Characterization of the Signal Peptide Processing and Membrane Association of Human Cytomegalovirus Glycoprotein O*

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Human cytomegalovirus (HCMV) has a structurally complex envelope that contains multiple glycoproteins. These glycoproteins are involved in virus entry, virus maturation, and cell-cell spread of infection. Glycoprotein H (gH), glycoprotein L (gL), and glycoprotein O (gO) associate covalently to form a unique disulfide-bonded tripartite complex. Glycoprotein O was recently discovered, and its basic structure, as well as that of the tripartite complex, remains uncharacterized. Based on hydrophobic analysis, we hypothesized that gO could adopt a type II transmembrane orientation. The data presented here, however, reveal that the single hydrophobic domain of gO functions as a cleavable signal peptide that is absent from the mature molecule. Although it lacks a membrane anchor, glycoprotein O is associated with the membranes of HCMV-infected cells. The sophisticated organization of the gHgLgO complex reflects the intricate nature of the multicomponent entry and fusion machinery encoded by HCMV.

The family *Herpesviridae* contains eight medically significant human pathogens. These viruses are large, enveloped double-stranded DNA viruses that establish lifelong latent infections within their hosts. Human cytomegalovirus (HCMV) causes multiple clinical sequelae in immunocompromised hosts. HCMV is able to enter and infect a wide variety of host cell types and can cause disease in most organs of the body. This broad tropism can be attributed in part to a complex set of viral envelope glycoproteins that play a vital role in the viral life cycle by mediating entry of the virus into host cells, cell-to-cell spread of infection, and maturation of virions. Most enveloped viruses, including influenza and human immunodeficiency virus, encode a single fusion glycoprotein that initiates attachment and fusion. In contrast, herpesviruses such as herpes simplex virus require a minimum of four glycoproteins for fusion. The multicomponent nature of herpesvirus fusion machinery provokes many questions about the mechanisms by which large viruses with multiple glycoproteins achieve membrane fusion.

HCMV enters host cells by a sequential cascade of molecular events involving multiple viral and cellular proteins, culminating with fusion of the envelope with the cellular plasma membrane. At least two HCMV envelope complexes are required for fusion, the homodimeric glycoprotein B (gB), and a heterologomeric complex thought for many years to be composed of glycoprotein H and glycoprotein L (5–7). Glycoprotein B, gH, and gL have homologs throughout the *Herpesviridae* family (7, 8), and in all cases tested these proteins function at the level of membrane fusion (9–13). Attempts to reconstitute the HCMV gH complex by coexpression of the gH and gL genes, open reading frames UL75 and UL115 of the HCMV genome, respectively (7, 14, 15), were unsuccessful (16, 17). This failure led to the discovery that the HCMV gH complex contains a third distinct gene product encoded by the *UL74* open reading frame, a protein now designated gO (18). Interestingly, the *UL74* gene has homologs in the genomes of the *β-Herpesvirinae* subfamily, which includes HCMV, human herpesvirus 6, and human herpesvirus 7 (18). Thus, while gHgL heterodimers predominate in other herpesviruses, the HCMV complex is an unusual tripartite complex composed of gHgLgO. This complex appears on the surface of HCMV-infected cells and in the envelopes of virions (18, 19). Glycoprotein H is a type I transmembrane protein with a 719-amino acid ectodomain and a short 6-amino acid cytoplasmic tail (15, 20), and expression of gH without gL results in endoplasmic reticulum retention (5, 6). gL lacks a membrane anchor and is disulfide-bonded to gH (5, 7, 15). In contrast to gH and gL, the membrane topology and organization of gO in the tripartite complex are unknown.

