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The Human Cytomegalovirus UL37 Immediate-Early Regulatory Protein Is an Integral Membrane N-Glycoprotein Which Traffics through the Endoplasmic Reticulum and Golgi Apparatus

HEBAH O. AL-BARAZI AND ANAMARIS M. COLBERG-POLEY*

Center for Virology, Immunology and Infectious Disease Research, Children's National Medical Center, Washington, DC 20010

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The human cytomegalovirus (HCMV) UL37 immediate-early gene is predicted to encode a type I membrane-bound glycoprotein, gpUL37. Following expression of the UL37 open reading frame in vitro, its signals for translocation and N-glycosylation were recognized by microsomal enzymes. Its orientation in the microsomes is that of a type I protein. gpUL37 produced in HCMV-infected human cells was selectively immunoprecipitated by rabbit polyvalent antiserum generated against the predicted unique domains of the UL37 open reading frame and migrated as an 83- to 85-kDa protein. Tunicamycin treatment, which inhibits N-glycosylation, increased the rate of migration of the UL37 protein to 68 kDa, verifying its modification by N-glycosylation in HCMV-infected cells. Consistent with this observation, gpUL37 was found to be resistant to digestion with either endoglycosidase F or H but sensitive to peptide *N*-glycosidase F digestion. These results suggested that gpUL37 is N-glycosylated and processed in both the endoplasmic reticulum (ER) and the Golgi apparatus. Direct demonstration of passage of gpUL37 through the ER and the Golgi was obtained by confocal microscopy. gpUL37 colocalized with protein disulfide isomerase, a protein resident in the ER, and with a Golgi protein. Subcellular fractionation of HCMV-infected cells demonstrated that gpUL37 is an integral membrane protein. Taken together, our results demonstrate that the HCMV gpUL37 immediate-early regulatory protein is a type I integral membrane N-glycoprotein which traffics through the ER and the Golgi network.

Human cytomegalovirus (HCMV) is a medically important herpesvirus which is the leading viral cause of birth defects and causes serious opportunistic infections in immunosuppressed patients (3). The first viral products to be synthesized during HCMV growth in human cells are encoded by the immediate-early (IE) genes which are located in the major IE, UL36-38, UL115-119, US3, TRS1, and IRS1 loci (reviewed in references 9 and 35). The IE transcripts encode regulatory proteins which alter the expression of cellular, as well as viral, genes and are presumed to be necessary for progression of the viral life cycle (reviewed in references 9 and 35). Consistent with this presumption is the recent finding that antisense oligonucleotides against either the HCMV UL36 or UL37 IE transcript inhibit HCMV DNA replication and block HCMV growth in permissive diploid human foreskin fibroblasts (HFF) (38, 44). In agreement with these results, products encoded by three HCMV IE loci, including the UL36-38 locus, as well as the major IE and TRS1/IRS1 genes, are required for HCMV DNA replication in transient transfection assays (39).

Of the IE products required for HCMV DNA synthesis, HCMV major IE proteins IE1 and IE2 are both nuclear phosphoproteins which are known to bind DNA and to interact with cellular transcription factors (2, 8, 16, 17, 21, 28, 29, 32, 37). In contrast, three of the remaining HCMV IE loci, UL37, US3, and UL119-117, predictably encode type I membrane-bound N-glycoproteins (7, 26, 30, 53).

The UL36-38 IE locus encodes multiple products (26, 46–48). Similar to other HCMV IE proteins, the UL36-38 IE proteins regulate nuclear gene expression (11, 49). Anders and coworkers recently demonstrated the regulation of HCMV early DNA replication promoters by UL36-38 proteins (23). Indeed, this regulatory activity may cause the requirement for the UL36-38 products to permit replication of HCMV DNA (38, 39, 44).

Motifs contained in UL36, UL37 exon 3, and UL38 open reading frames (ORF), rather than the genes themselves, are encoded by genes in the IE B locus of human herpesvirus 6 (HHV-6) (18, 36). Similar to the UL36-38 IE proteins, the HHV-6 proteins encoded by the IE B locus also have nuclear regulatory activity (36).

