mutant virus complemented with a retrovirus expressing pp28 suggested that pp28 has a role in envelopment of the particle (26). However, it was unclear if the deletion of pp28 resulted in a loss of tegument and/or envelope assembly and because progeny virions were not produced, the protein composition of particles produced by this recombinant virus also could not be analyzed. Although the stage at which virion assembly was blocked was not determined, this report convincingly demonstrated that pp28 has a critical role in the cytoplasmic

envelopment of HCMV (26). Similarly, our results also suggested that the replication impaired phenotype of the pp28 mutant virus, pp28STOP50, resulted from a defect in the assembly of enveloped particles. Analysis of this mutant virus was particularly informative because although it exhibited a replication impaired phenotype, it produced sufficient amounts of both intracellular and extracellular virus for characterization of its assembly, including the protein composition of the particle. The results of these studies indicated that the pp28STOP50 virus was replication impaired secondary to a defect in assembly based on findings that; (i) the viral genome was replicated to similar levels as replication competent wild type viruses, (ii) intracellular particles produced by the pp28STOP50 mutant contained similar amounts of the major capsid protein and viral DNA as particles purified from the replication competent pp28STOP61 virus, (iii) the infectivity of intracellular particles produced in pp28STOP50 infected cells was about 1.5 logs less than particles from pp28STOP61 infected cells suggesting that the defect in the pp28STOP50 virus was not secondary to a block in release of infectious virus, and (iv) extracelluar virions produced by cells infected with the pp28STOP50 mutant could readily enter permissive HF cells. Together with previous findings that demonstrated pp28 was expressed late in the replicative cycle of HCMV, these studies were consistent with earlier results that proposed a key role of pp28 in the cytoplasmic assembly of infectious HCMV (5, 15, 26).

The pp28STOP50 mutant virus expressed only 7 acidic aa of the 16 aa acidic cluster present in the 18 aa between aa 44-61 of the pp28STOP61 viral mutant, including the sequence GEDDD that is duplicated within this 16 aa sequence. However, the pp28STOP50 virus mutant was impaired in the production of infectious virus as compared to the pp28STOP61 virus indicating that the remaining stretch of acidic aa

between aa 44-50 in the pp28STOP50 mutant virus was insufficient to support wild type virus assembly. Previously, we have shown that a recombinant virus encoding a mutant pp28 protein constructed by deletion and transplantation of the 16 aa acidic cluster (aa 44-59) to the carboxyl terminus of pp28 was replication incompetent (24). The finding of context dependence for the function of the acidic cluster present between aa 44-59 in virus raised the possibility that the acidic domain of pp28 could have additional functions other than simply acting as intracellular trafficking motif that directed pp28 to a specific cellular compartment. This function was previously proposed for this stretch of acidic aa in pp28 based on the findings from an analysis of an acidic cluster in the amino terminal domain of herpes simplex virus homolog, UL11 (16). Clusters of aa in the cytoplasmic domains of viral glycoproteins and also present in UL11 have been reported to function in intracellular trafficking through interactions with cellular adaptor proteins such as PACS-1 (3, 20, 29, 31). Considerable variability in the sequence of aa found in a number of acidic clusters have been described and in many cases, phosphorylation of adjacent aa has been shown to regulate interactions with PACS-1 (3). In some cases, acidic cluster of 6-7 aa have been reported to be sufficient for interaction with cellular adaptor proteins and bipartite acidic clusters have described (3, 31). Thus, we expected that the remaining 7 acidic aa on the carboxyl terminus of pp28STOP50 should have been sufficient to interact with cellular adaptors such as PACS-1 and to support levels of replication that were similar to those of wild type virus. Yet the results in the current study argued that an intact acidic cluster (16 aa) within the carboxyl terminus of the mutant virus pp28STOP61 were required for levels of wild type virus replication. A study of the proprotein convertase, PC6B, described two acidic clusters in the cytoplasmic domain of this protein that functioned differently in intracellular sorting (31). The carboxyl terminal

acidic cluster of this protein interacted with PACS-1 but was not required for intracellular localization of the protein (31). The authors of this study postulated that interactions of PC6B with PACS-1 contributed to the intracellular localization of PC6B by directing the protein to the TGN, a step required for efficient PC6B localization that was shown to be absolutely dependent on the acidic cluster of aa more proximal to the amino-terminus of the protein (31). Additional mutations in the remaining aa in pp28STOP50 could determine if the contiguous sequence of acidic aa between aa 44-59 of pp28 functions as a bipartite sorting sequence similar to the acidic aa in PC6B. In addition, additional mutations in the remaining acidic aa at positions 44-50 could identify essential aa required for wild type replication and perhaps provide clues to the role of this domain in pp28 in virus assembly.

Characterization of the assembly of infectious virions provided evidence consistent with a defect in envelopment of the pp28STOP50 recombinant virus as compared to both wild type virus and the pp28STOP61 mutant virus. Several findings lead to this conclusion. First, electron microscopy indicated that only about 15% of intracellular particles in pp28STOP50 virus infected cells were enveloped as compared to over 40% of particles in pp28STOP61 virus infected cells. Analysis of the genome copy number and protein composition of intracellular particles purified from pp28STOP50 and pp28STOP61 virus infected cells indicated that both viruses produced a population of particles that contained similar quantities of viral DNA and major capsid protein (MCP). Furthermore, analysis of the intracellular particles for the tegument protein, pp65 (UL83), also revealed similar amounts of this protein in intracellular particles isolated from cells infected with both mutant viruses. In fact, intracellular particles from pp28STOP50 virus infected cells appeared to have a greater amount of pp65 than particles from

pp28STOP61 virus infected cells suggesting that a decrease in the acquisition of the pp28 tegument protein resulted in an accumulation of pp65 on intracellular particles, perhaps secondary to a block and/or delay in the normal assembly pathway. Together these findings suggested that the defect in the pp28STOP50 mutant virus was not associated with generalized defect in the production of DNA containing particles, capsids, or tegumentation of cytoplasmic particles, but rather a defect in a late step of assembly of the infectious particle, such as envelopment. A formal particle count was not carried out but under identical conditions of gradient purification, a similar viral DNA copy number and amount of MCP was detected in the peak gradient fractions for both viruses, thus arguing that cells infected with either virus produced a similar number capsid containing particles and that these particles also contained similar amounts of viral DNA. However, the intracellular particles purified from cells infected by these viruses differed significantly in the level of infectivity. Cells infected with the pp28STOP61 virus produced approximately 1.3 log more infectious intracellular particles than cells infected with the pp28STOP50 mutant virus, a finding consistent with the results from electron microscopy that suggested that only about 15% of intracellular particles in cells infected with the pp28STOP50 mutant virus were enveloped. In addition, intracellular particles purified from pp28STOP50 virus infected cells contained lesser amounts of the envelope glycoprotein B (and gM) relative to the MCP than intracellular particles purified from pp28STOP61 virus infected cells. Interestingly, the ratio of gB and gM to MCP was similar for both intracellular and extracellular particles from pp28STOP61 virus infected cells and from extracellular particles isolated from the supernatant of pp28STOP50 virus infected cells suggesting that a relatively fixed stoicheometry of gB (gM) and MCP in particles was associated with the infectivity of HCMV virions. The lower gB/MCP (and

gM/MCP) ratios in intracellular particles isolated from pp28STOP50 virus infected cells suggested that either all particles in this population contained decreased amounts of gB (gM) or more likely, that only a minor population of particles contained gB (gM) in amounts similar to particles from pp28STOP61 virus infected cells and that the remaining particles were not completely enveloped. Thus, these findings were consistent with the production of nearly equivalent numbers of intracellular particles by cells infected with the pp28STOP50 virus as compared to cells infected with the pp28STOP61 virus but a decrease in production of infectious intracellular particles as well as a decrease in the number of infectious particles released into the extracellular supernatant. When the percentage of total infectious particles present intracellularly is compared for these two viruses, this defect was also apparent. Of the total amount of infectious virus produced late in infection, approximately 55% of that produced by cells infected with the pp28STOP50 virus remained inside the cell whereas less than 20% of infectious viruses produced by the pp28STOP61 virus were present in the cell. Together, our findings were most consistent with a defect in the envelopment of the pp28STOP50 virus and also raised the possibility that during infection in HF cells, HCMV envelopment and virus release into the supernatant could be linked, i.e. defects in envelopment appeared to reduce the efficiency of virus release from the cell.

Interestingly, the defect that resulted in impaired replication of the pp28STOP50 virus did not result in a complete block in the production of infectious virus suggesting that a fraction of particles contained adequate amounts of the mutant pp28Mut50 protein to support envelopment and release of infectious particles into the extracellular media. As noted above, this conclusion was supported by the finding that infectious particles isolated from the extracellular supernatant contained a similar ratio of gB/MCP as did

extracellular virus isolated from the supernatant from cells infected with the pp28STOP61 mutant virus. However, the number of particles released from cells infected with the pp28STOP50 virus infected cells was reduced significantly based on the viral DNA content and MCP content of extracellular particles from the gradient fraction containing the peak infectivity. Although we cannot determine the specific infectivity and protein content of individual particles within this fraction, our results are compatible with a mechanism in which the majority of intracellular particles produced in cells infected with the pp28STOP50 virus mutant fail to become enveloped and were not released from the infected cell. The minority of particles that become enveloped appeared to acquire similar amounts of gB. Thus, it appeared that the defect in assembly of the pp28STOP50 mutant virus was related to decreased efficiency of envelopment presumably secondary to a loss in function of the mutant pp28Mut50 protein.

The loss of function of the pp28Mut50 protein that led to decreased envelopment of intracellular particles remains undefined. A failure to localize to the AC was a possibility based on our previous studies that demonstrated a failure of pp28 mutants expressing only aa 1-44 to localize in the AC (24). In cells infected with the recombinant pp28STOP50 virus, static imaging revealed that the pp28Mut50 protein localized to the AC, but less efficiently than either the pp28Mut61 protein or wild type pp28 (24). These results were confirmed in the current study utilizing FRAP to analyze protein trafficking in living cells. These studies indicated that the pp28Mut50 protein localized to the AC as efficiently as either the pp28Mut61 or wild type pp28 protein; however, the pp28Mut50 protein exited the AC over twice as rapidly as either the pp28Mut61 and wild type pp28 proteins. This finding indicated that at steady state, only a fraction of the pp28Mut50 protein localized in the AC as compared to either the pp28Mut61 or the wild type pp28

protein, a finding consistent with previous findings using static imaging (24). The decreased amount of the pp28Mut50 in the AC could in turn limit envelopment and assembly of infectious virus, particularly if the pp28 concentration in the AC was rate limiting in assembly of infectious particles.

Although our findings provided an explanation of the decreased production of infectious virions from pp28STOP50 mutant virus infected cells based on decreased amounts of the mutant pp28Mut50 protein in the AC, it was unclear why the mutant pp28Mut50 protein failed to accumulate in the AC. The results from studies using FRAP indicated that the mutant protein was efficiently localized to the AC, thus it is unlikely that interaction with adaptor proteins required for cellular trafficking in the secretory pathway was altered by this mutation. An obvious possibility was that the pp28Mut50 protein could not interact efficiently with cellular and/or viral proteins within the AC and thus exited the AC nearly as rapidly as it entered this compartment. Because this mutant was not completely defective in assembly, it was also likely that once a sufficient quantity of the pp28Mut50 mutant protein accumulated in the AC, assembly of an infectious particle could take place. Thus, it could be argued that a threshold amount of pp28 must be present in the AC to permit envelopment and assembly. The requirement for a threshold in pp28Mut50 mutant protein in AC for virion assembly could be secondary to productive interactions with other viral proteins, including multimerization with itself, or through interactions with cellular proteins. A corollary of such a mechanism would be that the defect in assembly of the pp28STOP50 virus could be overcome by delivering more mutant protein to the AC. Indeed this appears to be the case in that monolayers infected with a high moi of the pp28STOP50 mutant virus produce infectious virus nearly as efficiently as wild type virus.

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REFERENCES

1. **Andreoni, M., M. Faircloth, L. Vugler, and W. J. Britt.** 1989. A rapid microneutralization assay for the measurement of neutralizing antibody reactive with human cytomegalovirus. Journal of Virological Methods **23:**157-67.

2. **Baines, J. D., and B. Roizman.** 1992. The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. J Virol **66:**5168-5174.

3. **Bonifacino, J. S., and L. M. Traub.** 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annual Review of Biochemistry **72:**395-447.

4. **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.

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

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

7. **Britt, W. J., and C. A. Alford.** 1996. Cytomegalovirus, p. 2493-2523. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields Virology, Third Edition. Raven Press, New York.

8. **Britt, W. J., and S. Boppana.** 2004. Human Cytomegalovirus virion proteins. Human Immunology **65:**395-402.

9. **Dunn, W., C. Chou, H. Li, R. Hai, D. Patterson, V. Stolc, H. Zhu, and F. Liu.** 2003. Functional Profiling of the Human Cytomegalovirus Genome. Proceedings

of the National Academy of Sciences of the United States of America **100:**14223-14228.

10. **Fraile-Ramos, A., A. Pelchen-Matthews, T. N. Kledal, H. Browne, T. W. Schwartz, and M. Marsh.** 2002. Localization of HCMV UL33 and US27 in endocytic compartments and viral membranes. Traffic **3:**218-32.

11. **Fuchs, W., H. Granzow, B. G. Klupp, M. Kopp, and T. C. Mettenleiter.** 2002. The UL48 tegument protein of pseudorabies virus is critical for intracytoplasmic assembly of infectious virions. Journal of Virology. **76:**6729-42.

12. **Granzow, H., B. G. Klupp, W. Fuchs, J. Veits, N. Osterrieder, and T. C. Mettenleiter.** 2001. Egress of alphaherpesviruses: comparative ultrastructural study. Journal of Virology **75:**3675-84.

13. **Granzow, H., F. Weiland, A. Jons, B. G. Klupp, A. Karger, and T. C. Mettenleiter.** 1997. Ultrastructural analysis of the replication cycle of pseudorabies virus in cell culture: a reassessment. Journal of Virology. **71:**2072-82.

14. **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. Journal of Virology **74:**7720-9.

15. **Jones, T. R., and S. W. Lee.** 2004. An acidic cluster of human cytomegalovirus UL99 tegument protein is required for trafficking and function. Journal of Virology **78:**1488-502.

16. **Loomis, J. S., J. B. Bowzard, R. J. Courtney, and J. W. Wills.** 2001. Intracellular trafficking of the UL11 tegument protein of herpes simplex virus type 1. Journal of Virology **75:**12209-19.

17. **Mach, M., B. Kropff, M. Kryzaniak, and W. Britt.** 2005. Complex Formation by Glycoproteins M and N of Human Cytomegalovirus: Structural and Functional Aspects. J. Virol. **79:**2160-2170.

18. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. Journal of Virology. **76:**1537-47.

19. **Mocarski, E. S., Tan Courcelle, C.** 2001. Cytomegaloviruses and their replication, p. 2629-2673. *In* D. M. K. a. P. M. Howley (ed.), Fields Virology, 4th ed, vol. 2. Lippincott Williams and Wilkins, Philadelphia.

20. **Molloy, S. S., E. D. Anderson, F. Jean, and G. Thomas.** 1999. Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis. Trend Cell Biol **9:**28-35.

21. **Rubin, R.** 2002. Clinical approach to infection in the compromised host, p. 573-679. *In* R. Rubin, Young, LS (ed.), Infection in the Organ Transplant Recipient. Kluwer Academic Press, New York.

22. **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.

23. **Sanchez, V., E. Sztul, and W. J. Britt.** 2000. Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. Journal of Virology **74:**3842-51.

24. **Seo, J., and W. Britt.** 2006. Sequence requirements for localization of human cytomegalovirus tegument protein pp28 to the virus assembly compartment and for assembly of infectious virus. Journal of Virology **80:**5611-5626.

25. **Silva, M. C., J. Schroer, and T. Shenk.** 2005. Human cytomegalovirus cell-to-cell spread in the absence of an essential assembly protein. Proceedings of the National Academy of Sciences of the United States of America **102:**2081-6.

26. **Silva, M. C., Q. C. Yu, L. Enquist, and T. Shenk.** 2003. Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids. Journal of Virology. **77:**10594-605.

27. **Smith, J. D., and E. DeHarven.** 1973. Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. I. Sequence of viral replication. J Virol **12:**919-930.

28. **Stagno, S., and W. J. Britt.** 2005. Cytomegalovirus, p. In Press. *In* J. S. Remington and J. O. Klein (ed.), Infectious Diseases of the Fetus and Newborn Infant, 6th Edition. W.B. Saunders, Philadelphia.

29. **Wan, L., S. S. Molloy, L. Thomas, G. Liu, Y. Xiang, S. L. Rybak, and G. Thomas.** 1998. PACS-1 defines a novel gene family of cytosolic sorting proteins required for trans-Golgi network localization. Cell **94:**205-216.

30. **Whealy, M. E., J. P. Card, R. P. Meade, A. K. Robbins, and L. W. Enquist.** 1991. Effect of Brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J Virol **65:**1066-1081.

31. **Xiang, Y., S. S. Molloy, L. Thomas, and G. Thomas.** 2000. The PC6B cytoplasmic domain contains two acidic clusters that direct sorting to distinct trans-Golgi network/endosomal compartments. Molecular Biology of the Cell **11:**1257-73.

32. **Yu, D., M. C. Silva, and T. Shenk.** 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. Proceedings of the National Academy of Sciences of the United States of America. **100:**12396-401.

MULTIMERIZATION OF TEGUMENT PROTEIN PP28 WITHIN THE ASSEMBLY COMPARTMENT IS REQUIRED FOR CYTOPLASMIC ENVELOPMENT OF HUMAN CYTOMEGALOVIRUS

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ABSTRACT

Human cytomegalovirus (HCMV) UL99-encoded pp28 is an essential tegument protein for envelopment and production of infectious virus in cytoplasmic phase. Non-enveloped virions accumulate in the cytoplasm of cells infected with recombinant viruses with a UL99 deletion. Moreover, in a replication impaired virus encoding a carboxyl terminal deletion mutant of pp28, it appears that failure of accumulation of the mutant pp28 protein in the virus assembly compartment (AC) contributes to this loss of envelopment of virion. It suggests a post localization function of pp28 in envelopment of infectious HCMV. Here, we investigated the role of pp28 multimerization within the AC in the envelopment. Using a gel electrophoresis under less denaturing conditions (low concentration of SDS) and a pulse-chase analysis, we demonstrated that pp28 multimerized during viral infection. In addition, we mapped the interacting domains responsible for self-interaction using glutathione S-transferase (GST) pull-down assays in an *E.coli* system. Our data suggested that pp28 utilizes a single domain (aa 1-43) for the self-interaction. Moreover, using fluorescence resonance energy transfer (FRET) assays, we showed that the self-interaction occurred in the ERGIC in absence of other viral protein, and the first 30-35 aa of pp28 was sufficient for the self-interaction. In contrast, in transient expression/infection assays, the FRET indicated that the self-interaction took place in the AC and in the intracellular vesicles, and the first 50 aa of pp28 was required for the self-interaction in the AC. Although the first 35 aa of pp28 could multimerize in the ERGIC, this mutant molecule could not accumulate in the AC and support virus replication. These findings were consistent with our previous findings from the sequence requirements for localization of pp28 to the AC and for assembly of infectious virus. It also suggested that the accumulation of pp28 within the AC was a prerequisite for

multimerization of pp28 within the AC. Our interpretation was also supported by subcellular fractionation experiments. We argued that pp28 multimerization in the AC represent an essential step in the particle envelopment and production of infectious virions.

INTRODUCTION

Human cytomegalovirus (HCMV) is an important human pathogen associated with acute and chronic disease in both normal and immunocompromised populations (4, 32, 42, 43). The virus is the largest and most complex member of the family of human herpesviruses. The virion of HCMV consists of three distinct structures: a nucleocapsid containing a 230 kilobase pair of linear DNA genome that may encode over 200 open reading frames (ORFs), an envelope including an as yet undefined number of viral glycoproteins, and a tegument layer located between the capsid and envelope (28, 29, 46, 51, 53). In addition, the virion contains a minimum of 71 virus-encoded proteins and a large number of host-derived proteins (47).

HCMV assembly is a multistage and poorly understood process. Although all proposed models include well studied mechanisms of capsid assembly within the nucleus of infected cells, the final tegumentation and envelopment in the cytoplasm of infected cells remains poorly understood (26). Early ultrastructural studies of herpes simplex virus (HSV) and HCMV noted that nonenveloped cytoplasmic particles in HCMV-infected cells were coated with a thick tegument layer but that nonenveloped HSV particles often had the appearance of naked capsids (41). These and other findings suggested that tegument proteins could play a key role in the cytoplasmic phase of HCMV assembly and that the cytoplasmic assembly of HSV and HCMV could differ substantially.

Recently, several laboratories have approached the investigation of the tegumentation of HCMV by studying a tegument protein, pp28. This protein has 190 amino acids (aa) encoded by the UL99 ORF. It is a true late protein that is myristoylated and phosphorylated (20, 21, 35, 39). The pp28 protein is one of the most abundant constituents of the tegument layer and also is highly immunogenic (6, 27, 47). More importantly for studies of virus assembly, this protein is essential for the production of infectious virus and deletion of the UL99 ORF that encodes pp28 results in production of non-enveloped and non-infectious cytoplasmic virions suggesting a key role of this tegument protein in the assembly of an enveloped particles (3, 20, 39). The pp28 protein is localized to the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) in the absence of other viral proteins. Otherwise, it is localized to a cytoplasmic compartment designated the virus assembly compartment (AC) late in infection suggesting that viral functions are required for its localization to the AC (34, 35). The AC is thought to be a modified compartment of the cellular secretory system and possibly a compartment derived from late endosomes (34, 38, 45). Localization of pp28 to the AC is required for virus assembly and mutations that impair its localization in this AC also limit the production of infectious virus (38). Two previous studies have shown that only the first 60 aa of pp28 are sufficient for the generation of viruses exhibiting a phenotype of wild type virus replication (20, 38). In addition, these same studies demonstrated that deletion of an acidic aa cluster (aa 44-59) of pp28 lead to the loss of the recovery of infectious virus, suggesting that this stretch of aa is essential for assembly of infectious virions (20, 38). The role of this acidic cluster in the function of pp28 in the assembly of infectious HCMV remains unknown. We previously characterized a replication impaired virus mutant encoding only the first 50 aa (containing a half of the acidic aa cluster) of

pp28 (38). Our data indicated that the mutant pp28 protein encoded by this virus appeared to contribute to a loss of envelopment by delayed accumulation in the AC of infected cells, arguing that the role of pp28 in envelopment is expressed after its localization in the AC (38). Interestingly, several previous studies have shown that additional higher molecular weight form of pp28 is observed on SDS-PAGE/western blot analysis suggesting that this protein multimerizes in the infected cell. This finding raised the possibility that multimerization represented a post localization function of pp28 that could be essential for virion assembly.