Analysis of the amino acid sequence of gO reveals several interesting features, including 18 potential N-glycosylation sites and a single hydrophobic domain that begins 14 amino acids from the N terminus. Hydrophobic analysis of this segment shows a 20–22-amino acid region where we predicted to serve as both a signal peptide and a membrane anchor domain (18). This region is unusual for a signal peptide because it is inset from the amino terminus and it is long enough to span the bilayer as a membrane anchor. If uncleaved by signal peptidase, this region (a signal/anchor domain) would anchor gO in a type II membrane orientation with a cytosolic amino terminus and a carboxyl-terminal extracellular domain. In this work, we describe experiments demonstrating that, like glycoprotein L, glycoprotein O is a soluble protein that associates with cellular and viral membranes.

**EXPERIMENTAL PROCEDURES**

**Cells and Viruses**

Human foreskin fibroblasts and immortalized fibroblasts were cultured as previously described (21). Human cytomegalovirus strain AD169 was propagated and titered as previously described (21). 293T cells were grown in Dulbecco's modified Eagle medium supplemented with 1% penicillin-streptomycin-fungizone, 0.3% glutamine, and 10% fetal bovine serum (Harlan Biosciences).
HCMV gO Is a Soluble Envelope Glycoprotein

Plasmids

All polymerase chain reaction steps were performed using Pfu high fidelity polymerase (Stratagene). Oligonucleotides were synthesized at the University of Wisconsin Biotechnology Center DNA facility, where the final constructs were also verified by automated DNA sequencing. pCaggs.gO was produced by polymerase chain reaction amplification of the full-length gO coding region using primers 5’-GGAATTCCACCATGGGAGAAAGAGATG-3’ and 5’-AAACCGTGTCAGTTACTGCACACCCA-3’. The product was cut with EcoRI (5’) and XhoI (3’) and was inserted into the multiple cloning site of the pCaggs vector (donated by Y. Kawaoka, University of Wisconsin). The gO insert from pCaggs.gO was subcloned into pCDNA3 via EcoRI/XhoI. pCaggs.His-gO was generated by inserting into the pCaggs vector the region encoding amino acids 33–466 of gO. The ΔgO insert was amplified by polymerase chain reaction from pCDNA3.gO template using primers 5’-GGAATTCCACCATGGGAGAAAGAGATG-3’ and 5’-AAACCGTGTCAGTTACTGCACACCCA-3’. The product was cut with EcoRI (5’) and XhoI (3’) and was inserted into the multiple cloning site of the pCaggs vector (donated by Y. Kawaoka, University of Wisconsin). The gO insert from pCaggs.gO was subcloned into pCDNA3 via EcoRI/XhoI. pCaggs.His-gO was generated by subcloning His-gO from pCaggs into pCDNA3 via EcoRI/XhoI.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting were performed essentially as previously described (17, 18). Briefly, nitrocellulose membranes were washed in TBS containing 0.05% SDS, 0.5% Tween 20, and 20 mg/ml powdered skin milk. Horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce, and activity was detected using Renaissance enhanced chemiluminescence reagent (PerkinElmer Life Sciences). UL74 antiserum (recognizing glycoprotein O) was previously described (18). Immunoblots probed with UL74 antiserum were hybridized and washed in the presence of wash buffer (described above) and additional cell lysate (100 mM dish confluent 293-T, cos-7, or IF cells lysed in 1 ml of 1% SDS). Rabbit polyclonal antibody 6824 was previously described (17). Monoclonal antibody 27–78 was kindly provided by W. Britt. Murine monoclonal antibody recognizing Hsp90 was purchased from Transduction Laboratories. Rabbit polyclonal antiserum recognizing calreticulin was purchased from Stressgen. Rabbit polyclonal anti-His serum (His-probe) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Transfections

Plasmid DNA was purified by polyethylene glycol precipitation (22). Cells were transfected by either calcium phosphate precipitation (23) or genePorter lipid transfection reagent (Gene Transfer Systems) according to the manufacturer’s instructions. Calcium phosphate transfections were supplemented with 5 mM sodium butyrate.