Of the genes located in the UL36-38 locus, expression of the UL37 IE gene is the most stringently regulated in terms of its abundance and its kinetics of expression (26, 46–48). The UL37 transcript is expressed at an exceedingly low abundance, even compared with other HCMV IE gene transcripts. UL37 RNA is detectable by Northern blot analysis predominantly at IE times, and, characteristically for IE transcripts, its abundance is increased notably by treatment of HCMV-infected cells with inhibitors of protein synthesis (26, 46–48). Conversely, UL37×1 RNA, which is initiated at the same promoter as UL37 RNA, and UL36 RNA, which is initiated at a downstream promoter in the same locus, are much more abundant and present from IE times until early and late times of HCMV infection. Nevertheless, at IE times of infection, the UL37 transcript is rapidly transported to the cytoplasm and is associated with polysomes, demonstrating its availability for translation (48).

gpUL37, the product of the UL37 IE RNA, is predicted to be a type I membrane-bound glycoprotein (26). Figure 1 illustrates the features of the predicted UL37 ORF, which include a hydrophobic signal sequence, an acidic domain, 17 potential N-glycosylation sites, a basic domain, and a transmembrane-anchor sequence.

Despite its probable importance for HCMV biology and

* Corresponding author. Mailing address: Center for Virology, Immunology and Infectious Disease Research, Room 5720, Children's National Medical Center, 111 Michigan Ave., N.W., Washington, D.C. 20010. Phone: (202) 884-3984. Fax: (202) 884-3985. Electronic mail address: colberam@gwis2.circ.gwu.edu.

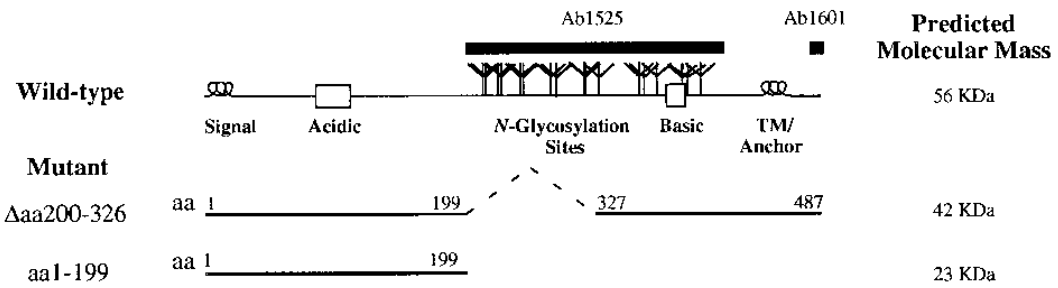

FIG. 1. wt and mutant gpUL37 proteins and immunogens for anti-gpUL37 serum production. The wt UL37 ORF is represented with the signal and transmembrane (TM) domains (coils), acidic and basic domains (open boxes), and 17 N-linked glycosylation signals (branches). Below the wt ORF are two mutants, one lacking aa 200 to 326 (Δaa200-326) and one lacking all of the sequences downstream from aa 199 (aa1-199). The immunogens used for rabbit antiserum production are shown.

perhaps because of its stringent regulation, synthesis of gpUL37 in HCMV-infected cells has not been experimentally demonstrated. We wished to examine gpUL37 production in HCMV-infected cells and to determine whether it is indeed a type I integral membrane N-glycoprotein which traffics through the endoplasmic reticulum (ER) and the Golgi network. For this purpose, we undertook in vitro studies of the translocation and processing of gpUL37, generated polyvalent antibodies against the unique domains of gpUL37, tested these antibodies against in vitro translation products, and used the antibodies to examine the modification and subcellular location of gpUL37 in transfected human cells and in permissive HCMV-infected cells. Our results demonstrated that the UL37 regulatory protein is synthesized in HCMV-infected cells as an integral membrane protein which is modified by N-glycosylation and traffics through the ER and the Golgi network.