A large number of reports have shown that multimerization of tegument or matrix proteins in structurally less complex viruses, such as small RNA viruses, plays an essential role in the viral assembly. Trimerization of matrix proteins has been proposed to lead to the formation of a protein lattice that can lead to membrane deformation and eventual budding or pinching off of that region of the membrane (19). Multimerization of myristoylated human immunodeficiency virus type 1 (HIV-1) gag protein has been linked to conformational changes and exposure of gag subdomains, including the myristoylated amino terminus leading to membrane association (myrsitic acid switch) (14, 33, 44). Similarly, the M protein of vesicular stomatitis virus (VSV), paramyxoviruses, Ebola virus, and influenza virus are membrane associated and may undergo a multimerization to create lattice like structures at the host cell plasma membrane (10, 15, 17, 24, 36, 37, 52). Although several studies have demonstrated the critical role of pp28 in envelopment of the infectious particle, it is unknown if specific post localization functions of pp28 such as multimerization, and/or interactions with other viral or cellular proteins in the AC contribute to the envelopment process.

In this study, we investigated the role of pp28 multimerization in the envelopment

of infectious HCMV. We utilized several distinct assays for pp28-pp28 multimerization. Initially, using biochemical assays, we confirmed the possible multimerization of pp28. We observed a what appeared to be a dimer form of pp28 on gel systems containing minimal amounts of SDS as well as on non-reducing gel system. This finding suggested that pp28 is multimerized, and that multimerization could be associated with intermolecular disulfide bonding. A pulse-chase analysis of virus infected cells demonstrated that pp28 multimerized during viral infection. We mapped the interacting domains for pp28 multimerization utilizing glutathione S-transferase (GST) pull-down assays. Our data suggested that pp28 takes advantage of a single domain (aa 1-43) for the self-interaction. These results were confirmed using fluorescence resonance energy transfer (FRET) assays. In addition, we defined the intracellular sites in which pp28 multimerization appears to take place. The self-interaction occurred in the ERGIC in absence of other viral protein, and the first 30-35 aa of pp28 was sufficient to interact with a full length of pp28 within the ERGIC. Interestingly, the first 25 aa of pp28 failed to interact with a full length of pp28, although they were co-localized in the ERGIC suggesting that the interacting domain for multimerization is distinct from a membrane binding or retention signal, and the multimerization does not affect pp28 membrane binding activity. In contrast to these results, the self-interaction appeared to occurr in the AC and in the intracellular vesicles in the virus infected cells, and the first 50 aa of pp28 was required to interact with a full length of pp28 in the AC. Although the first 35 aa of pp28 can multimerize in the ERGIC, this mutant molecule cannot accumulate in the AC and support virus replication. The result also suggested that the interacting domain for multimerization is distinct from a trafficking signal for authentic localization to the AC. This interpretation was further supported by cell fractionation experiments using

iodixanol gradients. We argued that the multimerization within in the AC plays a critical role in the envelopment process such as budding of infectious virus. The results of this study indicated that pp28 is multimerized through self-interaction in the AC during viral infection, and suggested that the pp28 multimerization within the AC as a critical post localization function of pp28 in the AC required for envelopment of infectious virus.

MATERIALS AND METHODS

Cells, viruses and antibodies.

Primary human foreskin fibroblasts (HF) were prepared, propagated, and infected as previously described (5, 8). HCMV strain AD169 was used for all experiments. Infectious stock was prepared from supernatants of infected HF cells which exhibited a 100% cytopathic effect and were titered as described previously (5, 8). COS-7 and HK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum and penicillin-streptomycin. Abs used in this study include, anti-pp28, mab 41-18; anti-pp65 (BB epitope), mab 28-19; anti-IE1 (UL123), mab (P63-27) (34); anti-EGFP, a rabbit antiserum (Invitrogen).

SDS-PAGE and immunoblotting.

Sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis (PAGE) under reducing, non-reducing, less denaturing conditions were carried out as described previously (1, 11). The resolving and stacking gels contained 0.1% SDS. Under reducing condition, samples were solubilized in sample buffer containing 2% SDS and 5% 2-mercaptoethanol and heated to 100°C. Under non-reducing condition, samples were solubilized in sample buffer containing 2% SDS without heating. Under less denaturing

condition, samples were solubilized in sample buffer containing 0.1% SDS without heating. The solubilized proteins were then subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Samples were analyzed by western blotting as described in previous publications and developed using enhanced chemiluminesence (Pierce Biotechnology, Rockford, Ill).

[^{35}S] Met/Cys labeling and sedimentation analysis of pp28 multimerization.

HCMV infected HF cells or HK293 cells transfected with an expression plasmid encoding pp28 were radiolabeled with 100 μCi of [^{35}S] methionine/cysteine (Met/Cys) per ml as previously described (1, 7, 8). Following solubilization in standard radioimmunoprecipitation assay buffer (0.1% NP-40, 1% deoxycholate, 0.1% SDS in Tris-buffered saline [TBS; pH 7.5, with 150 mM NaCl]), the labeled proteins were precipitated with mabs and the precipitated proteins were analyzed by SDS- PAGE as described in previous publications (1, 7, 8). The assay for multimerization has been described in detail in an earlier publication (7, 8). Briefly, monolayers of HCMV infected HF cells or HK293 cells transfected with an expression plasmid were washed and Met/Cys starved for 60 min. The cells were then incubated in Met/Cys-free media containing 100 μCi of [^{35}S] Met/Cys (New England Nuclear, Boston, Mass) per ml for 10 min, and then following extensive washing, the cells were either harvested by placing the dishes at - 80°C or chased with media containing Met and Cys and supplemented with 100 μg of cycloheximide per ml for 40 min. These cultures were harvested and placed at - 80°C. The labeled monolayers were solubilized in TBS containing 0.1% NP-40 at 4°C for 30 min. The solubilized proteins were then precleared by incubation with normal goat serum and staphylococcal Cowan I bacteria (Calbiochem, San Diego, Calif.) followed by

centrifugation for 15 min at 13,000 x *g*. The cleared lysate was applied to preformed 5 to 40% linear sucrose gradients and centrifuged for 20 h at 34,000 rpm in a Beckman SW41 rotor. The gradients were fractionated from the bottom into 1 ml fractions, and each fraction was precipitated with the specified antibody. The immune precipitates were collected by first adding rabbit anti-mouse IgG (Cappel Laboratories, Aurora, Ohio) followed by staphylococcal Cowan I bacteria. After extensive washing, the immune precipitates were analyzed by SDS-PAGE in 7.5% gels. Migration of the radiolabeled protein down the gradient, as reflected by recovery of the radiolabeled protein in fractions more proximal to the bottom of the gradient, indicated multimerization of the protein.

GST pull-down assay.

A series of GST-pp28 fusion proteins in which GST was fused with pp28 wild type, pp28 mutants expressing the first 33, 61, 90, and 123 aa of pp28, and three deletion mutants in which aa 26-33, aa 26-43, or aa 44-59, an acidic domain was deleted, and a BB epitope (RKTPRVTG fused to NH2 terminus) tagged wild type pp28 (BB-pp28) protein were expressed and induced in *E. coli* (BL21). Bacterial lysates containing recombinant proteins from 30-ml culture were prepared by using Bugbuster Protein Extraction Reagent (BPE reagent) (Novagen 70584-4) according to the manufacturer's instructions. Briefly, bacterial cell pellets were resuspended in 1 ml BPE reagent and incubated for 15 min at room temperature. The solubilized lysates were pelleted at 1,6000 x g for 20 min at 4°C. The lysate supernatants were used in pull downs. All binding steps were performed in a 1 ml reaction volume with BPE reagent. Thirty microliters of a 50% (vol/vol) slurry of glutathione-Sepharose beads (Amersham Biosciences product), prepared as described (9, 23), was added to each reaction mixture, as described below.

The supernatants of bacterial lysates containing equivalent amounts of GST-fusion protein (as normalized by staining polyacrylamide gels with Coomassie blue) were adsorbed to glutathione-Sepharose beads for 1h at room temperature. The beads were washed three times with BPE reagent and pelleted by centrifugation at 1000 x g for 5 min in a microcentrifuge. The supernatants of bacterial lysates containing BB-pp28 were precleared with glutathione-Sepharose beads 2 h at room temperature and then incubated with the purified GST proteins on glutathione-Sepharose beads for 1 h at room temperature. The beads were again pelleted and then washed with BPE reagent three times. The beads were resuspended in sample buffer containing 2% SDS and 5% 2-mercaptoethanol and heated to 100°C for 5 min, and pelleted. The supernatants were then subjected to SDS-PAGE in 10% gels. Gels were either stained with coomassie blue or visualized by western blotting with anti-pp28 mab (41-18) and anti-BB tag mab (28-19) followed by HRP conjugated goat anti-mouse Igs secondary ab and ECL. Mab 28-19 is reactive with peptide (RKTPRVTG) encoded by HCMV UL83.

Acceptor photobleaching fluorescence resonance energy transfer (FRET) by confocal microscopy.

For FRET assays, EGFP tagged pp28 protein and monomeric red fluorescence protein (DsRed) tagged pp28 protein were co-expressed in Cos-7 cells following transfection of a plasmid encoding the first 25 aa, 30 aa, 35 aa, 50 aa or 61 aa of pp28 or the full length of pp28 molecule fused at the C-terminus to EGFP and a plasmid encoding the full length of pp28 molecule. The transfected Cos-7 cells were grown on a 13 mm glass coverslip at 37°C in DMEM containing 10 % fetal calf serum. At day 2 post transfection, the Cos-7 cells on a coverslip were washed with phosphate buffered salt

solution (PBS, pH 7.4), and fixed in 2% paraformaldehyde for 10 min. A drop of mounting medium (50% glycerol in PBS) was added in the coverslip. The coverslip was mounted and sealed on slides. In addition, EGFP fused with DsRed (EGFP-DsRed) or GGA1 fused with EGFP (GGA1-EGFP) was prepared as a positive or negative control in transfected Cos-7 cells, respectively. FRET was processed using donor dequenching/acceptor photobleaching protocol on a confocal laser scanning microscope (Leica SP2, NJ). An excitation wavelength of 488 nm and an emission range of 500–550 nm, and an excitation wavelength of 561 nm and an emission range of 580–700 nm were used to acquire images of EGFP and DsRed, respectively. DsRed was photobleached by using full power of the 561 nm line on 0.3 regular set. An image of EGFP fluorescence and DsRed fluorescence after photobleaching was obtained by using the respective filter sets. Such data were collected from 5-10 different cells in different fields from the same coverslip. One to three regions of interest (ROI) in the photobleached area were selected per cell, and the mean EGFP fluorescence before and after photobleaching was obtained by using Leica confocal software (Leica SP2, NJ). FRET efficiency was calculated by using the following relationship: FRET efficiency (%) = [(*D*post -*D*pre)/*D*post] *100. Here, *D*post is the fluorescence intensity of the EGFP (donor; *D*) after photobleaching and *D*pre is the fluorescence intensity of the DsRed before photobleaching. A non-bleaching region was selected as an internal control on FRET analysis.

A similar experiment utilizing FRET was performed in transfected/infected cells. FRET analysis was performed at day 6 post infection in HF cells in which EGFP tagged pp28 protein and monomeric red fluorescence protein (DsRed) tagged pp28 protein were co-expressed following electroporation of a plasmid encoding the first 35 aa, 50 aa or 61 aa of pp28 or the full length of pp28 molecule fused at the C-terminus to EGFP and a

plasmid encoding the full length of pp28 molecule. Electroportated HF cells were infected with HCMV 48 hrs later at a multiplicity of infection (moi) of 0.1. The infected HF cells were grown on a 13 mm glass coverslip at 37°C in DMEM containing 10 % fetal calf serum. At day 6 post infection, the infected HF cells on a coverslip were washed with phosphate buffered salt solution (PBS, pH 7.4), fixed in 2% paraformaldehyde for 10 min and then stained with anti-IE-1 mab followed by FITC-labeled anti-mouse IgG to identify infected cells (green nuclei). A drop of mounting medium (50% glycerol in PBS) was added in the coverslip. The coverslip was mounted and sealed on slides. FRET was processed using donor dequenching/acceptor photobleaching protocol as described above.

Subcellular fractionation.

A 75-cm2 flask of HF cells was electroporated with 5 μg of an expression vector encoding pp28Mut35-EGFP or pp28Mut61-EGFP Twenty-four hours later, the cells were washed and infected with HCMV at a moi of 0.1. The HF cells were harvested on day 6 post infection by trypsinization, and the cell pellet was washed twice with cold PBS and then resuspended in 1 ml of homogenization buffer (0.25 M sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA). The cell suspension was repeatedly passed through a 23-gauge needle until there were no intact cells in the suspension, as determined by light microscopy, and a postnuclear supernatant was collected following centrifugation at 1,000 x *g* for 10 min. Subcellular fractionation was performed using a density gradient prepared from iodixanol (Optiprep; Sigma, St. Louis, Mo.) and ultracentrifugation using a modification of protocols described previously (38, 40, 48). A discontinuous gradient was prepared using 30%, 25%, 20%, 15%, and 10% (vol/vol) Optiprep solution. The gradient was allowed to

equilibrate vertically for 30 min at room temperature. The postnuclear supernatant was overlaid onto the discontinuous gradient and centrifuged at 100,000 x *g* in an SW41 rotor for 3 h at 4°C. Equal fractions were collected from the top of the gradient, and individual fractions were assayed for pp28Mut35-EGFP, pp28Mut61-EGFP and viral pp28 proteins by immunoblotting.

RESULTS

pp28 multimerizes during viral assembly.

Previous studies from our laboratory and other laboratories have reported that additional higher molecular weight form of pp28 could be observed in SDS-PAGE/western blot analysis of virus infected cells as well in cells transiently expressing pp28 in the absence of other viral protein (Fig. 1) (20, 38). This finding raised that possibility that pp28 multimerized duiring virion assembly. To investigate the possibility of pp28 self-interactions during virion assembly, we performed several assays. Initially, to confirm that the previously observed higher molecular weight form of pp28 were multimeric forms and not an aggregated or misfolded form of pp28, we carried out the experiments in which transiently expressed pp28 or viral pp28 was solubilized under reducing (2% SDS and 2-mercaptoethanol), non-reducing (2% SDS), and less denaturing (0.1% SDS) conditions. We observed a higher molecular weight form of pp28 when samples were disrupted in lower concentrations of SDS suggesting that this form was a multimer of pp28. It also suggested that the predominant form of the pp28 molecule was a dimer (90%) rather than a monomer (10%) when analyzed under less denaturing conditions. Moreover, the detection of higher molecular weight forms following SDS-PAGE in the absence of reducing agents suggested that the multimerization of pp28

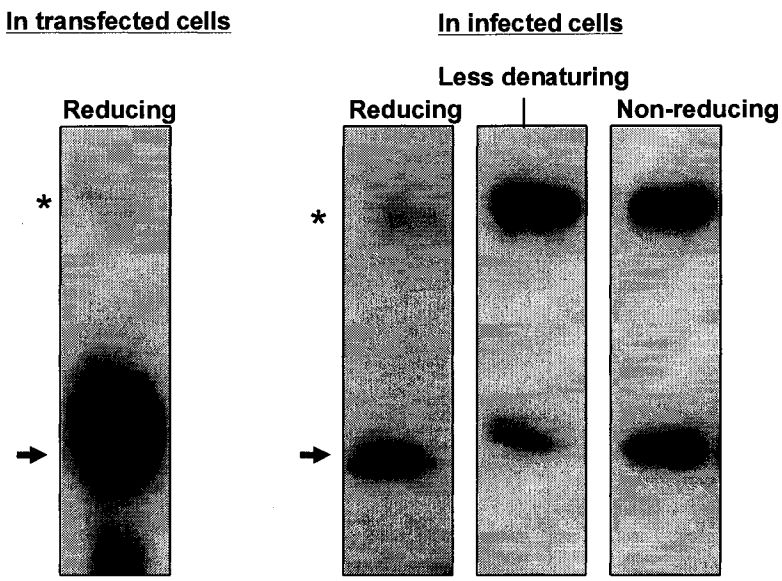

FIG. 1. Expression of multimeric forms of pp28. (A) HK293 cells were transfected with a plasmid encoding pp28, harvested at day 2 post transfection and (B) HF cells were infected with HCMV (AD169) at a moi of 0.1 and harvested at day 6 post infection. The cells were solubilized under reducing (2% SDS and 5% 2-mercaptoethanol), non-reducing (2% SDS), and less denaturing (0.1% SDS) conditions, subjected to SDS-PAGE in 10% gel and detected with anti-pp28 mab by western blot analysis as described in Materials and Methods. Arrows indicate the mass predicted (kilodaltons) from the amino acid sequence of pp28. An asterisk (*) indicates the migration of multimeric form of wild-type pp28.

could be facilitated through disulfide bonding at cysteine residues.

Next, we determined if multimerization of pp28 took place during virus infection of permissive cells by a pulse-chase analysis. Virus infected cells that had been pulse labeled with ^{35}S-met/cys were then chased in media containing unlabelled met/cys and cycloheximide. After cell lysis, labeled proteins were fractionated by centrifugation through sucrose density gradients and each fraction analyzed by immune precipitation and SDS-PAGE (2). Interestingly, in the later chase intervals, pp28 was detected in fractions of increased sucrose density as compared to pp28 synthesized during the pulse or at early chase intervals (Fig. 2A). The kinetics of formation of the higher molecular weight forms of pp28 argued against aggregation but did not rule out the possibility that pp28 associated with other cellular or viral proteins in the chase periods. This possibility was investigated by carrying out a similar experiment utilizing radiolabelling and pulse-chase analysis of transiently expressed pp28 in HK293 cells (Fig. 2B). Following pulse labeling and a 40 min chase interval, labeled proteins were fractionated as above. The amount of the precipitated protein was quantified by densitometry and expressed as a percentage of the total pp28 in the gradient (Fig. 2B). In the absence of other viral functions, pp28 appeared to multimerize as evidenced by its migration into the gradient with an increasing chase interval (Fig. 2B). These results indicated that pp28 multimerization did not require other viral proteins but did not rule out the possibility that pp28 mulitmerization followed interaction with a cellular protein.

The pp28 protein utilizes a single domain for self-interaction.

To map sequences required for the mulitmerization, we performed GST pull down assays. We created a series of GST-pp28 fusion proteins in which GST was fused with

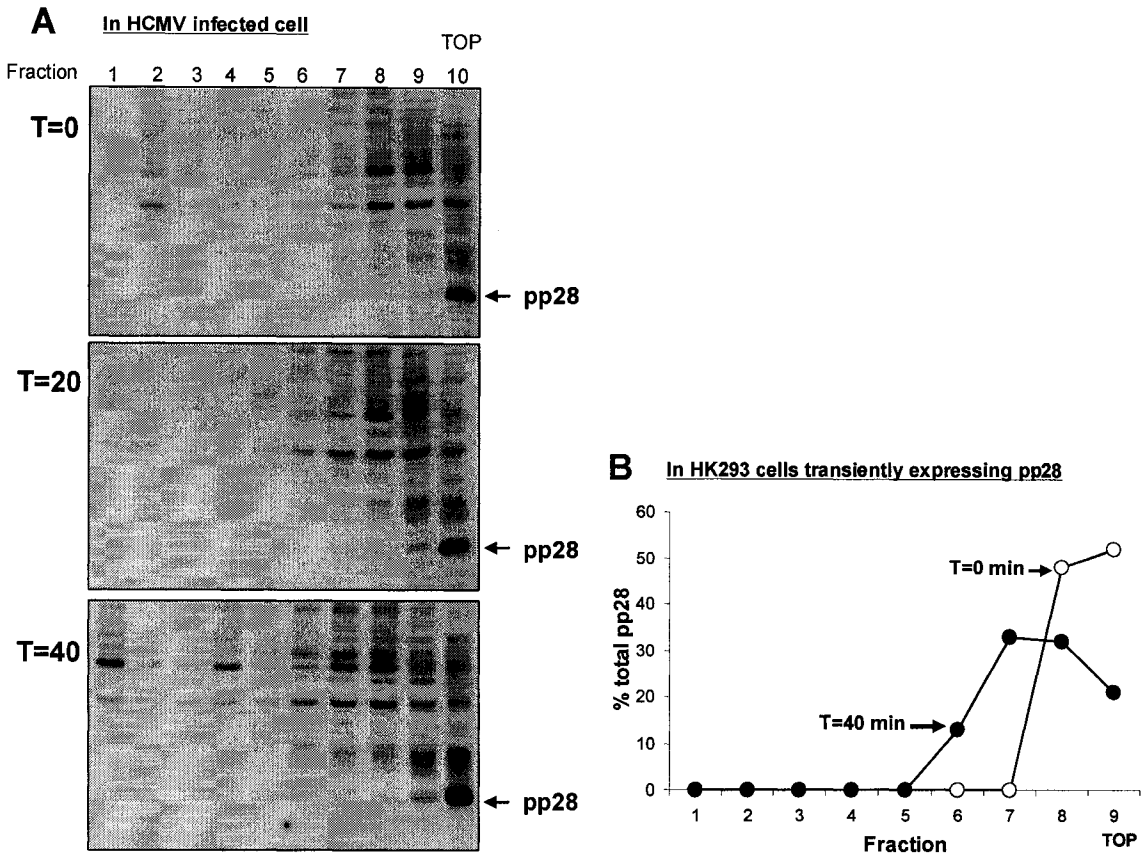

FIG. 2. Multimerization of pp28 through the self-interaction. (A) HF cells were infected with HCMV(AD169) at a moi of 0.1 and harvested at day 7 post infection. The cells were labeled with ^{35}S met/cys and then chased in cycloheximide containing media for indicated times as described in Materials and Methods. Cells were solubilized and fractionated on 5-40 % sucrose linear gradient as described. Gradient fractions collected from the bottom and each fraction precipitated with a anti-pp28 mab and washed in SDS containing RIPA buffer as described in Materials and Methods. Immune precipitates were analyzed by SDS-PAGE. Note the presence of pp28 in lower fractions of gradient as function of chase interval. (B) pp28 was transiently expressed in HK293 cells and pp28 fractionated in 5-40 % sucrose gradient as described above. Results shown as % of total protein in each fraction. Note with increasing chase interval (T=0 versus 40 min) pp28 found in lower fractions, consistent with increasing mass or multimerization.