Subcellular Fractionation

Preparation of Microsomes—Immortalized human fibroblasts were either mock-infected or infected with HCMV strain AD169 at a multiplicity of infection of 1. Six days postinfection, the cells were scraped into their media and collected by centrifugation. The cell pellet was suspended in hypotonic lysis buffer (20 mM HEPES, 1.5 mM MgCl2, 25 mM EDTA, 1x protease inhibitor mixture/PIC, pH 7.4) and Dounce-homogenized (final volume 15 ml). Nuclei were pelleted at 1500 x g for 10 min and discarded. The supernatant was centrifuged for 30 min at 18,000 x g to pellet microsomal membranes. Supernatant was precipitated with 10% trichloroacetic acid and constitutes the cytosolic fraction. Microsomal membranes were prepared from transiently transfected 293-T cells harvested 48 h post-transfection.

Alkaline Carbonate Extractions—Microsomal membranes were suspended in 100 μl of buffered sucrose (0.3 M sucrose plus 10 mM Tris, pH 7.5) on ice. The suspension was incubated on ice for 60 min in 5 ml of 0.1

Subcellular fractionation of transfected 293-T cells. 293-T cells were harvested 48 h after transfection with plasmids expressing HCMV gO (top three panels) or gH (bottom panel). Cells were fractionated as in Fig. 1, followed by SDS-PAGE and immunoblotting with antibodies as indicated to the right of each panel.

FIG. 1. Subcellular fractionation of HCMV-infected fibroblasts. Infected cells were harvested 7 days postinfection and separated into cytosolic (C), peripheral membrane (PM), and transmembrane (TM) fractions. The peripheral membrane fraction consists of proteins removed from membranes by alkaline carbonate treatment, including the ER chaperone protein calreticulin. Transmembrane proteins include those remaining in the membrane after alkaline carbonate treatment as demonstrated by the type I integral membrane proteins gH and gB. Protein fractions were subjected to SDS-PAGE followed by immunoblotting with antibodies to glycoprotein O, Hsp90, calreticulin, glycoprotein H, and glycoprotein B as indicated.

FIG. 2. Subcellular fractionation of transfected 293-T cells. 293-T cells were harvested 48 h after transfection with plasmids expressing HCMV gO (top three panels) or gH (bottom panel). Cells were fractionated as in Fig. 1, followed by SDS-PAGE and immunoblotting with antibodies as indicated to the right of each panel.
In Vitro Transcription/Translation and Immunoprecipitation

In vitro transcription and translation was performed using the TnT TT Quick coupled transcription/translation kit according to the manufacturer’s instructions (Promega). Immunoprecipitation was performed by diluting 45 μl of translation product in 1 ml of RIPA (150 mM NaCl, 50 mM Tris pH 8.0, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA) plus 0.5% bovine serum albumin. Samples were precleared with protein A beads, followed by incubation overnight with anti-His IgG. Complexes were precipitated with protein A beads, washed five times with RIPA plus bovine serum albumin, and eluted by boiling in RIPA plus reducing SDS-PAGE sample buffer.

RESULTS

Glycoprotein O Is a Membrane-associated Protein—To test the hypothesis that gO is a type II membrane protein, HCMV-infected cells were subjected to fractionation followed by alkaline carbonate extraction. This technique solubilizes peripheral membrane proteins while leaving transmembrane proteins associated with a membrane pellet (24). Immunoblotting of the resulting cytosolic, carbonate-extracted, and integral membrane proteins revealed that gO partitions in both the peripheral and integral membrane fractions (Fig. 1). A majority of gO consistently segregated with transmembrane proteins, while a lesser amount was detected in the peripheral membrane fraction. Controls for each fraction include Hsp90 (cytosol), calreticulin (soluble endoplasmic reticulum protein), and the cytomegalovirus-encoded proteins gH and gB (both type I integral membrane proteins), all of which were recovered in the appropriate fractions. Because gO was found in both membrane fractions in HCMV-infected cells, we hypothesized that the two types of membrane association may result from interactions of gO with other viral proteins.