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MATERIALS AND METHODS

Virus infections. HFF and HCMV (strain AD169) stocks were grown, and stock titers were determined as previously described (10). HFF cells were pretreated with cycloheximide (50 μg/ml) in Dulbecco's modified essential medium supplemented with 2% fetal calf serum for 15 or 30 min prior to and during HCMV infection. HFF cells were infected at a multiplicity of 5 PFU per cell for 18 h. To reverse the inhibitor block, cells were washed five times with phosphate-buffered saline (PBS). The cells were then starved for methionine for 1 h by using labeling medium (Dulbecco's modified essential medium without methionine with 0.5% fetal calf serum), and newly synthesized proteins were labeled metabolically for 2 h by addition of L-[^{35}S]methionine and L-[^{35}S]cysteine NEN Research Products) at 100 μCi/ml before harvesting in PBS. Where indicated, HFF cells were treated with tunicamycin (Boehringer Mannheim) at 10 μg/ml for 3 h starting at the time of release of the protein synthesis block.

Transfection of human cells. HeLa and HFF cells were transfected by calcium phosphate coprecipitation as previously described (11). Briefly, for each 10-cm-diameter cell culture dish containing a multiple-well glass slide, 10 μg of plasmid DNA and 10 μg of calf thymus DNA were precipitated, added to the medium covering the cells, and incubated at 37°C for 4 h in the presence of 3.5% CO_2. The cells were then treated for 2 min with 20% glycerol in PBS, washed twice with PBS, refed medium, and incubated for another 40 h. Cells were harvested by fixation in ice-cold methanol and air drying. Slides were stored at −20°C until use.

Plasmid DNA constructions. The wild-type (wt) and mutant gpUL37 used in these studies are shown in Fig. 1. wt gpUL37 was expressed from p414 (11). The wt UL37 cDNA contained in p414 was subcloned as an *Eco*RI fragment under control of the T7 promoter in vector pCITE-2a(+) (Novagen), thereby generating p591. A plasmid (p526) which encodes a mutant protein (Δaa200-326) lacking the first 11 N-linked glycosylation sites in the UL37 ORF was generated by insertion of an *Eco*RI linker from New England Biolabs (5′-CGG AAT TCC G-3′) into the *Sna*BI sites at nucleotides 50777 and 50395. DNA sequencing with the U.S. Biochemical Sequenase kit verified the insertion of a single linker which encodes three missense amino acids (aa), Gly-Ile-Pro. A gene (p559) encoding a truncation mutant of the UL37 protein (aa 1 to 199) was generated by insertion of a stop linker (5′-CTA GGC CTT AGC GGC CGC TAG-3′) between the *Sna*BI sites (nucleotides 50777 and 50395) in the UL37 gene. The inserts from p526 and p559 were cloned into the pCITE-2a(+) vector, generating p589 and p590, respectively. The expression clones for glycoprotein B (gB) (p370) and IE1 (pRR59) have already been described (11).

gpUL37 antisera. The immunogens used in the generation of rabbit antisera are shown in Fig. 1. Rabbit Ab1525 was prepared by repeated injection of a β-galactosidase fusion protein containing UL37 ORF aa 201 to 420 (Hazelton Laboratories, Denver, Pa.). Rabbit Ab1601 was generated by repeated injection of a synthetic peptide (DEGSSSTKNDVHRIV, UL37 ORF aa 473 to 487) from the gpUL37 carboxy-terminal sequences coupled to keyhole limpet hemocyanin. In each case, the corresponding preimmune serum, at the same dilution, was used as a control.

Other antibodies. A mouse monoclonal antibody against gB (CH-28) was obtained from Lenore Pereira (40). Mouse monoclonal anti-protein disulfide isomerase (PDI) and rabbit anti-calnexin antibodies were purchased from Stress-Gen Biotechnology Corp. Rabbit anti-histone H2B serum and a mouse anti-Golgi zone monoclonal antibody were purchased from Chemicon Corp.