pp28 wild type (GST-pp28WT), four truncated mutants expressing the first 33, 61, 90, and 123 aa of pp28 (GST-pp28Mut33, GST-pp28Mut61, GST-pp28Mut90, and GST-pp28Mut123, respectively), and three deletion mutants in which aa 26-33, aa 26-43, or aa 44-59, an acidic domain was deleted (GST-pp28Δ26-33, GST-pp28Δ26-43, and GST-pp28Δ44-59 (ac), respectively) as detailed in figure 3A. This panel of GST-pp28 fusion proteins was expressed in *E. coli,* and immobilized on glutathione beads. These immobilized GST-pp28 fusion proteins were used to perform a pull down of a soluble epitope tagged full length pp28 (BB-pp28) also expressed in *E. coli*. All GST-pp28 fusion proteins immobilized on beads were subjected to SDS-PAGE to normalize input amounts of GST-pp28 fusion proteins for pull down experiments (Fig. 3B). To verify antibody specificity of GST-pp28 fusion proteins on beads, we performed western blot analysis with a mab specific for pp28 or for BB-epitope. The anticipated forms of GST-pp28 fusion proteins on SDS-PAGE specifically reacted with the mab for pp28 but not with a mab that was specific for the BB-epitope (Fig. 3B). Interestingly, it was noted that GST-pp28Δ26-33 and GST-pp28Δ26-43 were not detected by a mab specific for the pp28, indicating that the epitope recognized by this mab included aa 26-33 of pp28. After establishing equivalent conditions for all GST-pp28 fusion proteins, these GST-pp28 fusion proteins were used to pull down a soluble BB-pp28 expressed in *E.coli*. After binding and washing, the beads were boiled and the eluted proteins subjected to SDS-PAGE and transfered to NC membranes. Probing the membrane with a mab specific for the BB epitope tag revealed that GST-pp28WT as well as all GST-pp28 mutants, but not GST alone bound soluble BB-pp28 expressed in *E.coli* (Fig. 3C). When the quantity of the bound pp28 was analyzed by densitometry, the amounts of BB-pp28 bound on GST-pp28 mutants were different indicating that multiple domains contribute to the wild type

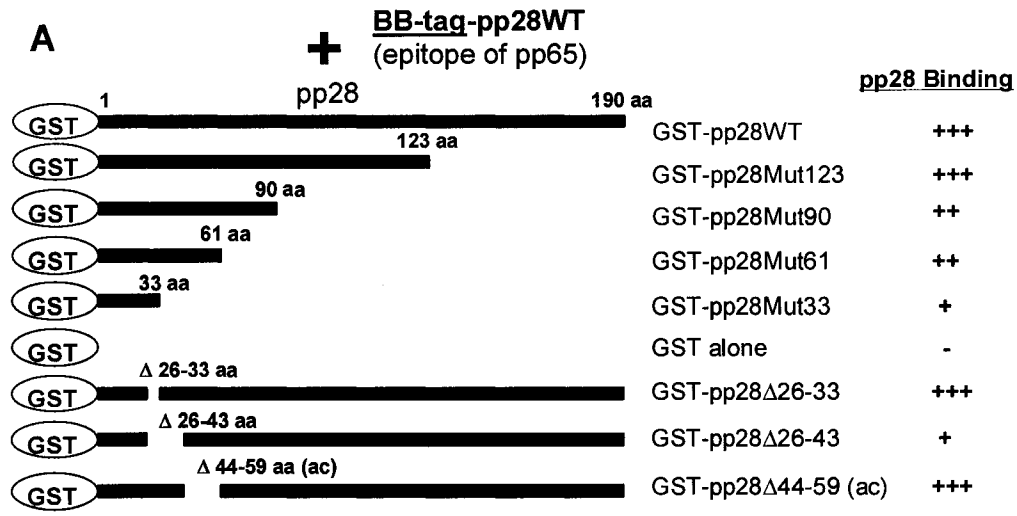

FIG. 3. Mapping of interacting domains of pp28 for the self-interaction by pull downs. (A) Schematic diagram of the GST-pp28 fusion proteins depicting the primary structure of the deletion mutants. Shaded horizontal bars indicate the residues expressed by the mutants. pp28 binding activity of each mutant, quantitated as described in the text, is presented at the right of the figure: +++ indicates activity 100 % of wild type, ++ indicates activity 70 to 90 % of wild type, + indicates activity less than 5 % of wild type, and - indicates activity 0 % of wild type.

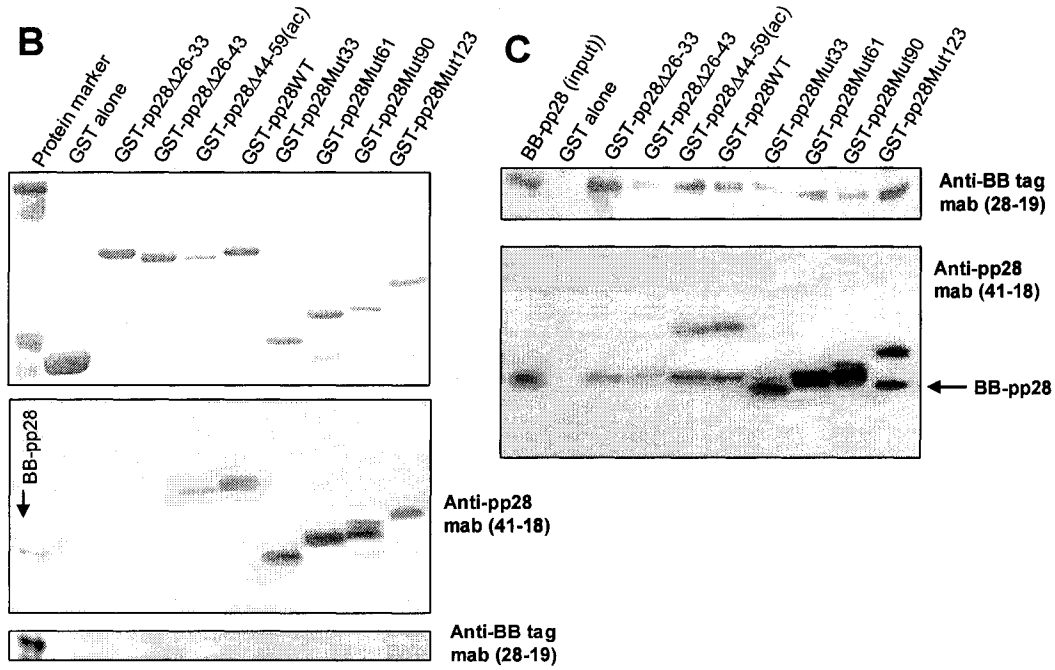

FIG. 3. (Continued) (B) SDS-PAGE (top) showing amounts of GST-pp28 fusion proteins on beads prior to pull downs, and immunoblots (middle and bottom) showing antibody specificity of GST-pp28 fusion proteins on beads. GST alone or GST-pp28 fusion proteins were purified from bacterial lysates by incubation with glutathione-agarose beads as described in Materials and Methods. Beads with associated proteins were subjected to SDS-PAGE and were visualized by western blotting with anti-pp28 mab and anti-BB tag mab as described in Materials and Methods. We noted that GST-pp28Δ26-33 and GST-pp28Δ26-43 were not detected by a anti-pp28 mab, indicating that the epitope recognized by this mab included aa 26-33 of pp28. (C) Immunoblots showing the pp28 binding activity of deletion mutants. The equal amounts of purified GST alone or GST-pp28 fusion proteins on beads in (B) were incubated with lysate from bacteria expressing BB tag-pp28. After extensive washing, bound proteins were subjected to SDS-PAGE and were visualized by western blotting with anti-pp28 mab (41-18) and anti-BB tag mab (28-19) as described above. The input lane shows 10% of the BB-pp28 fusion protein lysate added to the binding reaction.

level of pp28-pp28 interactions. The amounts of BB-pp28 bound on GST-pp28Mut33 and GST-pp28Δ26-43 were significantly less than that of GST-pp28WT and the other GST-pp28 mutants including GST-pp28Δ26-33 (Fig. 3C). The result indicated that the first 33 aa was not sufficient for the wild type level of pp28-pp28 interactions. Moreover, the region aa 34-43 contained aa sequences that were critical for the pp28-pp28 interactions. Thus, it appeared that the first 43 aa was contained an interacting domain including essential aa sequences for the self-interaction of pp28. In addition, GST-pp28Mut61 and GST-pp28Mut90 exhibited a slight decrease in the ability to interact with the BB-pp28 as compared with GST-pp28WT and GST-pp28Mut123, a mutant that binds with similar efficiency as wild type pp28 (Fig.3C). The result indicated that the first 61 aa of pp28 was sufficient for wild type pp28 interactions. Interestingly, GST-pp28Δ44-59 (ac) exhibited the wild type binding activity (Fig. 3C) suggesting that the acidic aa cluster has no direct role in the multimerization of pp28, although the region has a critical function for trafficking of pp28 and production of infectious virus as described previously (38). We also reprobed the membrane with a mab specific for the pp28 and the same patterns of the quantity of bound BB-pp28 were observed (Fig. 3C). In addition, this result confirmed that the quantity of GST-pp28 fusion proteins input in pull downs was similar.

pp28-pp28 interactions occur at the ERGIC in transiently expressed cells.

Previous studies from our laboratory have reported that pp28 is localized to the ERGIC in absence of other viral proteins (35, 38). Moreover, in the current study, we showed that pp28 multimerization did not require other viral proteins. In the following experiments, we used fluorescence resonance energy transfer (FRET) assays to

investigate the cellular sites of pp28-pp28 interactions in mammalian cells and to further determine sequence requirements for the self-interaction of pp28. Full length and a panel of deletion mutants of pp28 were fused with EGFP and co-expressed in Cos-7 cells together with a full length pp28 that was fused with monomeric red fluorescence protein (pp28-DsRed). The FRET was carried out utilizing a donor dequenching/acceptor photobleaching assay (Fig 4). This assay enabled us to detect pp28 interactions within membranes of transfected cells. For FRET assays, EGFP fused with DsRed (EGFP-DsRed) was used as a positive control in transfected Cos-7 cells. In addition, GGA1 fused with EGFP (GGA1-EGFP) was used as a negative control when co-expressed with pp28-DsRed in Cos-7 cells. We selected a part of ERGIC as region of interest (ROI1) for FRET, and non-bleaching region as an internal control (ROI2) after FRET (Fig.4A). The FRET efficiency of a full length pp28 or the first 61 aa of pp28 (pp28Mut61) (6-8%) was similar to that of EGFP-DsRed (7 %) (Fig.4B) indicating that the first 61 aa as well as full length of pp28 could strongly interact with the full length pp28 (pp28-DsRed). The result also showed that the pp28 self-interaction occurred in the ERGIC in the transfected cells indicating that self-association of pp28 did not require additional viral functions. The FRET efficiency of the deletion mutants containing the first 50, 35, and 30 aa of pp28 (3-5%) was lower than that of pp28Mut61 (Fig. 4B) indicating that although the first 30-35 aa of pp28 was sufficient for interaction with the full length of pp28, the first 61 aa of pp28 was required for wild type interactions. In contrast, the FRET efficiency of a construct containing only the first 25 aa of pp28 (pp28Mut25) was zero (0%) (Fig. 4B) indicating no interaction with pp28-DsRed even though pp28Mut25 could be co-localized with full length pp28 in the ERGIC by static immunofluorescence assays. This result also suggested that the binding or retention signal of pp28 to the ERGIC was distinct to the

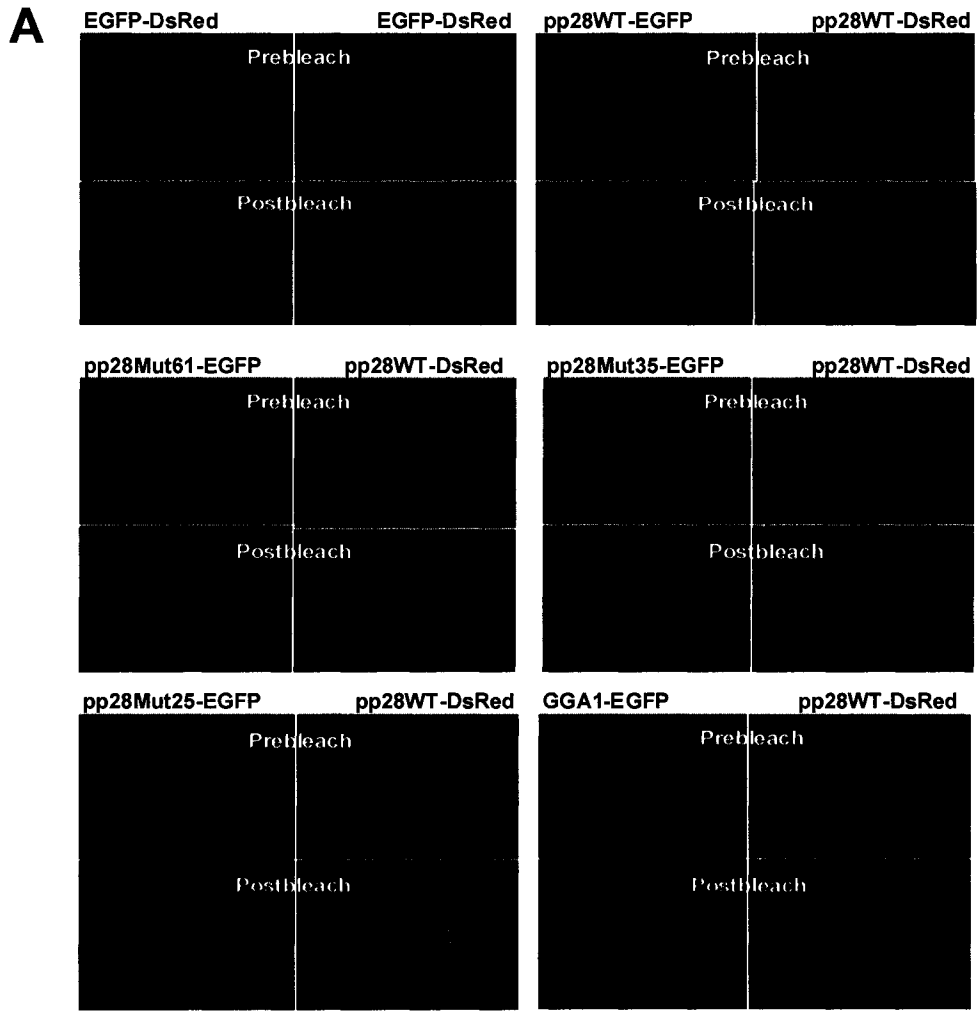

FIG. 4. Interactions of pp28 deletion mutants with the full length pp28 protein within the ERGIC in transfected Cos-7 cells. (A) Pre- and postbleaching of cells expressing EGFP and DsRed. Cos-7 cells were co-transfected with approximately 3 ug of two expression plasmids encoding wild type pp28-DsRed (pp28WT-DsRed) with wild type pp28-EGFP (pp28WT-EGFP) or mutant pp28-EGFP (pp28Mut61-EGFP, pp28Mut50-EGFP, pp28Mut35-EGFP, pp28Mut30-EGFP, or pp28Mut25-EGFP). The cells were harvested at day 2 post transfection, fixed with 2% paraformaldehyde, and analyzed by FRET assays as described in Materials and Methods. EGFP fused with DsRed (EGFP-DsRed) or GGA1 fused with EGFP (GGA1-EGFP) was prepared as a positive or negative control in FRET assays, respectively. ROI indicates region of interest (ERGIC). ROI 1, acceptor bleaching region; ROI 2, acceptor non-bleaching region as an internal control. Note the enhancement in the intensity of EGFP postbleaching.

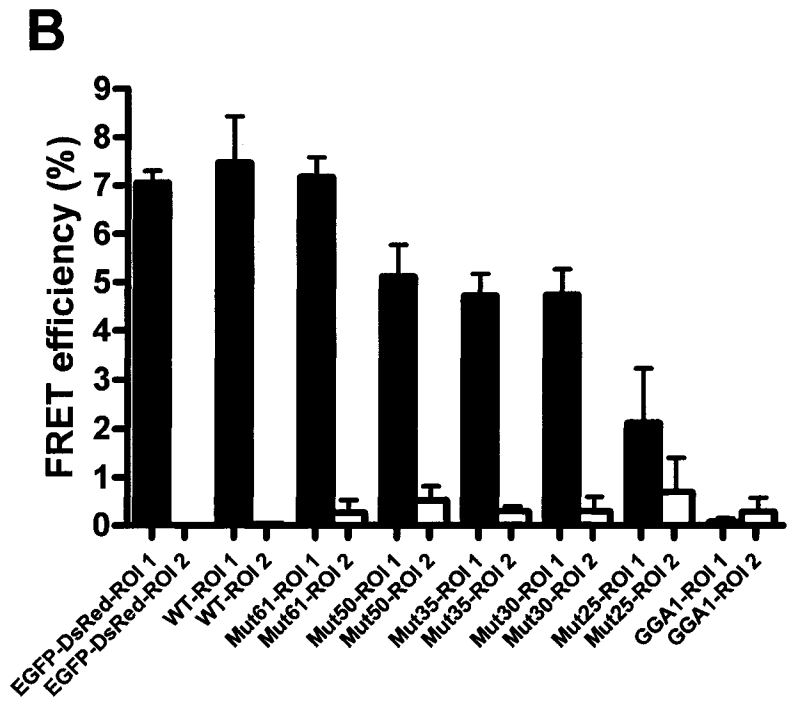

FIG. 4. (Continued) (B) Efficiency of FRET determined using Leica confocal microscope and software. Images (A) acquired on Leica confocal microscope and processed using donor dequenching/acceptor photobleaching protocol. FRET efficiency calculated by formula FRET efficiency (%) = [(*D*post -*D*pre)/*D*post] *100. *D*post is the fluorescence intensity of the EGFP (donor; *D*) after photobleaching and *D*pre is the fluorescence intensity of the DsRed before photobleaching. FRET efficiency statistically significant ($p < 0.001$) for all constructs except pp28Mut25 compared to internal control. FRET from pp28Mut25 significantly different from all other constructs ($p < 0.001$).

self-interacting domain of this protein. Interestingly, somewhat different results were noted between FRET assays and pull downs. The results of these experiments indicated that the pp28-pp28 interactions were influenced by differences in expression systems, mammalian cells and *E.coli*. In contrast to the result from GST-pp28 pull downs suggesting that the first 43 aa was required for the self-interaction, the FRET results indicated that the first 30-35 aa of pp28 were sufficient for pp28 self interactions within the ERGIC. Importantly, the FRET achieved with mutant forms of pp28 containing less than the first 61 aa was lower than wild type pp28. These results together with the results from pull downs indicated that the first 61 aa was required for wild type pp28 interactions.

pp28 multimerization is a post localization function of pp28 within the AC of cells infected with HCMV.

It has been reported that although the first 30-35 aa of pp28 was sufficient for localization of of pp28 to the ERGIC in transfected cells, the first 50 aa of pp28 was required for localization of pp28 to the AC and for assembly of infectious virus in virus infected cells (38). Furthermore, the first 61 aa of pp28 was required for wild type function of pp28 within the AC as measured by the assembly and release of wild type levels of infectious virus (38). To investigate the possible role of multimerization of pp28 in the AC or in other intracellular compartments in the process of virus assembly, we investigated the sequence requirement for pp28-pp28 interactions in different intracellular compartments of HCMV infected cells using FRET (Fig. 5). A DsRed tagged full length pp28 protein and an EGFP tagged pp28 protein were co-expressed in HF cells following electroporation of a plasmid encoding the first 61, 50, or 35 aa of the

pp28 molecule, each of which was fused at its C-terminus to EGFP (pp28Mut61-EGFP, pp28Mut50-EGFP, or pp28Mut35-EGFP). Electroporated HF cells were infected with HCMV 48 hrs later at a moi of 0.1. The FRET was performed on the regions of interest, AC (ROI1) and intracellular vesicles (ROI2 and 3) (Fig.5A). Significant level (>7%) of FRET was detected in the AC of cells expressing pp28Mut61, and lower level (3-4 %) was in the AC of cells expressing pp28Mut50 (Fig.5B). This result was consistent with results that were obtained in cells transiently expressing pp28 and pp28 mutants (Fig.4). However, the FRET efficiency of pp28Mut35 in the AC was significantly lower (< 1%) raising the possibility that although pp28Mut35 could multimerize in the ERGIC when transiently expressed in the absence of other viral proteins in transfected cells, it failed to localize and multimerize in the AC of the virus infected cells (Fig. 5B). This finding was consistent with our previous findings that described the sequence requirements for localization of pp28 to the virus assembly site and for assembly of infectious virus (38). These findings suggested that the accumulation of pp28 within the AC was a prerequisite for multimerization of pp28 and pp28 mutants and that multimerization was not sufficient for trafficking of pp28 to the AC. The FRET efficiency of pp28Mut61 or pp28Mut50 in the intracellular vesicles increased as compared to that seen in the AC (6-8%) (Fig. 5B) indicating that pp28 molecules in transiently expressed/infected cells interacted with each other in the intracellular vesicles as well as in the AC. Interestingly, the FRET efficiency of pp28Mut35 in the intracellular vesicles was significantly less (<1%) (Fig. 5B), although pp28Mut35 could be co-localized with full length pp28 at the vesicles. The results raised the possibility that in transient expression/infection assays, mutant and wild type pp28 molecules compete with wild type viral pp28 for self-interaction in the intracellular vesicles. Because wild type pp28 molecules interacted with pp28Mut50,