To examine gO in a system isolated from other viral gene products, a similar experiment was conducted using transiently transfected 293-T cells expressing gO (Fig. 2). Although it partitioned differentially in HCMV-infected cells, gO was solely detected in the soluble membrane content fraction of transfected cells. This result, shown in Figs. 1 and 2, demonstrated that gO is more strongly associated with membranes of infected cells than those of transfected cells, suggesting that the affinity of membrane association of gO differs with the context of expression.

The Signal Peptide of gO Is Cleaved from the Mature Molecule—Initial experiments suggested that gO was not a transmembrane protein. We further tested this hypothesis using two recombinant forms of gO (Fig. 3). One form, designated ΔgO, lacked the amino-terminal 32 amino acids comprising the putative signalanchor domain. A second construct, designated His-gO, encoded a six-residue polyhistidine tag in His-gO plasmids allows specific detection among wells. For alkaline carbonate extractions of microsomes from HCMV-infected cells, gO was found in both membrane fractions in HCMV-infected cells, we hypothesized that the two types of membrane association may result from interactions of gO with other viral proteins.

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cell lysates. If the signal peptide of gO were uncleaved, we would expect to see a difference in electrophoretic mobility corresponding to the 3-kDa size difference between the peptide backbones of gO and gO (Fig. 3). As shown in Fig. 4, the deglycosylated peptide backbones of gO and gO migrated similarly when subjected to SDS-PAGE, demonstrating that these proteins were the same 48-kDa size (Fig. 4A). Immunoblotting with anti-His serum demonstrated the presence of a polyhistidine tag on the control protein encoded by pHM6 (β-gal His), but not on His-gO.

DISCUSSION

The gHgL-gO complex undergoes multiple steps of assembly during its transit through the secretory pathway of HCMV-infected cells (19). With a mass greater than 240 kDa, the mature complex is a disulfide-linked heteroligomer of unknown stoichiometry (6, 7, 25, 26). Early studies of HCMV gH and gL showed that gH needs gL for proper trafficking through the secretory apparatus (5, 6). When gH is expressed in the presence of gL, complexes of the two proteins reach the cell surface but are not secreted into the medium. Removal of the C-terminal membrane anchor of gH results in secretion of soluble gHgL complexes, and when gL is expressed alone it can also be found in the medium (5, 6). These data led to the conclusion that gH contains a C-terminal membrane anchor and that gL is held in the viral envelope by its disulfide linkage(s) to gH. Beyond this basic characterization, little is known about the structure of the gHgL-gO complex.

Based on sequence analysis, we hypothesized that the 20-amino acid hydrophobic domain on the amino terminus of gO serves as a signal peptide and membrane anchor. In general, a signal peptide longer than 15–18 amino acids indicates that signal peptidase is unlikely to cleave the nascent chain, leaving an intact amino-terminal transmembrane domain (27). Several
viral envelope proteins, including influenza neuraminidase, are known to have this membrane orientation (28–30). As a novel member of the disulfide-linked gHgL complex, gO is unusual in that it is a viral envelope protein that does not need a transmembrane domain for membrane association. Covalent and/or noncovalent association with gH may suffice to tether gO to the viral envelope. Although we hypothesized, based on the amino acid sequence, that gO contained a membrane anchor, we addressed the question experimentally.

One established method for separating integral membrane proteins from soluble, membrane-associated proteins involves washing vesicular membranes in alkaline carbonate. Alkaline treatment converts membrane vesicles into sheets, releasing ER and Golgi contents into the supernatant and leaving transmembrane proteins in the membrane pellet (24). From previous work, it is known that processing of gO in the ER occurs prior to its association with gHgL complexes and that gO can be found as a monomer for several hours after synthesis (19). We propose that the subset of glycosylated gO that is not stably bound to gHgL complexes may be the same portion that can be extracted from membranes by alkaline carbonate treatment. Results in Fig. 2 show that gO, when expressed alone, can be completely extracted from membranes. Our results suggest that alkaline carbonate extraction solubilizes proteins from membrane vesicles in a context-specific fashion. The implication of such selective extraction is that proteins, such as gO, that stably associate with integral membrane proteins via noncovalent or covalent interactions cannot be reliably analyzed by alkaline extraction alone.