In vitro transcription-translation (IVT/T) and gel electrophoresis. The UL37 ORF was expressed in vitro by using Single Tube Protein System 2 (Novagen) as recommended by the manufacturer. In vitro-synthesized proteins were labeled with L-[^{35}S]Met (translation grade; NEN) and resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 10% polyacrylamide slab gels by following the method of Laemmli (27). ^{35}S-labeled proteins were then detected by fluorography with Enlightning (NEN). Molecular mass standards (Bio-Rad) were used to determine apparent molecular mass. Canine pancreatic microsomal membranes (Promega) were used at 8 equivalents per reaction. Where indicated, microsomes were pretreated with 0.05% Nikkol ($C_{12}E_8$; Calbiochem) for 10 min before use in IVT/T. Aliquots of the IVT/T reactions were digested for 1 h on ice with proteinase K (0.5 μg) in the presence or absence of 1% Triton X-100.

Immunoprecipitation. Cells were lysed with 30 volumes of lysis buffer (120 mM NaCl–50 mM Tris [pH 8.0]–1% Nonidet P-40–1% sodium deoxycholate–3.3 mM EDTA freshly supplemented with 0.3 mM phenylmethylsulfonyl fluoride [PMSF], 1.6 mM benzamide HCl, and 3.3 mM *N*-ethylmaleimide) at 4°C for 1 h. The lysate was cleared by centrifugation at 3,000 × *g* (10 min). Equal volumes of protein samples (~1 mg/ml as determined by the DC Protein assay [BioRad]) were precleared by reaction at 4°C with protein A-agarose beads (Boehringer Mannheim) precoated with preimmune serum. The precleared lysate was then immunoprecipitated with immune serum, prebound to protein A beads, and blocked with unlabeled, uninfected HFF lysates (1 mg/ml). Both reactions were performed at a volume ratio of 10:1:1 (lysate-beads-undiluted serum). For immunoprecipitation of fractionated cell lysates, unlabeled cellular fractions were used to block the antibody. The beads were washed four times with RIPA buffer (50 mM NaCl, 50 mM Tris [pH 7.5], 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), once with H_2O, and once with TBS (140 mM NaCl, 20 mM Tris [pH 7.5], 1% Tween 20). The immune complexes were eluted off the beads in 2× sample buffer (4% SDS, 20% glycerol, 120 mM Tris [pH 6.8], 0.01% bromphenol blue, 10% β-mercaptoethanol) and analyzed by SDS-PAGE.

Carbohydrate digestion. For digestion with endoglycosidase F (endo F; Boehringer Mannheim) and peptide *N*-glycosidase F (PNGase; Genzyme), immune complexes bound to beads were eluted with 0.5 M Tris buffer (pH 8) containing 0.5% SDS and 10% β-mercaptoethanol by heating at 95°C for 5 min. Aliquots of the same sample were either untreated, treated with endo F, or treated with PNGase in a buffer containing 1.25% *n*-octylglucoside (Boehringer Mannheim), 1 mM phenylmethylsulfonyl fluoride, and less than 0.2% SDS at 37°C for 72 h. Samples to be digested with endo H (NEN) were handled analogously, except that 0.1 M sodium citrate (pH 5.5)–0.5% SDS was used for the elution and reaction mixtures were treated with endo H in the absence of *n*-octylglucoside detergent. The digestions were terminated by addition of 6× sample buffer and followed by SDS-PAGE. The resolved [^{35}S]Met–[^{35}S]Cys-labeled proteins were detected by fluorography, as well as by scintillation counting, in which gel lanes were sliced into sequential 0.5-cm-long pieces and incubated overnight at 37°C in Econofluor-2 scintillation fluid and radioactivity was counted in a Beckman LS6500 counter.