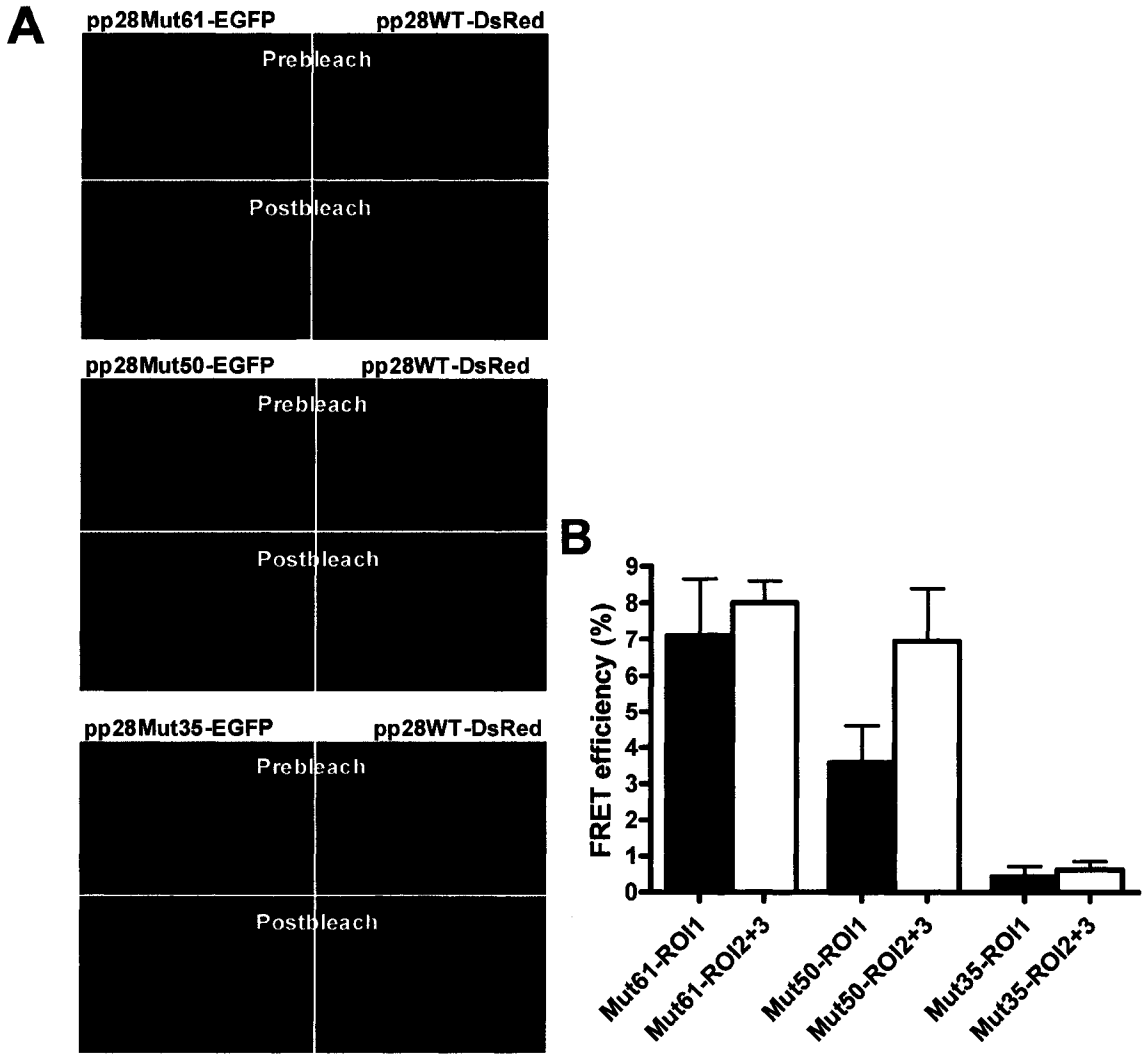

FIG. 5. Interactions of pp28 deletion mutants with the full length pp28 protein within the assembly compartment and intracellular vesicles in HCMV-infected HF cells. (A) Pre- and postbleaching of cells expressing EGFP and DsRed. HF cells were electroporated with approximately 5 ug of two expression plasmids encoding wild type pp28-DsRed (pp28WT-DsRed) with mutant pp28-EGFP (pp28Mut61-EGFP, pp28Mut50-EGFP, or pp28Mut35-EGFP) and infected 2 days later with HCMV at an moi of 0.1. The cells were harvested at day 6 post infection, fixed with 2% paraformaldehyde, stained with anti-IE-1 mab followed by FITC-labeled anti-mouse IgG to identify infected cells (green nuclei) and analyzed by FRET assays. ROI indicates region of interest. ROI 1, assembly compartment; ROI 2 and 3, intracellular vesicles. (B) Efficiency of FRET determined using Leica confocal microscope and software. FRET efficiency in ROI was calculated by formula FRET efficiency (%) = [(*D*post -*D*pre)/*D*post] *100 as described in Fig. 3. FRET efficiency in the assembly compartment and the intracellular vesicles statistically significant ($p < 0.001$) for pp28Mut61 and pp28Mut50 compared to pp28Mut35. In the assembly compartment, FRET from pp28Mut61 significantly different from pp28Mut50 ($p < 0.05$).

pp28Mut61, or viral pp28 molecules with similar affinity, the pp28Mut35 lacked a complete self-interacting domain and therefore interacted less efficiently as detected by FRET. These results together with the results from FRET assays in transfected cells argued that pp28 multimerization within the AC could be correlated with virus replication.

To verify our findings, we investigated the intracellular distribution of multimeric pp28 forms late in infection from cells transfected with pp28Mut35-EGFP or pp28Mut61-EGFP and then infected with HCMV. We selected these two molecules, pp28Mut35 and pp28Mut61 for this analysis for several reasons. In FRET assays, pp28Mut35 failed to accumulate and multimerize in the AC. In addition, it interacted weakly with wild type pp28 within intracellular vesicles suggesting that this mutant pp28 failed to efficiently compete with wild type viral pp28 for pp28 multimerization. The pp28Mut61 exhibited to wild type levels of accumulation in the AC, multimerized within the AC, and recombinant viruses constructed from this mutant replicated to wild type viral replication. Therefore, these two molecules allowed us to study the role of multimerization of pp28 in the AC. Postnuclear supernatants (PNS) were prepared from cells harvested late in infection (> 6 days) and subjected to centrifugation through discontinuous iodixanol (Optiprep) density gradients, and individual fractions were analyzed by western blotting following electrophoresis (Fig.6). Prior to the density gradients, we verified expression of pp28-EGFP fusion proteins and viral pp28 with an antibody specific for EGPF or pp28. PNS from cells transfected with pp28Mut35-EGFP or pp28Mut61-EGFP followed by infection of HCMV were subjected to analysis under solubilization conditions using low concentrations of SDS (0.1%). Monomeric and multimeric forms of pp28 molecules were detected by western blot with a polyclonal

antibody specific for EGFP. Following stripping of the same membrane, a pp28 specific mab detected the same form (Fig. 6A). As described earlier, the pp28 molecules were separated into monomer and dimers. According to the anticipated molecular weights of pp28 molecules, the results showed that monomeric form of pp28Mut61-EGFP migrated more slowly than viral pp28, and that the dimeric forms of pp28Mut61-EGFP (homodimer, GFP61+GFP61; heterodimer, GFP61+Vpp28) migrated more slowly than similar forms of the viral pp28. Interestingly, the monomeric form of pp28Mut35-EGFP migrated more slowly than viral pp28, while the dimeric forms of pp28Mut35-EGFP (homodimer, GFP35+GFP35; heterodimer, GFP35+Vpp28) migrated faster than the corresponding forms of viral pp28 (Fig. 6A). This result was confirmed in repeated experiments together with PNS from cells infected with HCMV as a control (Fig. 6B). These PNS were then subjected to the same analysis under solubilization conditions using low concentrations of SDS. The same patterns of monomeric and dimeric forms of pp28 molecules shown in figure 6A were detected following western blot with a mab specific for pp28. When we extended the electrophoresis to resolve higher molecular weight forms, the separation of possible dimers was more readily observed (Fig. 6B). The results suggested that dimerization of viral pp28 or pp28Mut61-EGFP increased their mass perhaps secondary to post translational modifications such as phosphorylation, ubiquination, or palmitoylation, while that of pp28Mut35-EGFP did not appear to increase in size. We noted that we could not distinguish a homodimer from a viral pp28 containing hetrodimer in the dimeric forms of pp28Mut61-EGFP or of pp28Mut35-EGFP in these experiments. Based on these migrations of pp28 molecules, we analyzed individual fractions from density gradients by western blotting with a mab specific for pp28 (Fig. 6C). We previously reported that the intracellular distribution of the AC

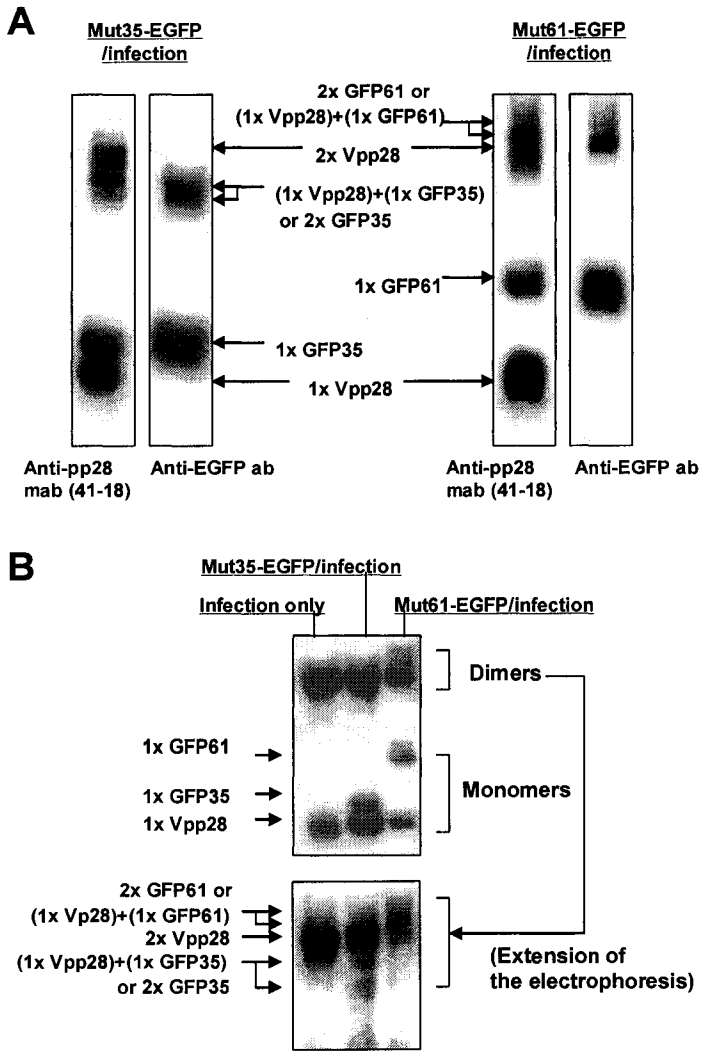

FIG. 6. Iodixanol density gradient fractionation of HCMV-infected HF cells transfected with pp28 mutants. HF cells were electroporated with approximately 5 ug of expression plasmids encoding pp28Mut61-EGFP or pp28Mut35-EGFP and infected 2 days later with HCMV at an moi of 0.1. The cells were harvested at day 6 post infection. (A) Expession of multimeric forms of pp28-EGFP fusion proteins and viral pp28. Postnuclear supernatants (PNS) were prepared from the harvested cells, solubilized under less denaturing (0.1% SDS) conditions, subjected to SDS-PAGE in 10% gel and detected with anti-pp28 mab and anti-EGFP polyclonal ab by western blot analysis as described in Materials and Methods. Note faster migration of dimeric forms of pp28Mut35-EGFP compared to dimeric form of viral pp28. The mass predicted (kilodaltons) from the amino acid sequence of pp28Mut35-EGFP is bigger than that of viral pp28. (B) Verification of migration in dimeric forms of pp28-EGFP fusion proteins and viral pp28. Results of panel A was confirmed by repeated experiments together with PNS from cells infected with HCMV as a control.

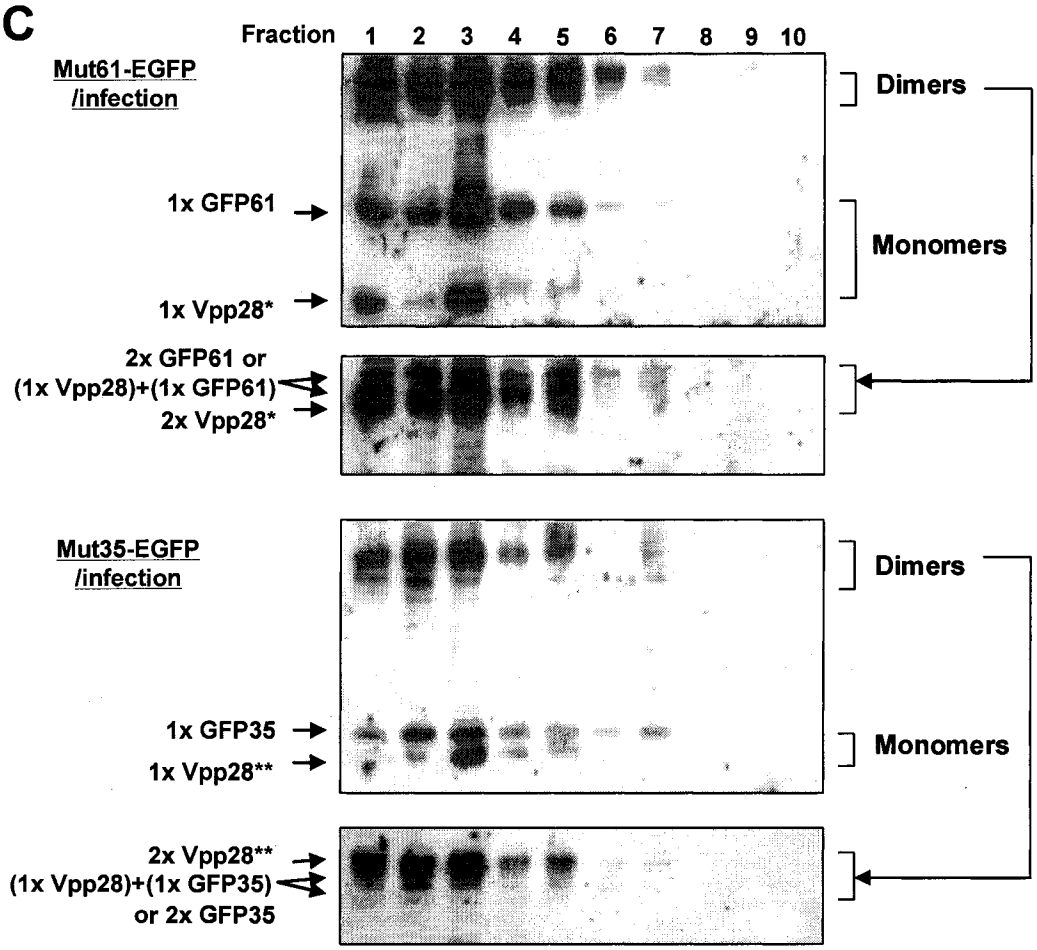

FIG. 6. (Continued) (C) Analysis of gradient fractions by western blotting. PNS was fractionated by centrifugation through iodixanol gradients as described in Materials and Methods. The gradient was fractionated by removing 1 ml fractions from the top; thus, fraction 1 represents the top of the gradient, and fraction 10 represents the bottom of the gradient. Gradient fractions were analyzed by western blot. Proteins were detected with a mab specific for pp28 as described Materials and Methods.

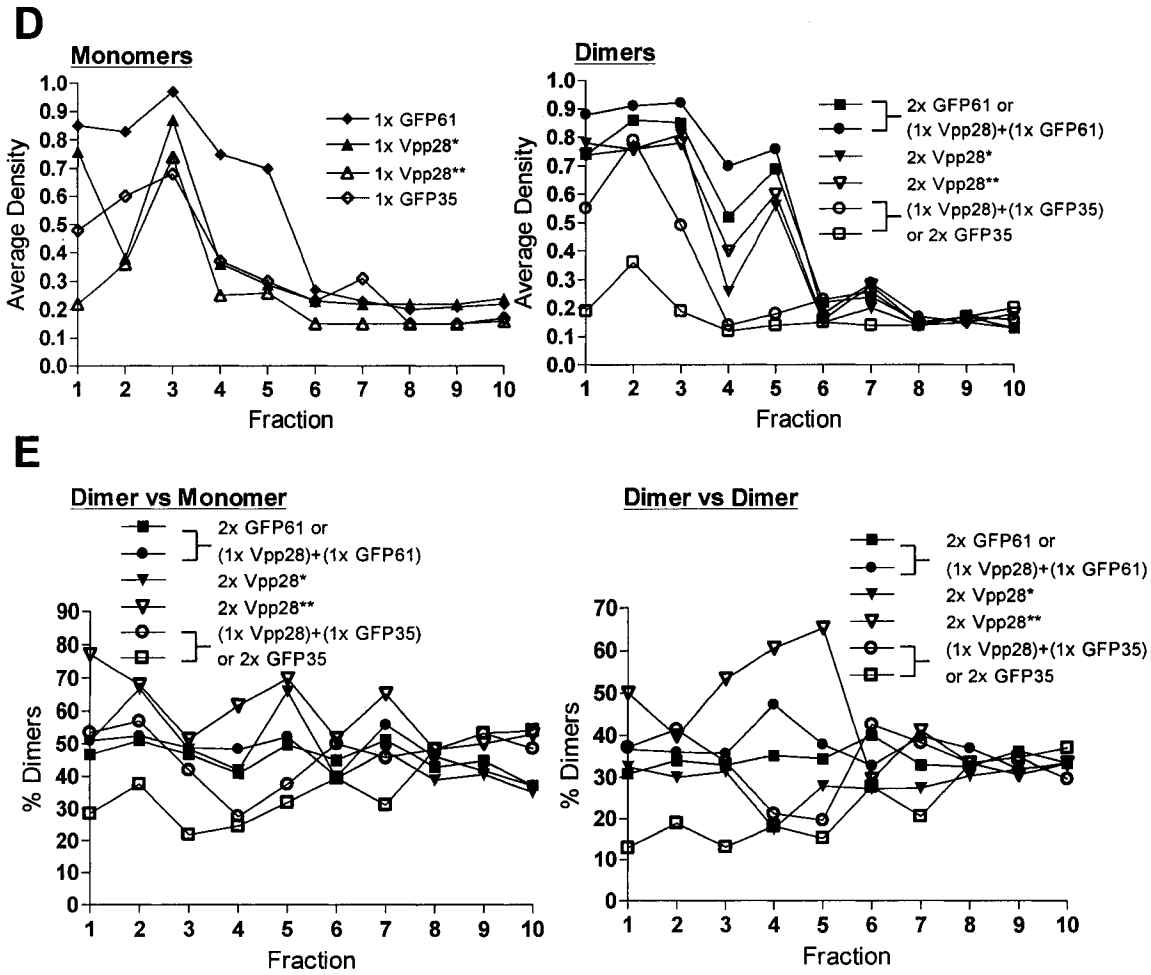

FIG. 6. (Continued) (D) Analysis of results of panel C by densitometry. Average density of signal from protein in all gradient fractions was measured. It represents a graphic comparison of the pattern of fractionation of virus-encoded pp28 to those of pp28Mut61-EGFP and pp28Mut35-EGFP in monomeric forms (left of D) and in dimeric forms (right of D). (E) Ratios of dimer to monomer and of dimer to total dimers in individual fraction. Average density in panel D was analyzed by the ratios of dimer/monomer [2xVpp28/1xVpp28, (Vpp28+GFP)/GFP, and 2xGFP/1xGFP] and dimer/total dimer [a dimer/(2xVpp28)+(Vpp28+GFP)+(2xGFP)] in each feaction and represented by a percentage of dimer (%) = [(dimer/(dimer+monomer)]*100 and (dimer/total dimer)*100. Vpp28, GFP61, and GFP35 indicate viral pp28, pp28Mut61-EGFP, and pp28Mut35-EGFP, respectively. 1x and 2x indicate monomer and dimer, respectively. An asterisk (*) and (**) indicates viral pp28 fractionated from HCMV-infected HF cells transfected with pp28Mut61-EGFP and pp28Mut35-EGFP, respectively.

containing the pp28 molecule was consistent with the distribution of the tetraspanin molecule CD63, a marker of late endosomal/lysosomal compartments (38). Using this as a marker of the AC, our gradients revealed that the AC partitioned into two broad peaks (38). The amount of protein detected in each fraction was quantified by densitometry (Fig. 6D). The monomers of pp28Mut35-EGFP, pp28Mut61-EGFP, and viral pp28 exhibited a similar distribution in the gradient with the majority of this protein migrating in the first 3 fractions of the gradient. In contrast, the dimeric forms of these molecules exhibited a different pattern of distribution. Dimers of pp28Mut61-EGFP or viral pp28 were distributed in two peaks, with one broad peak being associated with the first 3 fractions and a second peak migrating further into the gradient (fraction 5; Fig. 6D). This result suggested that the intracellular distributions of dimeric forms of viral pp28 and pp28Mut61-EGFP were consistent with the distribution of the AC under these gradient conditions. However, two dimeric forms, a homodimer of pp28Mut35-EGFP and pp28Mut35-EGFP, and a heterodimer of viral pp28 and pp28Mut35-EGFP, exhibited a different distribution in the gradient with the majority of this protein migrating in the first 2-3 fractions of the gradient similar to that of monomeric forms (Fig. 6D). The results suggested that a significant fraction of multimeric forms of pp28Mut61-EGFP and viral pp28 molecules could be found co-sedimenting with the AC, whereas the pp28Mut35 molecule multimerized in intracellular membranes that sedimented with membranes of the ER and Golgi (38).

To further investigate a role of pp28 multimerization during virus assembly, we analyzed the ratios of dimer/monomer and of dimer/total dimers for viral pp28 and pp28 mutants in each fraction of these gradients (Fig. 6E). The ratios of dimer/monomer showed that the majority of viral pp28 or pp28Mut61-EGFP molecules consisted of

dimers in fraction 5-7 (50-70%) indicating that the majority of the viral pp28 or pp28Mut61 in the AC was a dimeric form. In contrast, the majority of pp28Mut35-EGFP molecules was not dimers in fraction 1-3 (20-45%) indicating a favored form of pp28Mut35 in the cell was a monomeric form. Moreover, the ratios of a viral pp28 containing heterodimer of pp28Mut35-EGFP to a monomer ((GFP35+Vpp28)/1xGFP35) were different to that of a homodimer of pp28Mut35-EGFP to a monomer (2xGFP35/1xGFP35). This result indicated that pp28Mut35 interacted with pp28Mut35 or viral pp28 at a different binding affinity. In addition, the ratios of dimer/total dimers suggested that viral pp28 and pp28Mut61-EGFP have a similar proportion of dimers in fraction 5-7 (all 30%). This result indicated that viral pp28 and pp28Mut61 interacted with each other in the AC with a similar binding affinity. In contrast, viral pp28 and pp28Mut35-EGFP have a different proportion of dimers in fraction 1-5 (homodimer of viral pp28, 50-65%; heterodimer or homodimer of pp28Mut35-EGFP, 20-40% or 10-20%) indicating the binding affinity of viral pp28 to viral pp28 was higher than that of viral pp28 to pp28Mut35 in the intracellular vesicles, although the majority of viral pp28 dimers was distributed to the AC. This result together with the result from the ratios of dimer/monomer indicated that the binding affinity of pp28Mut35 to viral pp28 was higher than that of pp28Mut35 to pp28Mut35 in the intracellular vesicles, although the majority of pp28Mut35 was monomeric form and weakly interacted with pp28 molecules in the vesicles. Finally, the results indicated that the capacity of the mutant pp28 to multimerize in the AC was consistent with the capacity of the mutant pp28 to support virus replication. This finding together with the result from FRET assays suggested that pp28 multimerization as a post localization function in the AC (not in the intracellular vesicles) could represent an essential step in the particle envelopment and production of

infectious virions.