Faced with the experimental challenge of demonstrating the absence, rather than the presence, of a membrane-spanning domain, we created an epitope-tagged recombinant form of gO. The cumulative evidence from the His-tagged protein experiment and previous studies demonstrated that the signal peptide of gO is cleaved and that gO is a soluble protein. Thus, our working model of the gHgL-gO complex asserts that glycoproteins H, L, and O are held in the viral envelope by the single transmembrane domain of gH (Fig. 7). We also know that assembly of the complex occurs sequentially in the ER and that gO acquires terminal modifications while traversing the Golgi en route to the cell surface (19). It is not known, however, where final envelopment of the virus occurs.

The tripartite gHgL-gO complex is unusual among viral fusion glycoproteins and has to date been studied only in HCMV. The gH and gL components of the complex have characterized homologs among all herpesviruses examined (31–37), but gO homologs are found only in the β herpesviruses (18). Glycoproteins H and L are essential components of the virus (38) and are necessary for fusion of the viral envelope with the host cell membrane (9, 39, 40). In contrast, recent work has shown that gO is not essential for viral replication in tissue culture but that a gO knockout virus has a severely attenuated phenotype (38). Preliminary data from our laboratory also support involvement of gO in the fusion process, and studies are ongoing to elucidate the exact role that gO plays in viral entry and cell to cell spread of infection.

The Herpesviridae are unique among viruses in the complexity of their glycoprotein coats. Thus, models of membrane fusion derived from single glycoprotein viral systems are of only moderate utility in our efforts to dissect the mechanisms of viral entry and cell to cell spread of HCMV. Perhaps more relevant are models of intracellular vesicle transport and membrane fusion, such as neurotransmitter release from synaptic vesicles. The multiprotein interactions between v-SNAREs and t-SNAREs as well as the sequential nature of membrane docking and fusion steps closely resemble the stages of HCMV fusion with host cell membranes (41). Additionally, the core fusion complex of SNAP-29/syntaxin is composed of an integral membrane protein, syntaxin, which targets SNAP-29 to membranes (42). Several other proteins have been identified that bind to and positively or negatively regulate SNAREs in the neuron, including those proteins mediating calcium-triggered activation of fusion (41, 43, 44). Multiple additional proteins of uncharacterized function have also been identified in the envelope of HCMV (see Refs. 20 and 45; reviewed in Ref. 46) and may contribute to or regulate the fusion machinery in response to unknown triggering events. Examination of the mechanisms of intracellular membrane fusion may ultimately facilitate our understanding of the complex nature of HCMV-induced membrane fusion.

REFERENCES
1. Singher, C., and Jahn, G. (1996) Interivirology 39, 302–319
2. Turner, A., Bruun, B., Minson, T., and Browne, H. (1998) J. Virol. 72, 873–875
3. Compton, T., Nowlin, D. M., and Cooper, N. R. (1993) Virology 193, 834–841
4. Nowlin, D. M., Cooper, N. R., and Compton, T. (1991) J. Virol. 65, 3114–3121
5. Spafe, R. E., Perot, K., Scott, P. L., Nelson, J. A., Stinski, M. F., and Pachl, C. (1993) Virology 193, 855–861
6. Kaye, J. S., U. A. G., and Minson, A. C. (1992) J. Gen. Virol. 73, 2693–2696
7. Cranage, M. P., Smith, G. L., Bell, S. E., Hart, H., Brown, C., Bankier, A. T.,...