Indirect immunofluorescence assay. Fixed cells were washed with PBS and stained with the primary antibody diluted in 10% bovine serum albumin for 1 h at 37°C. The cells were washed thrice with PBS containing 1% Triton X-100 (5 min) and then stained for 1 h at 37°C with secondary antibodies (Leinco Tech), either immunoglobulin G (IgG) or Fab fragments, labeled with either fluorescein isothiocyanate (FITC) or Texas Red, and diluted in 10% bovine serum albumin. The slides were finally washed thrice with PBS containing 0.2% Tween 20 and once with water and air dried. The slides were mounted with coverslips by using a PBS-glycerol (1:1) solution. Analysis was performed with a Bio-Rad MRC1000 Confocal Laser Scanning Imaging System and recorded with a Mitsubishi CP2000 printer (Center for Microscopy and Image Analysis, George Washington University).

Cell fractionation. The fractionation of HFF cells was based upon method 2 of Radsak and coworkers (41), with the following modifications. All steps were performed in a cold room. The cell pellet was resuspended in 30 volumes of hypotonic buffer E (freshly supplemented with 0.3 mM phenylmethylsulfonyl fluoride, 1.6 mM benzamide HCl, and 3.3 mM *N*-ethylmaleimide) and sonicated three times, for 15 s each time. The nuclei were collected by centrifugation at 10,000 × *g* (10 min), and the supernatant was reserved. After resuspension of the pellet in 10 volumes of buffer E, the cells were further sonicated to ensure complete disruption of more than 90% of cells. The resuspended nuclear pellet was centrifuged through the sucrose cushion at 1,500 × *g* for 30 min. The nuclei were lysed in 30 volumes of lysis buffer (as in the immunoprecipitation protocol described above) by sonication. For recovery of membrane and cytosol fractions, the pooled postnuclear supernatant was brought to 0.5% Triton X-100 and incubated on ice for 10 min. The membrane fraction was collected by centrifugation at 100,000 × *g* for 1 h. The supernatant was collected as a cytosol, while the membrane pellet was lysed in 30 volumes of lysis buffer by sonication and cleared by centrifugation.

Western blot (immunoblot) analysis. Proteins in each fraction and total proteins were resolved by SDS–15% PAGE and transferred onto Immobilon-P membranes by using a semidry transfer apparatus in accordance with the manufacturer's (Integrated Separation Systems) instructions. Immunoreactive proteins on the blots were detected with a chemiluminescence detection system with the following modifications of the manufacturer's (Kirkegaard & Perry Laboratories) instructions. Briefly, the membrane was treated with milk diluent blocker, except that 0.15% Tween and 0.3% Tween 20 were added to the blocking solution and the wash solution, respectively. The dilution used for each primary antibody was empirically determined. Signals were detected upon exposure of the membrane blot to Hyperfilm-ECL film (Amersham) for 5 s to 20 min.

RESULTS

In vitro synthesis and N-glycosylation of the UL37 protein. To increase the efficiency of in vitro translation, we subcloned the UL37 ORF in expression vector pCITE-2a(+), which contains a cap-independent translation enhancer, the leader sequence from encephalomyocarditis virus RNA. By using this system, we efficiently synthesized the primary translation product of the UL37 ORF, pUL37, in vitro. Its apparent molecular mass was 60 kDa (Fig. 2, lane 2). This result corresponds well to the predicted value of 56 kDa for the UL37 ORF and is consistent with our previous results (47).

To determine whether the signal sequence and N-linked glycosylation sites of the UL37 ORF are recognized by mammalian enzymes, we added canine pancreatic microsomal membranes to the IVT/T reaction mixture. These membranes contain signal peptidase and core glycosylating activities which recognize glycosylation signals and covalently bind sugar moieties to the protein backbone specifically via N linkage (52). Indeed, pUL37 was modified by microsomal enzymes since, in addition to pUL37, two more-slowly migrating species of 83 to 85 and 120 kDa were produced following the addition of microsomes to the IVT/T reaction mixture (lane 3). Pretreatment of the microsomes with Nikkol, a nonionic detergent which disrupts the lipid bilayer, resulted in elimination of the 83 to 85-kDa species (referred to henceforth as gpUL37) but not of untranslocated pUL37 (60 kDa) or of a larger UL37 species of 120 kDa (lane 4). Microsomes treated with Nikkol lose N-glycosylation activity but retain signal peptidase activity. Our results demonstrated that the pUL37 signal sequence and N-glycosylation signals are correctly recognized and modified in vitro to generate glycosylated gpUL37 with an apparent molecular mass of 83 to 85 kDa.