DISCUSSION

The role of multimerization of tegument proteins in the herpesvirus assembly has not been studied. However, numerous reports in the literature have documented that the multimerization of tegument or matrix proteins in structurally less complex viruses, such as small RNA viruses plays an essential roles in the viral assembly. The matrix proteins in vesicular stomatitis virus (VSV), paramyxoviruses, Ebola virus, influenza virus, Rous sarcoma virus (RSV), and human immunodeficiency virus type 1 (HIV-1) are membrane associated and undergo a multimerization to create lattice like structures which then lead to the eventual budding or pinching off of that region of the membrane (10, 13, 15-17, 19, 22, 24, 33, 36, 37, 44, 52). In this study, we have provided evidence that multimerization of the HCMV tegument protein, pp28 appeared to be required for viral assembly.

Previously our studies and those from other laboratories have reported that additional higher molecular weight form of pp28 can be observed in SDS-PAGE/western blot analysis of pp28 (20, 38). However, these findings of higher molecular weight forms of pp28 could also suggest that pp28 could form mulitmers or that misfolded forms of pp28 could aggegrate into higher molecular species. In the experiments presented in this report, we confirmed that the higher molecular weight forms are multimers of pp28 by several methods. Initially, we found a higher molecular weight form of pp28 molecule could be detected under less denaturing (0.1% SDS) conditions of protein solubilization. In addition, the detection of higher molecular weight forms following SDS-PAGE in the absence of reducing agents suggested that the multimerization of pp28 could be mediated by intra- and inter-chain disulfide bonding. We also confirmed the multimerization of

pp28 in virus infected cells by a pulse-chase analysis and sedimentation in sucrose gradients (2). Furthermore, the pulse-chase analysis from transiently expressed pp28 molecule indicated that pp28 can multimerize through self-interactions in absence of other viral proteins.

The domains mediating pp28 multimerization have been mapped by use of several techniques. Initially we used a yeast two-hybrid screen to identify a large region consisting of the N-terminal third (the first 61 aa) of pp28 as the region mediating pp28-pp28 interactions (data not shown). In addition, the results from two-hybrid analysis confirmed that pp28 multimerizes through self-interaction. In the experiments using GST pull down assays, a deletion of pp28 in which aa 26-43 was deleted from this 190 aa protein resulted in a significant decrease in its interaction with the full length of pp28. This finding indicated that the first 43 aa of pp28 contained a aa sequence that was essential for wild type levels of self-interactions of pp28. However, the pp28 binding activity of other deletions, Δaa 124-190, Δaa 26-33, or Δaa 44-59 (ac) was comparable to that of the full length of pp28 indicating that the C-terminal third of pp28, aa 26-33, and N-terminal acidic aa cluster were not essential for wild type pp28 self-interaction. It was of interest to note that although the acidic aa cluster has a critical function for trafficking of pp28 and production of infectious virus as described previously (38), this domain has no direct role in the multimerization of pp28. In addition, the other deletions, Δaa 62-190 or Δaa 91-190 of pp28 exhibited a little decrease in the ability to interact with the full length of pp28. The result indicated that the first 61 aa was sufficient for the self-interaction of pp28. Finally, our findings from experiments using pull down assays suggested that pp28 utilizes a single domain (aa 1-43) for the self-interaction and greatly refined the mapped domain (aa 1-61) from the two-hybrid analysis (data not shown).

In contrast to the finding of higher molecular weight forms of pp28 following expression in mammalian cells as shown in figure 1, higher molecular weight forms were not detected on the SDS-PAGE/western blot analysis during pull down assays using GST-pp28 fusion proteins and BB-pp28. These findings suggested that pp28 expressed in mammalian cell underwent post translational modification such as phosphorylation, palmitoylation, and ubiquitination, which does not occur in *E.coli*, and thus the modification at the specific residues within the interacting domains possibly affected to pp28-pp28 interactions. Alternatively, in pull down assays, the pp28 multimers were denatured completely by the presence of efficient detergents such as SDS and a reducing agent, 2-mercaptoethanol during its analysis.

Using fluorescence resonance energy transfer (FRET), we have addressed a question relevant to the cytoplasmic assembly of HCMV. In which compartment of the infected cell does the virion structural protein, pp28 multimerize? We found that pp28-GFP and pp28-RFP proteins showed significant FRET at the ERGIC following transient expression in absence of other viral proteins. Moreover, FRET assays of pp28 mutants containing C-terminal deletions transiently expressed in Cos-7 cells suggested that the first 30-35 aa of pp28 was sufficient for interaction with the full length of pp28, although the first 61 aa of pp28 was required for wild type interactions. Together with the results from the experiments carried out under non-reducing conditions, this finding raised the possibility that disulfide bonding at cysteines (Cys 6, 10, and 11) of N-terminal region could be required for multimerization of pp28. It has been reported that cysteines within the interacting domain of viral structural proteins such as the NC domain of the Gag polyprotein and antigenic domain 1 of HCMV gB, are required for multimerization of the proteins (2, 25, 30, 31). However, the low FRET efficiency of pp28Mut25 containing

these cysteine residues suggested that although disulfide bonding could be required for self-interactions, it is not sufficient for mulitmerization of this molecule. In addition, considering the correlation between multimerization of pp28 at the ERGIC and the requirement of myristoylation at Gly 2 of pp28 for the authentic localization (3, 38), we questioned the possibility that pp28 multimerization could play a role in pp28-membrane binding. It has been reported that mutations in SP1 that inhibit Gag multimerization reduce membrane binding (22), and dimerization of the RSV MA increased its capacity to associate with membrane in vitro (13). In addition, structural studies with HIV-1 MA have demonstrated that the multimerization leads to greater exposure of the myristate moiety, thereby presumably increasing membrane binding ability (44). However, the FRET assays of pp28 mutants containing C-terminal deletions showed that in contrast to the first 30-35 aa of pp28, the first 25 aa of pp28 could not interact with the full length of pp28, although they are co-localized at the ERGIC in transfected cells. Moreover, in our previous findings from static immunofluorescence assays, unlike non-myristoylated pp28 mutant, pp28 mutant expressing the first 25 aa trafficked and bound to the membrane of ERGIC in transfected cells (3, 38). Therefore, the binding or retention signal of pp28 to the ERGIC is distinct to the self-interacting domain of this protein, and thereby pp28 multimerization is not required for pp28-membrane binding.

When the results from FRET assays were compared with the results from pull downs, it was of interest to note that the pp28-pp28 interactions were possibly influenced on by differences such the presence of post translational modifications in eukaryotic expressed pp28. In contrast to the result from pull downs that the first 43 aa of pp28 was required for the self-interaction, the FRET results indicated that the first 30-35 aa of pp28 were sufficient for pp28 self-interactions within the ERGIC. Alternatively, these different

results could be explained by differences in sensitivity of these distinct assays

In contrast to these finding in transient expression assays, FRET assays of pp28 in transiently expressed/infected cells revealed that pp28-GFP and pp28-RFP proteins interact with each other in the AC as well as in the intracellular vesicles. Moreover, FRET assays of pp28 mutants containing C-terminal deletions in transiently expressed/infected HF cells suggested that at least the first 50 aa of pp28 is required for the multimerization of pp28 in the AC. In addition, pp28Mut50 could strongly interact with the full length of pp28 in the intracellular vesicles similarly to pp28Mut61 that is competent to wild type interactions and virus replication. These findings suggested two possibilities. The first is that pp28 may form multimeric intermediates in the intracellular vesicles, and these intermediate complexes could then traffic to the AC. Alternatively, pp28 multimers could form as a result of accumulation of pp28 within the AC. Consistent with the latter possibility was the finding that although pp28Mut35 likely multimerized in the ERGIC of the transfected cells, it failed to accumulate in the AC and as a result did not multimerize in the AC of the virus infected cells. Moreover, this finding was consistent with our previous findings from the sequence requirements for localization of pp28 to the virus assembly site and for assembly of infectious virus (38) suggesting that the accumulation of pp28 is a prerequisite for multimerization of this protein in the AC. Unexpectedly, and in contrast to results from FRET analysis of pp28Mut50 and pp28Mut61, FRET assays of pp28Mut35 in the intracellular vesicles as well as in the AC of transiently expressed/infected cells exhibited little evidence supportive of pp28-pp28 interactions. The results suggested that in transient expression/infection assays, mutant and wild type pp28 molecules compete with wild type viral pp28 for self-interaction in the intracellular vesicles. However, wild type pp28 molecules interacts with pp28Mut50,

pp28Mut61, or viral pp28 molecules with similar affinity, while it interacts with pp28Mut35 much less efficiently because of the lack of a complete self-interacting domain and therefore does not exhibit significant FRET. It was also of interest to note that truncation mutants such as pp28Mut35 likely multimerize at the ERGIC yet fail to localize in the AC. Thus, this mutant molecule cannot accumulate in this AC and support virus replication when expressed in the absence of wild type virus. It suggested that the effect of pp28 multimerization on virus replication such as assembly and release might be distinct from a role in pp28 trafficking. Our previous finding from fluorescence recovery after photobleaching (FRAP) assays with mutants pp28Mut35 and pp28Mut50 supported this interpretation and indicated that at equilibrium, these molecules exited the AC at a faster rate than wild type pp28 and pp28Mut61, perhaps accounting for its defect in replication (J. of Virol. in press). It has been reported that the retroviral matrix protein, gag protein alone is sufficient to organize the particle for budding (12, 18, 49, 50). Our results allowed us to exclude at least one possibility that pp28 multimerization in the ERGIC plays a role in the viral budding, and argue that the multimerization in the AC plays a critical role in the envelopment process such as budding of infectious virus. Moreover, the subcellular fractionation analysis using density gradients supported our argument that pp28 multimerization taking place in the AC plays a role in the viral assembly. We previously reported that the intracellular distribution of the AC containing the pp28 molecule was consistent with the distribution of the tetrapanin molecule CD63, a marker of late endosomal/lysosomal compartment (38). The intracellular distributions of multimeric forms of viral pp28 wild type and pp28 mutants expressing the first 61 aa were consistent with that of the AC, while the intracellular distributions of multimeric forms of pp28 mutants expressing the first 35 aa were not. Moreover, the results indicated

that a predominant proportion of pp28Mut61 and viral pp28 molecules multimerize in the AC at the similar binding affinity, whereas that of pp28Mut35 molecule exist as monomers in the intracellular vesicles due to its lower binding affinity with viral pp28 or pp28Mut35. Finally, the results indicated that the capacity of the mutant pp28 to multimerize in the AC was consistent with the capacity of the mutant pp28 to support virus replication. When these data are combined with the FRET analysis, we suggest that pp28 multimerization as a post localization function in the AC (not in the intracellular vesicles such as ERGIC and Golgi as observed in FRET assays) plays an essential role in the particle envelopment and production of infectious virions rather than in membrane binding or intracellular trafficking. Here, we could also propose that once pp28 molecule containing self-interacting domains accumulates in the AC, the pp28 protein is sufficient for function of multimerization in viral assembly. Ongoing studies will attempt to define the role of specific aa residues residing within the first 50 aa of pp28 in the self-interaction. It will allow us to define further a precise role of pp28 multimerization during viral infection.

REFERENCES

1. **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.

2. **Britt, W., M. Jarvis, D. Drummond, and M. Mach.** 2004. Antigenic domain 1 (AD-1) is required for oligomerization of human cytomegalovirus glycoprotein B. Journal of Virology **in press**.

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

4. **Britt, W. J., and C. A. Alford.** 1996. Cytomegalovirus, p. 2493-2523. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields Virology, Third Edition. Raven Press, New York.

5. **Britt, W. J., and D. Auger.** 1986. Synthesis and processing of the envelope gp55-116 complex of human cytomegalovirus. J Virol **58:**185-191.

6. **Britt, W. J., and S. Boppana.** 2004. Human Cytomegalovirus virion proteins. Human Immunology **65:**395-402.

7. **Britt, W. J., M. A. Jarvis, D. D. Drummond, and M. Mach.** 2005. Antigenic domain 1 is required for oligomerization of human cytomegalovirus glycoprotein B. Journal of Virology **79:**4066-79.

8. **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.

9. **Burniston, M. T., A. Cimarelli, J. Colgan, S. P. Curtis, and J. Luban.** 1999. Human immunodeficiency virus type 1 Gag polyprotein multimerization requires the nucleocapsid domain and RNA and is promoted by the capsid-dimer interface and the basic region of matrix protein. Journal of Virology **73:**8527-40.

10. **Chong, L. D., and J. K. Rose.** 1993. Membrane association of functional vesicular stomatitis virus matric protein in vivo. J Virol **67:**407-414.

11. **Cohen, G. H., V. J. Isola, J. Kuhns, P. W. Berman, and R. J. Eisenberg.** 1986. Localization of discontinuous epitopes of herpes simplex virus glycoprotein D: use of a nondenaturing ("native" gel) system of polyacrylamide gel electrophoresis coupled with Western blotting. J Virol **60:**157-66.

12. **Conte, M. R., M. Klikova, E. Hunter, T. Ruml, and S. Matthews.** 1997. The three-dimensional solution structure of the matrix protein from the type D retrovirus, the Mason-Pfizer monkey virus, and implications for the morphology of retroviral assembly. EMJO J **16:**5819-5826.

13. **Dalton, A. K., P. S. Murray, D. Murray, and V. M. Vogt.** 2005. Biochemical characterization of rous sarcoma virus MA protein interaction with membranes. J Virol **79:**6227-38.

14. **Demirov, D. G., and E. O. Freed.** 2004. Retrovirus budding. Virus Research **106:**87-102.

15. **Fujiyoshi, Y., N. P. Kume, K. Sakata, and S. B. Sato.** 1994. Fine structure of influenza A virus observed by electron cryo-microscopy. EMBO J **13:**318-326.

16. **Garoff, H., R. Hewson, and D. J. E. Opstelten.** 1998. Virus maturation by

budding. Microbiol Mol Biol Rev **62:**1171-1190.

17. **Gaudin, Y., J. Sturgis, M. Doumith, A. Barge, B. Robert, and R. W. H. Ruigrok.** 1997. Conformational flexibility and polymerization of vesicular stomatitis virus matrix protein. J Mol Biol **274:**816-825.

18. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde.** 1989. Assembly and release of HIV-1 precursor $Pr55^{gag}$ virus-like particles from recombinant baculovirus-infected insect cells. Cell **59:**103-112.

19. **Hill, C. P., D. Worthylake, D. P. Bancroft, A. M. Christensen, and W. I. Sundquist.** 1996. Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. Proc Natl Acad Sci USA **93:**3099-3104.

20. **Jones, T. R., and S. W. Lee.** 2004. An acidic cluster of human cytomegalovirus UL99 tegument protein is required for trafficking and function. Journal of Virology **78:**1488-502.

21. **Kerry, J. A., M. A. Priddy, C. P. Kohler, T. L. Staley, D. Weber, T. R. Jones, and R. M. Stenberg.** 1997. Translational regulation of the human cytomegalovirus pp28 (UL99) late gene. J Virol **71:**981-987.

22. **Liang, C., J. Hu, J. B. Whitney, L. Kleiman, and M. A. Wainberg.** 2003. A structurally disordered region at the C terminus of capsid plays essential roles in multimerization and membrane binding of the gag protein of human immunodeficiency virus type 1. J Virol **77:**1772-83.

23. **Luban, J., K. B. Alin, K. L. Bossolt, T. Humaran, and S. Goff.** 1992. Genetic assay for multimerization of retroviral gap polyproteins. J Virol **66:**5157-5160.

24. **Markwell, M. A. K., and C. F. Fox.** 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking. J Virol **33:**152-166.

25. **McDermott, J., L. Farrell, R. Ross, and E. Barklis.** 1996. Structural analysis of human immunodeficiency virus type 1 Gag protein interactions, using cysteine-specific reagents. J Virol **70:**5106-14.

26. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. Journal of Virology. **76:**1537-47.

27. **Meyer, H., A. Bankier, M. P. Landini, and R. Ruger.** 1988. Identification and procaryotic expression of the gene coding for the highly immunogenic 28-kilodalton structural phosphoprotein (pp28) of human cytomegalovirus. J Virol **62:**2243-2250.

28. **Mocarski, E. S., Tan Courcelle, C.** 2001. Cytomegaloviruses and their replication, p. 2629-2673. *In* D. M. K. a. P. M. Howley (ed.), Fields Virology, 4th ed, vol. 2. Lippincott Williams and Wilkins, Philadelphia.

29. **Murphy, E., D. Yu, J. Grimwood, J. Schmutz, M. Dickson, M. A. Jarvis, J. A. Nelson, R. M. Myers, and S. T.E.** 2003. Coding capacity of laboratory and clinical strains of human cytomegalovirus. Proceedings of the National Academy of Sciences of the United States of America **100:**14976-14981.

30. **Rein, A., D. E. Ott, J. Mirro, L. O. Arthur, W. Rice, and L. E. Henderson.** 1996. Inactivation of murine leukemia virus by compounds that react with the zinc finger in the viral nucleocapsid protein. J Virol **70:**4966-72.

31. **Rice, W. G., J. G. Supko, L. Malspeis, R. W. Buckheit, Jr., D. Clanton, M. Bu, L. Graham, C. A. Schaeffer, J. A. Turpin, J. Domagala, R. Gogliotti, J. P. Bader, S. M. Halliday, L. Coren, R. C. Sowder, 2nd, L. O. Arthur, and L. E. Henderson.** 1995. Inhibitors of HIV nucleocapsid protein zinc fingers as candidates for the treatment of AIDS. Science **270:**1194-7.

32. **Rubin, R.** 2002. Clinical approach to infection in the compromised host, p. 573-679. *In* R. Rubin, Young, LS (ed.), Infection in the Organ Transplant Recipient. Kluwer Academic Press, New York.

33. **Saad, J. S., J. Miller, J. Tai, A. Kim, R. H. Ghanam, and M. F. Summers.** 2006. Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly.[see comment]. Proceedings of the National Academy of Sciences of the United States of America **103:**11364-9.

34. **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.

35. **Sanchez, V., E. Sztul, and W. J. Britt.** 2000. Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. Journal of Virology **74:**3842-51.

36. **Schnell, M. J., L. Buonocore, E. Boritz, H. P. Ghosh, R. Chernish, and J. K. Rose.** 1998. Requirement for a nonspecific glycoprotein cytoplasmic domain sequence to drive efficient buddign of vesicular stomatitis virus. EMBO J **17:**1289-1296.

37. **Scianimanico, S., G. Schoehn, J. Timmins, R. H. Ruigrok, H. D. Klenk, and W. Weissenhorn.** 2000. Membrane association induces a conformational change in the Ebola virus matrix protein. EMBO Journal **19:**6732-41.

38. **Seo, J., and W. Britt.** 2006. Sequence requirements for localization of human cytomegalovirus tegument protein pp28 to the virus assembly compartment and for assembly of infectious virus. Journal of Virology **80:**5611-5626.

39. **Silva, M. C., Q. C. Yu, L. Enquist, and T. Shenk.** 2003. Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids. Journal of Virology. **77:**10594-605.

40. **Sims, A. C., J. Ostermann, and M. R. Denison.** 2000. Mouse hepatitis virus replicase proteins associate with two distinct populations of intracellular membranes. Journal of Virology **74:**5647-54.

41. **Smith, J. D., and E. DeHarven.** 1973. Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. I. Sequence of viral replication. J Virol **12:**919-930.

42. **Stagno, S., and W. J. Britt.** 2005. Cytomegalovirus, p. In Press. *In* J. S. Remington and J. O. Klein (ed.), Infectious Diseases of the Fetus and Newborn Infant, 6th Edition. W.B. Saunders, Philadelphia.

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

44. **Tang, C., E. Loeliger, P. Luncsford, I. Kinde, D. Beckett, and M. F. Summers.** 2004. Entropic switch regulates myristate exposure in the HIV-1 matrix protein.[see comment]. Proceedings of the National Academy of Sciences of the United States of America **101:**517-22.

45. **Tooze, J., M. Hollinshead, B. Reis, K. Radsak, and H. Kern.** 1993. Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. Eur J Cell Biol **601:**163-178.

46. **Trus, B. L., W. Gibson, N. Cheng, and A. C. Steven.** 1999. Capsid structure of simian cytomegalovirus from cryoelectron microscopy: evidence for tegument attachment sites.[erratum appears in J Virol 1999 May;73(5):4530]. Journal of Virology. **73:**2181-92.

47. **Varnum, S. M., D. N. Streblow, M. E. Monroe, P. Smith, K. J. Auberry, L. Pasa-Tolic, D. Wang, D. G. Camp, K. Rodland, S. Wiley, W. Britt, T. Shenk, R. D. Smith, and J. A. Nelson.** 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. Journal of Virology **78:**10960-10966.

48. **Wentz-Hunter, K., R. Kubota, X. Shen, and B. Y. Yue.** 2004. Extracellular myocilin affects activity of human trabecular meshwork cells. Journal of Cellular Physiology **200:**45-52.

49. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral Gag proteins. AIDS **5:**639-654.

50. **Wills, J. W., R. C. Craven, and J. A. Achacoso.** 1989. Creation and expression of myristylated forms of Rous sarcoma virus gag protein in mammalian cells. J Virol **63:**4331-4343.

51. **Yu, X., S. Shah, I. Atanasov, P. Lo, F. Liu, W. J. Britt, and Z. H. Zhou.** 2005. Three-dimensional localization of the smallest capsid protein in the human cytomegalovirus capsid. Journal of Virology **79:**1327-32.

52. **Zhao, H., M. Ekstrom, and H. Garoff.** 1998. The M1 and NP proteins of influenza A virus form homo- but not heterooligomeric complexes when coexpressed in BHK-21 cells. Journal of General Virology **79:**2435-46.

53. **Zhou, Z. H., M. Dougherty, J. Jakana, J. He, F. J. Rixon, and W. Chiu.** 2000. Seeing the herpesvirus capsid at 8.5 A. Science. **288:**877-80.

CONCLUSIONS

The virion of HCMV consists of an icosahedral nucleocapsid containing a large, double-stranded linear DNA genome, which is surrounded by a tegument layer that is also in contact with a lipid containing envelope that consists of > 15 viral glycoproteins (98).

HCMV morphogenesis is a complex and poorly understood process. The proposed mechanisms of HCMV assembly are based on studies of alpha herpesviruses, herpes simplex virus (HSV), varicella-zoster virus (VZV), and pseudorabies virus (PRV) (115). However, approaches to study this process have focused on either capsid or envelope morphogenesis with little emphasis on tegumentation (117, 130, 156). While capsid assembly in the nucleus is relatively well understood, the mechanism for acquisition of the final envelope in cytoplasmic phase poorly described.

The assembly of herpesviruses has been studied using several approaches. Genetic approaches such as the creation of null or temperature sensitive mutants have been successful for the study of the role of specific proteins in HSV-1 morphogenesis. However, these mutagenic approaches based on homologous recombination in eukaryotic cells have been limited to genetic manipulation of only the most rapidly replicating herpesviruses, such as HSV and PRV (126). The generation of recombinant HCMV has proved difficult because permissive cell types are few primary cells, low viral yield, and prolonged replication (117, 130, 156). Recently, the propagation of herpesviruses as infectious clones in bacterial artificial chromosomes (BAC) has permitted the

experimental manipulation of viral genomes. This technology has allowed application of the tools of prokaryotic molecular biology to the study of viral genetics, thereby facilitating the genetic analysis of many herpesviruses including HCMV (12, 19, 23, 114, 149, 170).

Imaging techniques such as electron microscopy have provided information about the location of capsid, tegument, and envelope assembly using antibodies to specific viral and cellular proteins. However, these approaches have provided only static images of particles and assembly intermediates with little information about other characteristics such as the dynamics and direction of intracellular protein trafficking. In addition, they have been dependent on the generation of virus specific immunologic reagents. Recently, improvement of fluorescence microscopic techniques has allowed more complete investigation of herpesvirus assembly. As an example, the assembly process including intracellular distributions or transport kinetics of specific proteins and protein-protein interactions has been analyzed in live cells rather than in static images. The introduction of this methodology has allowed determination of virus specific protein localization as a function of time post-infection.

Our approach for understanding the mechanisms of HCMV morphogenesis was to address the final envelopment of the virion from the point of view of tegumentation in the cytoplasm. We focused on a tegument protein, pp28. It has been reported that the HSV-1 homolog of this protein, UL11 is myristoylated, traffics in the cytoplasm, and is essential for wild type levels of virus replication *in vitro* (3, 102, 107, 108). In addition, early studies suggested that UL11 was a nuclear protein which has been suggested to play a role in envelopment of HSV-1 virions at the nuclear membrane (3). Furthermore, more recent studies of the PRV UL11 have demonstrated that like HSV UL11, it is also

required for virus replication and that viruses with deletions in UL11 are defective in secondary cytoplasmic envelopment of tegumented capsids (96). Our hypothesis is that pp28 plays a similar role in the final tegumentation and envelopment of the virion in the cytoplasm during HCMV infection. By using a combination of genetic, imaging and biochemical techniques, we attempted to define not only the key role of pp28 in the viral assembly process but also its specific domains for intracellular trafficking and protein-protein interactions, and for production of infectious virions. Our findings have allowed us to propose a general assembly pathway in cytoplasmic phase, which will be dissected in detail in future studies.

The first approach for examination of the role of pp28 in the HCMV assembly was the generation of recombinant HCMV in which the pp28 gene was deleted or mutated at specific sites without altering the expression of other viral genes. In this study, we used a recombination strategy for mutagenesis of BACs containing HCMV genome. As previously described (18), this methodology took advantage of a lambda phage recombination system (*RED locus)* expressed from a temperature-sensitive promoter that enables the use of linear single stranded DNA to target 'genes-of-interest' carried on BACs maintained in *E. coli* (36, 169). Moreover, because all manipulations were carried out in bacteria, the introduction and analysis of mutations in the viral genome were rapidly performed. The recombinant viruses produced in this recombination system, could be used to study mechanisms of HCMV morphogenesis.

Our analysis of the recombinant viruses indicated that HCMV pp28 is essential for the production of infectious virus and that mutation at the myristoylation site on pp28 is associated with the lack of production of infectious virus (18). Previous findings from our laboratory have shown that wild type pp28 is observed in the ERGIC in transfected

cells and in the assembly compartment (AC) in HCMV infected cells (135), but the non-myristoylated mutant distributes in a diffuse pattern within the nucleus and cytoplasm. Our data suggested that authentic intracellular localization of pp28 and not only the expression of this protein are required for virus assembly (18).

Because localization of tegumented capsids, tegument proteins, and envelopment proteins to the site of virion envelopment is a prerequisite for the assembly of the infectious particle, characterization of intracellular trafficking of essential virion structural proteins should offer insights into the assembly of HCMV. We concentrated our studies on characterization of the intracellular trafficking of the essential tegument protein, pp28, in particular, the identification of specific domains which target this protein to sites of virus assembly. Initially, we analyzed the amino acid (aa) composition of the pp28 protein by comparing with that of the HSV UL11, a homologue of pp28. Interestingly, the aa similarity between pp28 and HSV UL11 resided within the amino terminal half of the pp28 protein. Similar to UL11, pp28 is myristoylated at glycine 2, is palmitoylated, has several cysteine residues within the first 13 aa, and contains an aspartate- and glutamate-rich acidic cluster in the region of aa 60. Despite the aa similarity at amino terminus of these proteins, and in contrast to HSV UL11 which traffics between Golgi and plasma membrane (PM), pp28 is only present in the cytoplasm and is localized to the ERGIC in the absence of other viral proteins (135). It suggested that the remainder (aa 61-190) of pp28, which has no aa similarity to that of UL11, includes a specific domain responsible for the intracellular trafficking. Alternatively, the amino terminus of pp28 has a functionally different trafficking signal, yet short domains within this region show aa composition that are similar to that of UL11. It was of interest to note that pp28 has a very long aa acidic cluster, 16 aa (DEGEDDDDGEDDDNEE) as

compared to the 7 aa acidic cluster (DIESEEE) of HSV UL11, suggesting a potentially different role in assembly.

Our results from experiments using the carboxyl terminal deletion mutants of pp28 indicated that only the first 30 aa of pp28 were required for wild type pp28 intracellular trafficking and co-localization with ERGIC proteins. These findings indicated that sequences within the first 30 aa were required for ERGIC localization in absence of other viral proteins. In addition, the non-myristoylated mutant of pp28 trafficked throughout the cytoplasm and even entered the nucleus suggesting that membrane association of pp28 was necessary but not sufficient for its localization in the ERGIC. Interestingly, our studies utilizing both imaging and density gradient fractionation assays indicated that the first 35 aa of pp28 localized with other virion structural proteins to the AC early in infection, but localization to the AC at late times required a minimum of 50 aa. More importantly, the recombinant virus expressing only the first 50 aa was replication competent and this mutant pp28 localized to the AC in virus infected cells. The correlation between pp28 localization to the AC and the assembly of infectious virions strongly argued that virus replication and production of infectious virus was dependent on pp28 localization within the AC. This finding also suggested that the function(s) of the remaining carboxyl terminal 140 aa of this 190 aa protein were non-essential for the replication of this virus in permissive HF cells. These regions of pp28 could be domains that interacted with virion proteins that are not essential for infectivity, or host cell proteins that are destined for incorporation into the virion. Our finding was similar to the results of other studies from Jones et al. that described a virus mutant expressing only the first 57 aa of pp28 that was replication competent, while a recombinant virus expressing the first 43 aa of the pp28 exhibited a

null phenotype (88). Their results suggested that acidic aa between positions 44-57 were responsible for localization of pp28 to sites of assembly, perhaps through interactions with cellular adaptor proteins such as phosphofurin acidic cluster sorting protein-1 (PACS-1) (88). PACS-1 acts as a connector protein, linking acidic cluster motifs to the adaptor complex AP-1, and this function of PACS-1 is required for the retrieval of integral membrane proteins, such as furin, from endosomal compartments to the TGN (40). In addition, several studies have reported that an acidic cluster of aa in the cytoplasmic tail of gB is required for efficient internalization from the cell surface, perhaps through interactions with PACS-1 (54, 163). We confirmed the requirement of this acidic aa cluster for wild type trafficking of pp28 and recovery of infectious virus by creating an additional two mutants in which the acidic domain (aa 44-59) was deleted or was excised and transplanted to the carboxyl terminus of the molecule. Importantly, the result indicated that the role of the acidic cluster in pp28 in localization to the AC and virus assembly was context dependent. Even though two independent studies have shown that the amino terminal acidic cluster of pp28 plays a critical role in the localization of pp28 to the AC late in infection, the mechanism through which this sequence of aa functions to direct this molecule to the AC remains to be determined.

A recent report described that inhibition of PACS-1 expression resulted in only a 2-3 fold decrease in HCMV production (39) whereas mutations in the pp28 that alter its intracellular trafficking resulted in the loss of infectious virus production. Thus, it could be argued that if the amino terminal cluster of aa (aa 44-59) of pp28 interacts with PACS-1, then this interaction likely has only a limited role in localization of pp28 to the AC. Alternatively, the acidic aa cluster could interact with cellular adaptor proteins other than PACS-1; however, this motif of pp28 lacks other requisite signals such as adjacent

dileucines that function as signals recognized by cellular adaptor proteins responsible for trafficking between compartments of the secretory pathway (10, 123).

We are presently pursuing experiments to distinguish between the possibilities of trafficking and post-localization functions of this acidic domain of pp28 (aa 44-59). We generated chimeric molecules in which well described sorting signals such as an acidic dileucine sequences (EEESEERDDHLL), an AP-2 endocytosis signal (NPVY), an acidic cluster of PACS-1 binding motif on furin (SDSEEDE), and a tyrosine motif (YQAL) were grafted onto aa 1-43 of pp28, a mutant which does not localize to the AC, and the chimeric protein fused to EGFP. Our preliminary studies using these constructs indicated that chimeric pp28 molecules containing sequences from an acidic dileucine sequences and an AP-2 endocytosis signal trafficked similarly as wild type pp28 in a transient expression/infection assay. In contrast, the chimeric pp28 expressing an acidic cluster of PACS-1 binding motif and a tyrosine motif caused significant redistribution of wild type pp28 into widely distributed vesicular structures. The nature of these vesicular structures is unclear, but it is possible that this chimeric protein altered the formation of the AC or retargeted pp28 to another compartment of the infected cell. These preliminary findings demonstrated that we could target the mutant expressing the first 43 aa of pp28 to the AC using heterologous trafficking sequences and suggested that we can use these constructs to determine if the acidic aa cluster in wild type pp28 serves only as a trafficking signal or alternatively, plays a role in virion envelopment within the AC. These constructs will be introduced into UL99 of the viral genome by recombination and infectious virus will be recovered from these recombinant BACs as described previously. In the case of chimeric pp28 mutants that fail to localize in the AC, failure to recover infectious viruses from recombinant BACs encoding these chimeras will be consistent with our previous

findings indicating localization of pp28 to the AC is a prerequisite for virus replication. Alternatively, if pp28 is redirected to other cellular compartments such as the PM or TGN and infectious virus is produced, this result would argue for a dominant role of the first 43 aa of pp28 in organizing the process of virion assembly, regardless of the cellular compartment, and confirm that aa 44-59 act only as a context dependent sorting signal. Conversely, if none of the chimeric pp28 mutants that localize to the AC also supports virus replication when recombined into the viral genome, this result would suggest that specific aa sequences between aa 44-59 have a role other than simply acting as a trafficking signal in pp28 dependent envelopment. Our preliminary results from studies of the chimeric pp28 molecules containing sequences from an acidic dileucine sequences and an AP-2 endocytosis signal suggested that at least one defined intracellular trafficking signal will replace the function of aa 44-59 of pp28 in localization to the AC. Thus, we believe that we will be able to definitively assign a function to this domain of pp28 and begin to investigate the role of sequences within the first 43 aa of this tegument protein in the process of envelopment.

Our studies to further define the trafficking of pp28 and pp28 mutants to the AC unexpectedly revealed that the secretory pathway was morphologically remodeled during virus infection resulting in the loss of ribbon-like stacks of the Golgi and TGN and formation of a juxtanuclear, more spherically shaped organelle. These observations suggested that the formation of the AC was associated with remodeling of the secretory compartment with the eventual displacement of host cell proteins of the secretory compartment to the periphery of this structure. Previous studies from our laboratory reported that the AC was localized in a cellular compartment that failed to co-localize with markers of the Golgi, TGN, lysosome or aggresome (134). In current our studies, we

have demonstrated that CD63, a luminal late endosome marker, co-localizes with HCMV structural proteins within the AC and that Rab7, a Rab found in late endosome following maturation from early endosomes, is also found in the AC. Thus, it suggested to us that the previously defined AC was derived from late endosomes localized to a juxtanuclear position in infected cells. In addition, studies of the dynamics in the morphological changes of the secretory compartment indicated that only the pp28 mutants that localized to the compact AC that formed late in infection supported virus replication. Our experiments using density gradient fractionation and biochemical identification of specific cell markers confirmed the nature of the AC containing the pp28 protein and the different intracellular distributions of wild type pp28 and mutant forms of pp28 late in infection.

Based on our findings, we could propose the mechanism(s) that lead to pp28 trafficking from the ERGIC to the AC in virus infected cells. HCMV infection and late gene expression could remodel the cellular secretory compartments resulting in the approximation of the proximal compartments such as the ERGIC with more distal compartments including the TGN. Thus, viral protein trafficking through the secretory pathway could be less compartmentalized and could occur following mixing of closely approximated viral protein containing vesicles. Alternatively, the dependence of pp28 localization to the AC on expression of late gene products raised the possibility that interactions between pp28 and other viral proteins result in the redistribution of pp28 from the ERIGIC to more distal sites in the secretory pathway. The latter mechanism has been observed in assembly pathways of other viruses, including HIV and Mason-Pfizer monkey virus. Interactions between viral matrix proteins and envelope proteins of HIV have been suggested from imaging studies (79). Perhaps more relevant to our studies of

HCMV have been observations that interactions between envelope proteins and the gag protein of Mason-Pfizer virus (MPV) are required for virus assembly at the PM. In the absence of envelope glycoprotein expression, the MPV gag protein remains in a pericentriolar endosomal compartment (141, 142). However, interactions between the MPV gag protein and the envelope glycoprotein within a pericentriolar endosomal compartment results in transport of the immature capsids to the PM for final assembly and budding (141, 142). Inhibition of retrieval of endocytosed vesicles containing envelope glycoproteins by inhibitors of the endocytic pathway, including expression of dominant negative Rab 11, lead to decreased virus production and retention of the gag protein in the pericentriolar compartment (141, 142). These examples of viral protein interactions required for virus envelopment and assembly are consistent with the hypothesis that localization of essential HCMV tegument proteins (pp28) to the AC could be dependent on interactions with viral glycoproteins. In transient expression assays, pp28 can be relocalized from the ERGIC to the Golgi following coexpression with HCMV glycoproteins (data not shown) suggesting that pp28 could traffic with viral glycoproteins in virus infected cells. This protein interaction could provide coordinated transport of this essential tegument protein with virion glycoproteins to the AC.

Our findings also suggested to us an important question in the viral assembly that the sequence requirement for the authentic intracellular trafficking of pp28 is sufficient for normal level of function of the protein essential for assembly of infectious virus in the AC. It was of interest that the pp28STOP50 recombinant virus that expressed only the first 50 aa of pp28 replicated less efficiently than the wild type virus, even though it could be localized to the AC in infected cells and was incorporated into the virion. This finding suggested that the replication defect in pp28STOP50 virus was unrelated to

authentic intracellular trafficking of pp28 but perhaps secondary to a loss or decrease in another function of pp28 such as interactions with virus-encoded or host cell proteins that contribute to the efficiency of virus assembly. Alternatively, the defect of pp28STOP50 virus could be secondary to a defect in the kinetics of localization of the mutant pp28 protein to the AC such that late in infection, a smaller fraction of pp28Mut50 localized to the AC and was available for assembly of infectious virions. To address these possibilities, we concentrated on characterization of the pp28STOP50 virus.

Although a recent report described that cells infected with recombinant viruses in which pp28 was deleted failed to produce enveloped particles (145), it was unclear if the deletion of pp28 resulted in a loss of tegument and/or envelope assembly. Even the protein composition of particles produced by this recombinant virus could not be analyzed, because progeny virions were not produced. In contrast to the pp28 deletion mutant virus, the pp28STOP50 virus was useful to investigate the role of pp28 in viral assembly. Although it exhibited a replication impaired phenotype, it produced sufficient amounts of both intracellular and extracellular virus for characterization of its assembly including the protein composition of the particle. Our analysis for the pp28STOP50 virus provided strong evidence that pp28 has a role in envelopment of the particle. Our investigations revealed that the defect in virus production associated with the pp28STOP50 virus was not directly related to virus entry, spread between cells or a defect in genome replication. Electron microscopic studies indicated that a decreased number of intracellular particles in pp28STOP50 virus infected cells were enveloped. Analysis of protein composition of viral particles exhibited that intracellular particles produced by the pp28STOP50 mutant contained similar amounts of the major capsid protein and viral DNA as particles from the replication competent pp28STOP61 virus. In

addition, the infectivity assay of pp28STOP50 intracellular particles suggested that the defect in the pp28STOP50 virus was not secondary to a block in release of infectious virus. These observations indicated to us that the replication impaired phenotype of the pp28STOP50 virus was not associated with defects in the production of DNA containing particles, capsids, or tegumentation of cytoplasmic particles, but rather a defect in a late step of assembly of the infectious particle, such as envelopment.

Interestingly, the defect in replication of the pp28STOP50 virus did not result in a complete block in the production of infectious virus suggesting that a fraction of particles contained adequate amounts of the mutant pp28 protein to support envelopment and release of infectious particles into the extracellular media (Fig. 1). We could suggest a mechanism in which the minority of intracellular particles produced in cells infected with the pp28STOP50 virus become enveloped and appeared to acquire similar amounts of viral glycoprotein, although the majority of particles produced in cells infected with this virus fail to become enveloped and are not released from the infected cell (Fig. 1).

In addition, our imaging studies using fluorescence recovery after photobleaching (FRAP) indicated that the mutant pp28 protein encoded by this pp28STOP50 virus failed to efficiently accumulate in the AC. The decreased amount of the mutant pp28 protein in the AC could limit envelopment and assembly of infectious virus, particularly if the pp28 concentration in the AC was rate limiting in assembly of infectious particles (Fig. 1). Thus, it appeared that the defect in assembly of the pp28STOP50 mutant virus was related to decreased efficiency of envelopment presumably secondary to a loss in function of the mutant pp28Mut50 protein. Together with previous findings from our laboratory and others (88, 145), these studies suggested that pp28 must accumulate in the AC for efficient envelopment of the particle and provided evidence for a direct role of

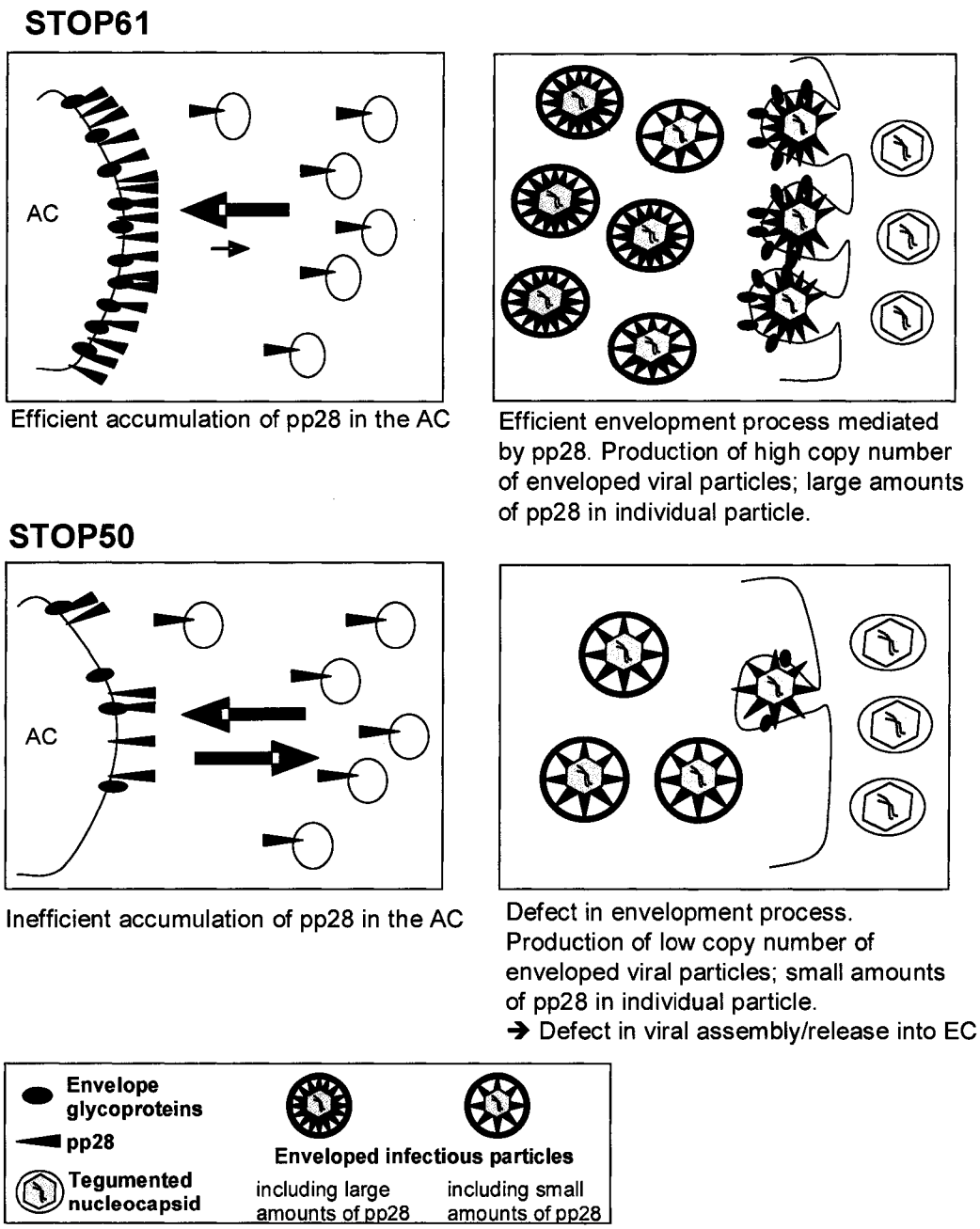

FIG. 1. Summary diagram of the cytoplasmic assembly/release of the pp28STOP61 and pp28STOP50 viruses.

this tegument protein in envelopment and cytoplasmic assembly of infectious virus.

However, these findings did not address the possibility that aa 1-43 of pp28 are also required for essential functions of pp28 such as self-interactions leading to multimerization and/or interactions with other viral proteins or cellular proteins in the AC. These self-interactions or interactions with other viral proteins could be required for particle envelopment and production of infectious virions, but independent of pp28 localization to the AC. Additional studies provided information about the role of multimerization in the intracellular localization and trafficking of pp28, virion envelopment, and production of infectious virus. Our observations indicated that pp28 could multimerize through self-interaction both in transiently expressed cells and in virus infected cells. In addition, we defined the sequence requirements for this essential function of pp28. Pull down assays from *E.coli* system suggested that the the first 43 aa containing a critical interacting sequence (aa 34-43) of pp28 was required for the self-interaction. However, the first 61 aa of pp28 was required for wild type interactions. The results were confirmed using fluorescence resonance energy transfer (FRET) assays of pp28 transiently expressed in Cos-7 cells. The FRET assays suggested that the first 30-35 aa of pp28 was sufficient for interaction with the full length of pp28. Somewhat differences in sequence requirements for the self-interaction suggested that pp28 expressed in mammalian cell underwent post translational modification such as phosphorylation, palmitoylation, and ubiquitination, which does not occur in E.coli, and thus the modification at the specific residues within the interacting domains possibly affects pp28-pp28 interactions. However, consistently with the results from pull downs, the first 61 aa of pp28 was required for wild type interactions. It was also of interest that the first 25 aa of pp28 could not interact with the full length of pp28, although they are

co-localized at the ERGIC in transfected cells. Moreover, in our previous findings from static immunofluorescence assays, unlike non-myristoylated pp28 mutant, pp28 mutant expressing the first 25 aa trafficked and bound to the membrane of ERGIC in transfected cells. It suggested that the binding or retention signal of pp28 to the ERGIC is distinct from the self-interacting domain of this protein, and thereby pp28 multimerization is not required for pp28-membrane binding. In contrast to these finding in transient expression assays, FRET assays of pp28 in transiently expressed/infected cells revealed that pp28-GFP and pp28-RFP proteins interact with each other in the AC as well as in the intracellular vesicles. Moreover, FRET assays of pp28 in transiently expressed/infected cells revealed that at least the first 50 aa of pp28 is required for the multimerization of pp28 in the AC. In addition, pp28Mut50 could strongly interact with the full length of pp28 in the intracellular vesicles as compared to pp28Mut61 that is competent to wild type interactions. These findings suggested two possibilities. The first is that pp28 may form multimeric intermediates on the intracellular vesicles, and these intermediate complexes could then traffic to the AC. Alternatively, pp28 multimers appearing in both compartments might result from accumulation of pp28 within the AC. Consistent with the latter possibility was the finding that although pp28Mut35 likely multimerized in the ERGIC of the transfected cells, it failed to accumulate to the AC and multimerize in the AC of the virus infected cells. Moreover, this finding was consistent with our previous findings from the sequence requirements for localization of pp28 to the virus assembly site and for assembly of infectious virus suggesting that the accumulation of pp28 is a prerequisite for multimerization of this protein in the AC. Unexpectedly, and in contrast to results from FRET analysis of pp28Mut50 and pp28Mut61, FRET assays of pp28Mut35 in the intracellular vesicles as well as in the AC of transiently

expressed/infected cells exhibited little evidence supportive of pp28-pp28 interactions. The results suggested that in transient expression/infection assays, mutant and wild type pp28 molecules compete with wild type viral pp28 for self-interaction in the intracellular vesicles. However, wild type pp28 molecules interacts with pp28Mut50, pp28Mut61, or viral pp28 molecules with similar affinity, while it interacts with pp28Mut35 much less efficiently because of the lack of a complete self-interacting domain and therefore does not exhibit significant FRET. It was also of interest to note that truncation mutants such as pp28Mut35 likely multimerize at the ERGIC yet fail to localize in the AC. Thus, this mutant molecule cannot accumulate in this AC and support virus replication when expressed in the absence of wild type virus. It suggested that the effect of pp28 multimerization on virus replication such as assembly and release might be distinct from a role in pp28 trafficking. Our previous finding from fluorescence recovery after photobleaching (FRAP) assays with mutants pp28Mut35 and pp28Mut50 supported this interpretation and indicated that at equilibrium, these molecules exited the AC at a faster rate than wild type pp28 and pp28Mut61, perhaps accounting for its defect in replication. Here, we could propose that once pp28 molecule containing self-interacting domains accumulates in the AC, the pp28 protein is sufficient for function of multimerization in viral assembly (Fig. 2). Moreover, our experiments using density gradients confirmed that multimerization of pp28 takes place in the AC. Therefore, our data suggested that multimerization of pp28 in the AC (not in the ERGIC as observed in transient expression assays in the absence of other viral proteins) could represent an essential step in the particle envelopment and production of infectious virions.

In spite of our findings, it was unclear why the mutant pp28Mut50 protein failed to accumulate in the AC. The pp28STOP50 mutant virus expressed only a half of the

acidic cluster present in between aa 44-59 of pp28. The defect in replication of pp28STOP50 virus indicated that the remaining stretch of acidic aa between aa 44-50 in the pp28STOP50 mutant virus was insufficient to support wild type virus assembly. An extension of the proposed function of pp28 during virus assembly would be that once pp28 is localized to the AC by targeting signals located between aa 44-50, other post-localization functions such as interactions with other viral proteins and/or host cell proteins are required for accumulation of the protein in the AC (Fig. 2). An obvious possibility was that the pp28Mut50 protein could not interact efficiently with cellular and/or viral proteins within the AC and thus exited the AC nearly as rapidly as it entered this compartment. Because this mutant was not completely defective in assembly, it was also likely that once a sufficient quantity of the pp28Mut50 mutant protein accumulated in the AC, assembly of an infectious particle could take place. Thus, it could be argued that a threshold amount of pp28 must be present in the AC to permit envelopment and assembly. The requirement for a threshold in pp28Mut50 mutant protein in AC for virion assembly could be secondary to productive interactions with other viral proteins or cellular proteins. It was also unclear whether domains or sequences within these 50 aa are also responsible for other as yet undefined functions of pp28 in virion assembly after its localization to the AC. We are investigating the post localization functions of this pp28 protein during virus assembly.

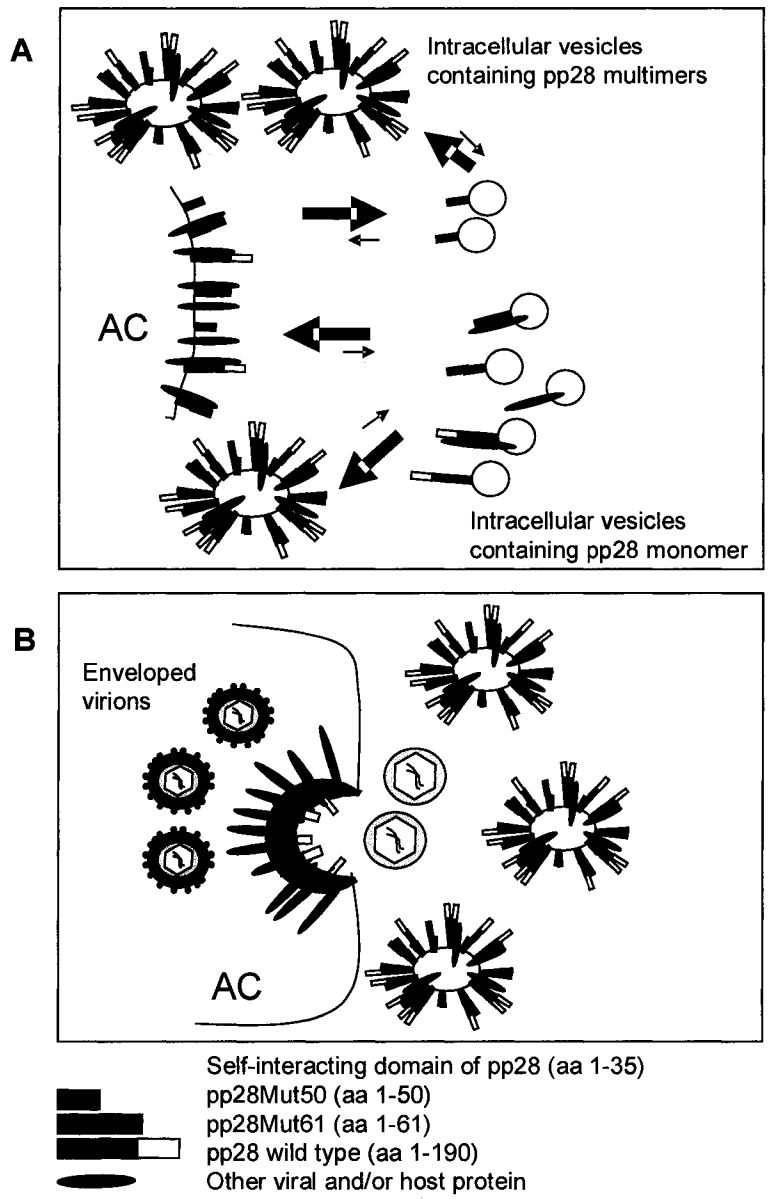

FIG. 2. A model of accumulation and multimerization of pp28 in the AC for the viral replication. (A) Accumulation of pp28 mutants in the AC and in the intracellular vesicles. pp28Mut35 or pp28Mut50 containing a self-interacting domain accumulates and multimerizes in the intracellular vesicles, but not efficiently in the AC. pp28Mut61 or pp28 wild type accumulates and multimerizes in the AC and in the intracellular vesicles. Arrows depict kinetics in/out of the compartment. Thick arrows indicate a predominant direction in the kinetics. (B) Multimerization of pp28 in the AC for the viral assembly. Accumulation of pp28 by possible interactions with other viral and/or host proteins in the AC is a prerequisite for multimerization of pp28 in the AC. Multimerization of pp28 results in budding of subviral particles into the AC and thus production of enveloped virions.

GENERAL LIST OF REFERENCES

1. **Adair, R., E. R. Douglas, J. B. Maclean, S. Y. Graham, J. D. Aitken, F. E. Jamieson, and D. J. Dargan.** 2002. The products of human cytomegalovirus genes UL23, UL24, UL43 and US22 are tegument components. J Gen Virol **83:**1315-24.

2. **Ahn, J. H., W. J. Jang, and G. S. Hayward.** 1999. The human cytomegalovirus IE2 and UL112-113 proteins accumulate in viral DNA replication compartments that initiate from the periphery of promyelocytic leukemia protein-associated nuclear bodies (PODs or ND10). J Virol **73:**10458-71.

3. **Baines, J. D., and B. Roizman.** 1992. The UL11 gene of herpes simplex virus 1 encodes a function that facilitates nucleocapsid envelopment and egress from cells. J Virol **66:**5168-5174.

4. **Baldick, C. J., Jr., A. Marchini, C. E. Patterson, and T. Shenk.** 1997. Human cytomegalovirus tegument protein pp71 (ppUL82) enhances the infectivity of viral DNA and accelerates the infectious cycle. Journal of Virology. **71:**4400-8.

5. **Baldick, C. J., Jr., and T. Shenk.** 1996. Proteins associated with purified human cytomegalovirus particles. Journal of Virology. **70:**6097-105.

6. **Battista, M. C., G. Bergamini, M. C. Boccuni, F. Campanini, A. Ripalti, and M. P. Landini.** 1999. Expression and characterization of a novel structural protein of human cytomegalovirus, pUL25. J Virol **73:**3800-3809.

7. **Bechtel, J. T., and T. Shenk.** 2002. Human cytomegalovirus UL47 tegument protein functions after entry and before immediate-early gene expression. Journal of Virology. **76:**1043-50.

8. **Birkenbach, M., X. Tong, L. E. Bradbury, T. F. Tedder, and E. Kieff.** 1992. Characterization of an Epstein-Barr virus receptor on human epithelial cells. J Exp Med **176:**1405-14.

9. **Bodaghi, B., T. R. Jones, D. Zipeto, C. Vita, L. Sun, L. Laurent, F. Arenzana-Seisdedos, J. L. Virelizier, and S. Michelson.** 1998. Chemokine sequestration by viral chemoreceptors as a novel viral escape strategy: withdrawal of chemokines from the environment of cytomegalovirus-infected cells. Journal of Experimental Medicine **188:**855-66.

10. **Bonifacino, J. S., and L. M. Traub.** 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annual Review of Biochemistry **72:**395-447.

11. **Boppana, S. B., R. F. Pass, W. J. Britt, S. Stagno, and C. A. Alford.** 1992. Symptomatic congenital cytomegalovirus infection: neonatal morbidity and mortality. Pediatr Infect Dis J **11:**93-99.

12. **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.

13. **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:**5364-72.

14. **Bresnahan, W. A., I. Boldogh, E. A. Thompson, and T. Albrecht.** 1996. Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1. Virology **224:**150-60.

15. **Bresnahan, W. A., and T. E. Shenk.** 2000. UL82 virion protein activates expression of immediate early viral genes in human cytomegalovirus-infected cells. Proc Natl Acad Sci U S A **97:**14506-11.

16. **Brignati, M. J., J. S. Loomis, J. W. Wills, and R. J. Courtney.** 2003. Membrane association of VP22, a herpes simplex virus type 1 tegument protein. Journal of Virology **77:**4888-98.

17. **Britt, W.** 2005. Virus entry,establishment of infection, spread in host, mechanisms of tissue damage. *In* A. Arvin, Campadielli-Fume, G., Mocarski, E., Roizman, B., Whitley, R., Yamanishi, K (ed.), Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge University Press, London.

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

19. **Britt, W. J.** 2000. Infectious clones of herpesviruses: a new approach for understanding viral gene function. Trends in Microbiology **8:**262-5.

20. **Britt, W. J., and C. A. Alford.** 1996. Cytomegalovirus, p. 2493-2523. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields Virology, Third Edition. Raven Press, New York.

21. **Britt, W. J., and M. Mach.** 1996. Human cytomegalovirus glycoproteins.

Intervirology **39:**401-12.

22. **Browne, H., S. Bell, T. Minson, and D. W. Wilson.** 1996. An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. J Virol **70:**4311-4316.

23. **Brune, W., C. Menard, U. Hobom, S. Odenbreit, M. Messerle, and U. H. Koszinowski.** 1999. Rapid identification of essential and nonessential herpesvirus genes by direct transposon mutagenesis. Nature Biotech **17:**60-64.

24. **Cai, X., and B. R. Cullen.** 2006. Transcriptional origin of Kaposi's sarcoma-associated herpesvirus microRNAs. J Virol **80:**2234-42.

25. **Cai, X., A. Schafer, S. Lu, J. P. Bilello, R. C. Desrosiers, R. Edwards, N. Raab-Traub, and B. R. Cullen.** 2006. Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. PLoS Pathog **2:**e23.

26. **Cantrell, S. R., and W. A. Bresnahan.** 2006. Human cytomegalovirus (HCMV) UL82 gene product (pp71) relieves hDaxx-mediated repression of HCMV replication. J Virol **80:**6188-91.

27. **Cantrell, S. R., and W. A. Bresnahan.** 2005. Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication. J Virol **79:**7792-802.

28. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchison, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr Top Microbiol Immunol **154:**125-170.

29. **Chee, M. S., S. C. Satchwell, E. Preddie, K. M. Weston, and B. G. Barrell.** 1990. Human cytomegalovirus encodes three G protein-coupled receptor homologues.[comment]. Nature. **344:**774-7.

30. **Chen, D. H., H. Jiang, M. Lee, F. Liu, and Z. H. Zhou.** 1999. Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus. Virology. **260:**10-6.

31. **Chen, D. H., H. Jiang, M. Lee, F. Liu, and Z. H. Zhou.** 1999. Three-dimensional visualization of tetument/capsid interactions in the intact human cytomegalovirus. Virology **260:**10-16.

32. **Chong, L. D., and J. K. Rose.** 1993. Membrane association of functional vesicular stomatitis virus matric protein in vivo. J Virol **67:**407-414.

33. **Chrisp, P., and S. P. Clissold.** 1991. Foscarnet. A review of its antiviral activity,

pharmacokinetic properties and therapeutic use in immunocompromised patients with cytomegalovirus retinitis. Drugs **41:**104-129.

34. **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.

35. **Conte, M. R., M. Klikova, E. Hunter, T. Ruml, and S. Matthews.** 1997. The three-dimensional solution structure of the matrix protein from the type D retrovirus, the Mason-Pfizer monkey virus, and implications for the morphology of retroviral assembly. EMJO J **16:**5819-5826.

36. **Copeland, N. G., N. A. Jenkins, and D. L. Court.** 2001. Recombineering: a powerful new tool for mouse functional genomics. Nature Reviews Genetics. **2:**769-79.

37. **Cosson, P.** 1996. Direct interaction between the envelope and matrix proteins of HIV-1. EMBO J **15:**5783-5788.

38. **Courcelle, C. T., J. Courcelle, M. N. Prichard, and E. S. Mocarski.** 2001. Requirement for uracil-DNA glycosylase during the transition to late-phase cytomegalovirus DNA replication. J Virol **75:**7592-601.

39. **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. Journal of Virology. **77:**11105-13.

40. **Crump, C. M., Y. Xiang, L. Thomas, F. Gu, C. Austin, S. A. Tooze, and G. Thomas.** 2001. PACS-1 binding to adaptors is required for acidic cluster motif-mediated protein traffic. EMBO Journal. **20:**2191-201.

41. **Crumpacker, C.** 1996. Ganciclovir. N Engl J Med **335:**721-729.

42. **Cullen, B. R.** 2006. Viruses and microRNAs. Nat Genet **38 Suppl:**S25-30.

43. **Dahle, A. F., KB; Wright, JD; Boppana, SB; Britt, WJ; Pass, RF.** 2000. Longitudinal Investigation of Hearing Disorders in Children with Congenital Cytomegalovirus. J. Am Acad Audiol **11:**283-290.

44. **del Rio, T., H. C. Werner, and L. W. Enquist.** 2002. The pseudorabies virus VP22 homologue (UL49) is dispensable for virus growth in vitro and has no effect on virulence and neuronal spread in rodents. Journal of Virology. **76:**774-82.

45. **Demirov, D. G., and E. O. Freed.** 2004. Retrovirus budding. Virus Research **106:**87-102.

46. **Dijkstra, J. M., N. Visser, T. C. Mettenleiter, and B. G. Klupp.** 1996. Identification and characterization of pseudorabies virus glycoprotein gM as a nonessential virion component. J Virol **70:**5684-5688.

47. **Dittmer, D., and E. S. Mocarski.** 1997. Human cytomegalovirus infection inhibits G1/S transition. J Virol **71:**1629-34.

48. **Drew, W. L., L. Mintz, R. C. Miner, M. Sands, and B. Ketterer.** 1981. Prevalence of cytomegalovirus infection in homosexual men. J Infect Dis **143:**188-192.

49. **Drobyski, W. R., K. K. Knox, D. R. Carrigan, and R. C. Ash.** 1991. Foscarnet therapy of ganciclovir-resistant cytomegalovirus in marrow transplantation. Transplantation **52:**155-157.

50. **Dubovi, E., and R. R. Wagner.** 1977. Spatial relationships of the proteins of vesicular stomatitis virus: induction of reversible oligomes by cleavable protein cross-linking and oxidation. J Virol **22:**500-509.

51. **Elliott, G., G. Mouzakitis, and P. O'Hare.** 1995. VP16 interacts via its activation domain with VP22, a tegument protein of herpes simplex virus, and is relocated to a novel macromolecular assembly in coexpressing cells. Journal of Virology **69:**7932-41.

52. **Esposito, S., A. Renzulli, L. Agozzino, K. Thomopoulos, M. Piccolo, C. Maiello, A. Della Corte, and M. Cotrufo.** 1999. Late complications of heart transplantation: an 11-year experience. Heart & Vessels **14:**272-6.

53. **Faulds, D., and R. C. Heel.** 1990. Ganciclovir. A review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy in cytomegalovirus infections. Drugs **39:**597-638.

54. **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.

55. **Fortunato, E. A., A. K. McElroy, I. Sanchez, and D. H. Spector.** 2000. Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. Trends in Microbiology **8:**111-9.

56. **Fortunato, E. A., and D. H. Spector.** 1999. Regulation of human cytomegalovirus gene expression. Advances in Virus Research **54:**61-128.

57. **Fowler, K. B., F. P. McCollister, A. J. Dahle, S. Boppana, W. J. Britt, and R. F. Pass.** 1997. Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection. J Pediatr **130:**624-630.

58. **Fraile-Ramos, A., A. Pelchen-Matthews, T. N. Kledal, H. Browne, T. W. Schwartz, and M. Marsh.** 2002. Localization of HCMV UL33 and US27 in endocytic compartments and viral membranes. Traffic **3:**218-32.

59. **Freeman, W. R., D. E. Henderly, W. L. Wan, and e. al.** 1987. Prevalence, pathophysiology, and treatment of rhegmatogenous retinal detachment in treated cytomegalovirus retinitis. Am J Ophthalmol **15:**527-536.

60. **Fujiyoshi, Y., N. P. Kume, K. Sakata, and S. B. Sato.** 1994. Fine structure of influenza A virus observed by electron cryo-microscopy. EMBO J **13:**318-326.

61. **Furukawa, H., T. Kuroiwa, and S. Mizushima.** 1983. DNA injection during bacteriophage T4 infection of Escherichia coli. J Bacteriol **154:**938-45.

62. **Gao, J. L., and P. M. Murphy.** 1994. Human cytomegalovirus open reading frame US28 encodes a functional beta chemokine receptor. J Biol Chem **269:**28539-42.

63. **Garoff, H., R. Hewson, and D. J. Opstelten.** 1998. Virus maturation by budding. Microbiology & Molecular Biology Reviews **62:**1171-90.

64. **Garoff, H., R. Hewson, and D. J. E. Opstelten.** 1998. Virus maturation by budding. Microbiol Mol Biol Rev **62:**1171-1190.

65. **Gaudin, Y., J. Sturgis, M. Doumith, A. Barge, B. Robert, and R. W. H. Ruigrok.** 1997. Conformational flexibility and polymerization of vesicular stomatitis virus matrix protein. J Mol Biol **274:**816-825.

66. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde.** 1989. Assembly and release of HIV-1 precursor $Pr55^{gag}$ virus-like particles from recombinant baculovirus-infected insect cells. Cell **59:**103-112.

67. **Gibson, W.** 1996. Structure and assembly of the virion. Intervirology **39:**389-400.

68. **Gilbert, M. J., S. R. Riddell, B. Plachter, and P. D. Greenberg.** 1996. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. Nature **383:**720-2.

69. **Goldmacher, V. S.** 2002. vMIA, a viral inhibitor of apoptosis targeting mitochondria. Biochimie **84:**177-85.

70. **Granzow, H., B. G. Klupp, W. Fuchs, J. Veits, N. Osterrieder, and T. C. Mettenleiter.** 2001. Egress of alphaherpesviruses: comparative ultrastructural study. Journal of Virology **75:**3675-84.

71. **Granzow, H., F. Weiland, A. Jons, B. G. Klupp, A. Karger, and T. C. Mettenleitner.** 1997. Ultrastructural analysis of the replication cycle of

pseudorabies virus in cell culture: a reassessment. J Virol **71:**2072-2082.

72. **Grattan, M. T., C. E. Moreno-Cabral, V. A. Starnes, P. E. Oyer, E. B. Stinson, and N. E. Shumway.** 1989. Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. JAMA **261:**3561-3566.

73. **Gretch, D. R., B. Kari, L. Rasmussen, R. C. Gehrz, and M. F. Stinski.** 1988. Identification and characterization of three distinct families of glycoprotein complexes present in the envelopes of human cytomegalovirus. J Virol **62:**875-881.

74. **Gribaudo, G., L. Riera, T. L. Rudge, P. Caposio, L. F. Johnson, and S. Landolfo.** 2002. Human cytomegalovirus infection induces cellular thymidylate synthase gene expression in quiescent fibroblasts. J Gen Virol **83:**2983-93.

75. **Grose, C.** 1990. Glycoproteins encoded by varicella-zoster virus: biosynthesis, phosphorylation, and intracellular trafficking. Ann Rev Microbiol **44:**59-80.

76. **Hayashi, M. L., C. Blankenship, and T. Shenk.** 2000. Human cytomegalovirus UL69 protein is required for efficient accumulation of infected cells in the G1 phase of the cell cycle. Proceedings of the National Academy of Sciences of the United States of America. **97:**2692-6.

77. **Heggeness, M. H., P. R. Smith, and P. W. Choppin.** 1982. In vitro assembly of the nonglycosylated membrane protein (M) of Sendai virus. Proc Natl Acad Sci USA **79:**6232-6236.

78. **Henderly, D. E., and L. M. Jampol.** 1991. Diagnosis and treatment of cytomegalovirus retinitis. J AIDS **4:**S6-S10.

79. **Hermida-Matsumoto, L., and M. D. Resh.** 2000. Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. Journal of Virology **74:**8670-9.

80. **Hill, C. P., D. Worthylake, D. P. Bancroft, A. M. Christensen, and W. I. Sundquist.** 1996. Crystal structures of the trimeric human immunodeficiency virus type 1 matrix protein: implications for membrane association and assembly. Proc Natl Acad Sci USA **93:**3099-3104.

81. **Homman-Loudiyi, M., K. Hultenby, W. Britt, and C. Soderberg-Naucler.** 2003. Envelopment of human cytomegalovirus occurs by budding into Golgi-derived vacuole compartments positive for gB, Rab 3, trans-golgi network 46, and mannosidase II.[erratum appears in J Virol. Arch. 2003 Jul;77(14):8179]. Journal of Virology. **77:**3191-203.

82. **Hosenpud, J. D.** 1999. Coronary artery disease after heart transplantation and its relation to cytomegalovirus. American Heart Journal **138:**S469-72.

83. **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.

84. **Jabs, D. A., C. Enger, and J. G. Bartlett.** 1989. Cytomegalovirus retinitis and acquired immunodeficiency syndrome. Arch Ophthalmol **107:**75-80.

85. **Jarvis, M. A., K. N. Fish, C. Soderberg-Naucler, D. N. Streblow, H. L. Meyers, G. Thomas, and J. A. Nelson.** 2002. Retrieval of human cytomegalovirus glycoprotein B from cell surface is not required for virus envelopment in astrocytoma cells. Journal of Virology. **76:**5147-55.

86. **Jault, F. M., J. M. Jault, F. Ruchti, E. A. Fortunato, C. Clark, J. Corbeil, D. D. Richman, and D. H. Spector.** 1995. Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest. J Virol **69:**6697-6704.

87. **Jones, F., and C. Grose.** 1988. Role of cytoplasmic vacuoles in varicella-zoster virus glycoprotein trafficking and virion envelopment. J Virol **62:**2701-2711.

88. **Jones, T. R., and S. W. Lee.** 2004. An acidic cluster of human cytomegalovirus UL99 tegument protein is required for trafficking and function. Journal of Virology **78:**1488-502.

89. **Jones, T. R., E. J. Wiertz, L. Sun, K. N. Fish, J. A. Nelson, and H. L. Ploegh.** 1996. Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. Proceedings of the National Academy of Sciences of the United States of America **93:**11327-33.

90. **Kalejta, R. F., and T. Shenk.** 2002. Manipulation of the cell cycle by human cytomegalovirus. Frontiers in Bioscience. **7:**d295-306.

91. **Kari, B., W. Li, J. Cooper, R. Goertz, and B. Radeke.** 1994. The human cytomegalovirus UL100 gene encodes the gC-II glycoproteins recognized by group 2 monoclonal antibodies. J Gen Virol **75:**3081-3086.

92. **Karlin, S., E. S. Mocarski, and G. A. Schachtel.** 1994. Molecular evolution of herpesviruses: genomic and protein sequence comparisons. J Virol **68:**1886-902.

93. **Kolesnikova, L., H. Bugany, H. D. Klenk, and S. Becker.** 1825. VP40, the matrix protein of Marburg virus, is associated with membranes of the late endosomal compartment. Journal of Virology **76:**1825-38.

94. **Komanduri, K. V., M. N. Viswanathan, E. D. Wieder, D. K. Schmidt, B. M. Bredt, M. Jacobson, and J. M. McCune.** 1998. Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. Nature Med **4:**953-956.

95. **Kopp, M., H. Granzow, W. Fuchs, B. Klupp, and T. C. Mettenleiter.** 2004. Simultaneous deletion of pseudorabies virus tegument protein UL11 and glycoprotein M severely impairs secondary envelopment. Journal of Virology **78:**3024-34.

96. **Kopp, M., H. Granzow, W. Fuchs, B. G. Klupp, E. Mundt, A. Karger, and T. C. Mettenleiter.** 2003. The pseudorabies virus UL11 protein is a virion component involved in secondary envelopment in the cytoplasm. Journal of Virology. **77:**5339-51.

97. **Landini, M. P., B. Severi, G. Furlini, and D. G. L. Badiali.** 1987. Human cytomegalovirus structural components: intracellular and intraviral localization of p28 and p65-69 by immunoelectron microscopy. Virus Res **8:**15-23.

98. **Landolfo, S., M. Gariglio, G. Gribaudo, and D. Lembo.** 2003. The human cytomegalovirus. Pharmacol Ther **98:**269-97.

99. **Lee, J. Y., A. Irmiere, and W. Gibson.** 1988. Primate cytomegalovirus assembly: evidence that DNA packaging occurs subsequent to B capsid assembly. Virology. **167:**87-96.

100. **Liu, B., and M. F. Stinski.** 1992. Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements. J Virol **66:**4434-4444.

101. **Ljungman, P.** 2001. Prophylaxis against herpesvirus infections in transplant recipients. Drugs. **61:**187-96.

102. **Loomis, J. S., J. B. Bowzard, R. J. Courtney, and J. W. Wills.** 2001. Intracellular trafficking of the UL11 tegument protein of herpes simplex virus type 1. Journal of Virology **75:**12209-19.

103. **Loomis, J. S., R. J. Courtney, and J. W. Wills.** 2003. Binding partners for the UL11 tegument protein of herpes simplex virus type 1. Journal of Virology **77:**11417-24.

104. **Lu, M., and T. Shenk.** 1996. Human cytomegalovirus infection inhibits cell cycle progression at multiple points, including the transition from G1 to S. J Virol **70:**8850-7.

105. **Lu, M., and T. Shenk.** 1999. Human cytomegalovirus UL69 protein induces cells to accumulate in G1 phase of the cell cycle. J Virol **73:**676-683.

106. **Lundstrom, K. H., D. H. Bamford, E. T. Palva, and K. Lounatmaa.** 1979. Lipid-containing bacteriophage PR4: structure and life cycle. J Gen Virol **43:**583-92.

107. **MacLean, C. A., B. Clark, and D. J. McGeoch.** 1989. Gene UL11 of herpes simplex virus type 1 encodes a virion protein which is myristylated. J Gen Virol **70:**3147-3157.

108. **MacLean, C. A., A. Dolan, F. E. Jamieson, and D. J. McGeoch.** 1992. The myristylated virion proteins of herpes simplex virus type 1: investigation of their role in the virus life cycle. J Gen Virol **73:**539-547.

109. **Markwell, M. A. K., and C. F. Fox.** 1980. Protein-protein interactions within paramyxoviruses identified by native disulfide bonding or reversible chemical cross-linking. J Virol **33:**152-166.

110. **Martin, D. R., A. Yuryev, K. R. Kalli, D. T. Fearon, and J. M. Ahearn.** 1991. Determination of the structural basis for selective binding of Epstein-Barr virus to human complement receptor type 2. J Exp Med **174:**1299-311.

111. **McDonald, K., T. S. Rector, E. A. Braulin, S. H. Kubo, and M. T. Olivari.** 1989. Association of coronary artery disease in cardiac transplant recipients with cytomegalovirus infection. Am J Cardiol **64:**359-362.

112. **McLauchlan, J., and F. J. Rixon.** 1992. Characterization of enveloped tegument structures (L particles) produced by alphaherpesviruses: integrity of the tegument does not depend on the presence of capsid or envelope. J Gen Virol **73:**269-276.

113. **McVoy, M. A., and S. P. Adler.** 1994. Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. J Virol **68:**1040-51.

114. **Messerle, M., I. Crnkovic, W. Hammerschmidt, H. Ziegler, and U. Koszinowski.** 1997. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci USA **94:**14759-14763.

115. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. Journal of Virology. **76:**1537-47.

116. **Mettenleiter, T. C., B. G. Klupp, and H. Granzow.** 2006. Herpesvirus assembly: a tale of two membranes. Curr Opin Microbiol **9:**423-9.

117. **Mocarski, E. S.** 1996. Cytomegaloviruses and their replication, p. 2447-2492. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields Virology, Third Edition. Lippincott-Raven, Philadelphia.

118. **Mocarski, E. S., G. W. Kemble, J. M. Lyle, and R. F. Greaves.** 1996. A deletion mutant in the human cytomegalovirus gene encoding IE1 (491aa) is replication defective due to a failure in autoregulation. Proc Natl Acad Sci USA

93:11321-11326.

119. **Mocarski, E. S., Tan Courcelle, C.** 2001. Cytomegaloviruses and their replication, p. 2629-2673. *In* D. M. K. a. P. M. Howley (ed.), Fields Virology, 4th ed, vol. 2. Lippincott Williams and Wilkins, Philadelphia.

120. **Muranyi, W., J. Haas, M. Wagner, G. Krohne, and U. H. Koszinowski.** 2002. Cytomegalovirus recruitment of cellular kinases to dissolve the nuclear lamina.[comment]. Science. **297:**854-7.

121. **Nichols, W. G., and M. Boeckh.** 2000. Recent advances in the therapy and prevention of CMV infections. Journal of Clinical Virology. **16:**25-40.

122. **O'Sullivan, C. E., W. L. Drew, D. J. McMullen, R. Miner, J. Y. Lee, R. A. Kaslow, J. G. Lazar, and M. S. Saag.** 1999. Decrease of cytomegalovirus replication in human immunodeficiency virus infected-patients after treatment with highly active antiretroviral therapy.[comment]. Journal of Infectious Diseases. **180:**847-9.

123. **Ohno, H., R. C. Aguilar, D. Yeh, D. Taura, T. Saito, and J. S. Bonifacino.** 1998. The medium subunits of adaptor complexes recognize distinct but overlapping sets of tyrosine-based sorting signals. Journal of Biological Chemistry **273:**25915-21.

124. **Parent, L. J., R. P. Bennett, R. C. Craven, T. D. Nelle, N. K. Krishna, J. B. Bowzard, C. B. Wilson, B. A. Puffer, R. C. Montelaro, and J. W. Wills.** 1995. Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus gag proteins. J Virol **69:**5455-5460.

125. **Penfold, M. E., and E. S. Mocarski.** 1997. Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis. Virology **239:**46-61.

126. **Post, L. E., and B. Roizman.** 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. Cell **25:**227-232.

127. **Rhee, S. S., H. Hui, and E. Hunter.** 1990. Preassembled capsids of type D retroviruses contain a signal sufficient for targeting specifically to the plasma membrane. J Virol **64:**3844-3852.

128. **Rixon, F. J., C. Addison, and J. McLauchlan.** 1992. Assembly of enveloped tegument structures (L particles) can occur independently of virion maturation in herpes simplex virus type 1-infected cells. Journal of General Virology. **73:**277-84.

129. **Roizman, B.** 1996. Herpesviridae, p. 2221-2230. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields Virology, Third Edition. Lippincott-Raven, Philadelphia.

130. **Roizman, B., and A. E. Sears.** 1996. Herpes simplex viruses and their replication, p. 2231-2295. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Virology, Third Edition. Lippincott-Raven, Philadelphia.

131. **Rossmann, M. G., V. V. Mesyanzhinov, F. Arisaka, and P. G. Leiman.** 2004. The bacteriophage T4 DNA injection machine. Curr Opin Struct Biol **14:**171-80.

132. **Saad, J. S., J. Miller, J. Tai, A. Kim, R. H. Ghanam, and M. F. Summers.** 2006. Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly.[see comment]. Proceedings of the National Academy of Sciences of the United States of America **103:**11364-9.

133. **Salvant, B. S., E. A. Fortunato, and D. H. Spector.** 1998. Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription. Journal of Virology. **72:**3729-41.

134. **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.

135. **Sanchez, V., E. Sztul, and W. J. Britt.** 2000. Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. Journal of Virology **74:**3842-51.

136. **Sarisky, R. T., and G. S. Hayward.** 1996. Evidence that the UL84 gene product of human cytomegalovirus is essential for promoting oriLyt-dependent DNA replication and formation of replication compartments in cotransfection assays. J Virol **70:**7398-413.

137. **Schmidt, G. M., A. Kovacs, J. A. Zaia, D. A. Horak, and a. l. et.** 1988. Ganciclovir/immunoglobulin combination therapy for the treatment of human cytomegalovirus-associated interstitial pneumonia in bone marrow allograft recipients. Transplantation **46:**905-907.

138. **Schnell, M. J., L. Buonocore, E. Boritz, H. P. Ghosh, R. Chernish, and J. K. Rose.** 1998. Requirement for a nonspecific glycoprotein cytoplasmic domain sequence to drive efficient buddign of vesicular stomatitis virus. EMBO J **17:**1289-1296.

139. **Scianimanico, S., G. Schoehn, J. Timmins, R. H. Ruigrok, H. D. Klenk, and**

W. Weissenhorn. 2000. Membrane association induces a conformational change in the Ebola virus matrix protein. EMBO Journal **19:**6732-41.

140. **Severi, B., M. P. Landini, and E. Govoni.** 1988. Human cytomegalovirus morphogenesis: an ultrastructural study of the late cytoplasmic phases. Arch Virol **98:**51-64.

141. **Sfakianos, J. N., and E. Hunter.** 2003. M-PMV capsid transport is mediated by Env/Gag interactions at the pericentriolar recycling endosome. Traffic **4:**671-80.

142. **Sfakianos, J. N., R. A. LaCasse, and E. Hunter.** 2003. The M-PMV cytoplasmic targeting-retention signal directs nascent Gag polypeptides to a pericentriolar region of the cell. Traffic **4:**660-70.

143. **Shamu, C. E., C. M. Story, T. A. Rapoport, and H. L. Ploegh.** 1999. The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. J Cell Biol **147:**45-58.

144. **Sherman, G., and S. L. Bachenheimer.** 1988. Characterization of intranuclear capsids made by ts morphogenic mutants of HSV-1. Virology **163:**471-80.

145. **Silva, M. C., Q. C. Yu, L. Enquist, and T. Shenk.** 2003. Human cytomegalovirus UL99-encoded pp28 is required for the cytoplasmic envelopment of tegument-associated capsids. Journal of Virology. **77:**10594-605.

146. **Sinzger, C., M. Kahl, K. Laib, K. Klingel, P. Rieger, B. Plachter, and G. Jahn.** 2000. Tropism of human cytomegalovirus for endothelial cells is determined by a post-entry step dependent on efficient translocation to the nucleus. Journal of General Virology. **81:**3021-35.

147. **Skaletskaya, A., L. M. Bartle, T. Chittenden, A. L. McCormick, E. S. Mocarski, and V. S. Goldmacher.** 2001. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proceedings of the National Academy of Sciences of the United States of America **98:**7829-34.

148. **Skeppner J.N., A. W., H. Browne, A. Minson.** 2001. Herpes Simplex virus nucleocapsids mature to progeny virus by an envelopment-deenvelopment-reenvelopment pathway. Journal of Virology **75:**5697-5702.

149. **Smith, G. A., and L. W. Enquist.** 1999. Construction and transposon mutagenesis in *Escherichia coli* of a full-length infectious clone of pseudorabies virus, an alphaherpesvirus. J Virol **73:**6405-6414.

150. **Smith, J. D., and E. DeHarven.** 1973. Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. I. Sequence of viral replication. J Virol **12:**919-930.

151. **Song, Y. J., and M. F. Stinski.** 2002. Effect of the human cytomegalovirus IE86 protein on expression of E2F-responsive genes: a DNA microarray analysis. Proc Natl Acad Sci U S A **99:**2836-41.

152. **Spear, P. G.** 2004. Herpes simplex virus: receptors and ligands for cell entry. Cell Microbiol **6:**401-10.

153. **Spear, P. G., and R. Longnecker.** 2003. Herpesvirus entry: an update. J Virol **77:**10179-85.

154. **Stagno, S., and W. J. Britt.** 2005. Cytomegalovirus, p. In Press. *In* J. S. Remington and J. O. Klein (ed.), Infectious Diseases of the Fetus and Newborn Infant, 6th Edition. W.B. Saunders, Philadelphia.

155. **Stasiak, P. C., and E. S. Mocarski.** 1992. Transactivation of the cytomegalovirus ICP36 gene promoter requires the alpha gene product TRS1 in addition to IE1 and IE2. J Virol **66:**1050-8.

156. **Steven, A. C., and P. G. Spear.** 1997. Herpes capsid assembly and envelopment, p. 312-351. *In* W. Chiu, R. M. Burnett, and R. L. Garcea (ed.), Structural Biology of Viruses. Oxford University Press, New York.

157. **Story, C. M., M. H. Furman, and H. L. Ploegh.** 1999. The cytosolic tail of class I MHC heavy chain is required for its dislocation by the human cytomegalovirus US2 and US11 gene products. Proc Natl Acad Sci U S A **96:**8516-21.

158. **Sylwester, A. W., B. L. Mitchell, J. B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P. R. Sleath, K. H. Grabstein, N. A. Hosken, F. Kern, J. A. Nelson, and L. J. Picker.** 2005. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med **202:**673-85.

159. **Tang, C., E. Loeliger, P. Luncsford, I. Kinde, D. Beckett, and M. F. Summers.** 2004. Entropic switch regulates myristate exposure in the HIV-1 matrix protein.[see comment]. Proceedings of the National Academy of Sciences of the United States of America **101:**517-22.

160. **Theiler, R. N., and T. Compton.** 2001. Characterization of the signal peptide processing and membrane association of human cytomegalovirus glycoprotein O. Journal of Biological Chemistry. **276:**39226-31.

161. **Tooze, J., M. Hollinshead, B. Reis, K. Radsak, and H. Kern.** 1993. Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. Eur J Cell Biol **601:**163-178.

162. **Torriani, F. J., W. R. Freeman, J. C. Macdonald, M. P. Karavellas, D. M. Durand, D. D. Jeffrey, P. R. Meylan, and R. D. Schrier.** 2000. CMV retinitis

recurs after stopping treatment in virological and immunological failures of potent antiretroviral therapy. Aids **14:**173-80.

163. **Tugizov, S., E. Maidji, J. Xiao, and L. Pereira.** 1999. An acidic cluster in the cytosolic domain of human cytomegalovirus glycoprotein B is a signal for endocytosis from the plasma membrane. J Virol **73:**8677-8688.

164. **Varnum, S. M., D. N. Streblow, M. E. Monroe, P. Smith, K. J. Auberry, L. Pasa-Tolic, D. Wang, D. G. Camp, K. Rodland, S. Wiley, W. Britt, T. Shenk, R. D. Smith, and J. A. Nelson.** 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. Journal of Virology **78:**10960-10966.

165. **Wade, M., T. F. Kowalik, M. Mudryj, E. S. Huang, and J. C. Azizkhan.** 1992. E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. Mol Cell Biol **12:**4364-74.

166. **Whealy, M. E., J. P. Card, R. P. Meade, A. K. Robbins, and L. W. Enquist.** 1991. Effect of Brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J Virol **65:**1066-1081.

167. **Wills, J. W., and R. C. Craven.** 1991. Form, function, and use of retroviral Gag proteins. AIDS **5:**639-654.

168. **Wills, J. W., R. C. Craven, and J. A. Achacoso.** 1989. Creation and expression of myristylated forms of Rous sarcoma virus gag protein in mammalian cells. J Virol **63:**4331-4343.

169. **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. Proceedings of the National Academy of Sciences of the United States of America **97:**5978-83.

170. **Yu, D., G. A. Smith, L. W. Enquist, and T. Shenk.** 2002. Construction of a self-excisable bacterial artificial chromosome containing the human cytomegalovirus genome and mutagenesis of the diploid TRL/IRL13 gene. Journal of Virology **76:**2316-28.

171. **Zhao, H., and H. Garoff.** 1998. The M1 and NP proteins of influenza A virus form homo- but not heterooligomeric complexes when coexpressed in BHK-21 cells. J Gen Virol **79:**2435-2446.

172. **Zhu, H., Y. Shen, and T. Shenk.** 1995. Human cytomegalovirus IE1 and IE2 proteins block apoptosis. Journal of Virology **69:**7960-70.

173. **Zhu, Z., M. D. Gershon, Y. Hao, R. T. Ambron, C. A. Gabel, and A. A.**

Gershon. 1995. Envelopment of varicella-zoster virus: targeting of viral gylcoproteins to the trans-Golgi network. J Virol **69:**7951-7959.