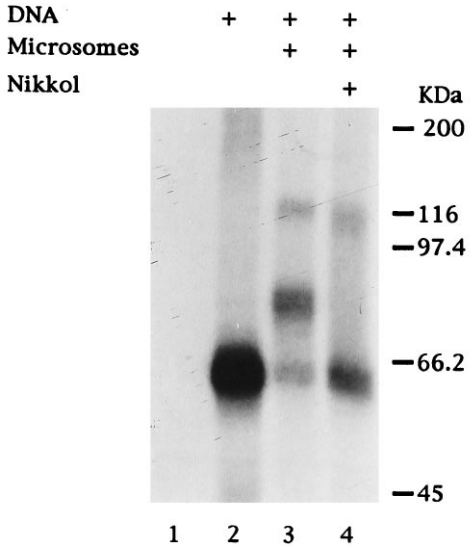

FIG. 2. In vitro synthesis and processing of gpUL37. pUL37 was synthesized and labeled with [^{35}S]Met by IVT/T. The reactions were either unprogrammed (lane 1) or programmed with wt UL37 DNA (lanes 2 to 4). Microsomal membranes, either untreated (lane 3) or pretreated with 0.05% Nikkol (lane 4), were included in the reaction mixtures. Proteins were resolved by SDS–10% PAGE and visualized by fluorography. The migration positions of molecular mass standards are indicated.

Association and orientation of gpUL37 in microsomes. To verify the association of gpUL37 with membrane vesicles and determine its orientation in the vesicles, in vitro-synthesized and translocated gpUL37 was digested with a protease (Fig. 3). Proteinase K digestion of gpUL37 increased its apparent rate of migration (Fig. 3A, lane 5) compared with that of undigested gpUL37 (lane 3). This change in migration was more apparent when the samples were analyzed side by side following more extensive electrophoresis (Fig. 3B). Proteinase K-digested gpUL37 had an apparent molecular mass of ~78 kDa (lane 2). This decrease in mass is consistent with removal of the predicted cytoplasmic tail of gpUL37 (28 aa) and protection of the amino-terminal 459 aa. Consistent with the results in Fig. 2, the 60- and 120-kDa species were degraded by protease digestion, indicating that these species are located outside of the microsomal vesicles.

Addition of Triton X-100 to the IVT/T reaction mixtures, which disrupts the membrane vesicles, resulted in digestion of all pUL37 species (Fig. 3A, lanes 6 and 7), indicating their susceptibility to protease digestion and, consequently, their identity as proteins. Taken together, our results establish that gpUL37 is a type I membrane-anchored protein such that the bulk of the protein is protected in the lumen of the microsomes while a short cytoplasmic tail is externally exposed.

Synthesis and posttranslational processing of gpUL37 in HCMV-infected HFF cells. To examine the production and processing of gpUL37 in HCMV-infected cells, we used Ab1525 to immunoprecipitate lysates from uninfected or HCMV-infected cells (Fig. 4). Ab1525 was generated against UL37 unique domains (Fig. 1) and, consequently, detects UL37 products but not the predicted UL37×1 protein, which shares the amino-terminal 162 aa with the UL37 ORF (7, 26).

We used inhibitors of protein synthesis to increase the abundance of the UL37 IE transcript and to enhance the detection of gpUL37 in HCMV-infected cells. Total HCMV-infected cell lysates showed the presence of a prominent protein of 72 kDa, which is consistent with the molecular mass of the IE1 protein

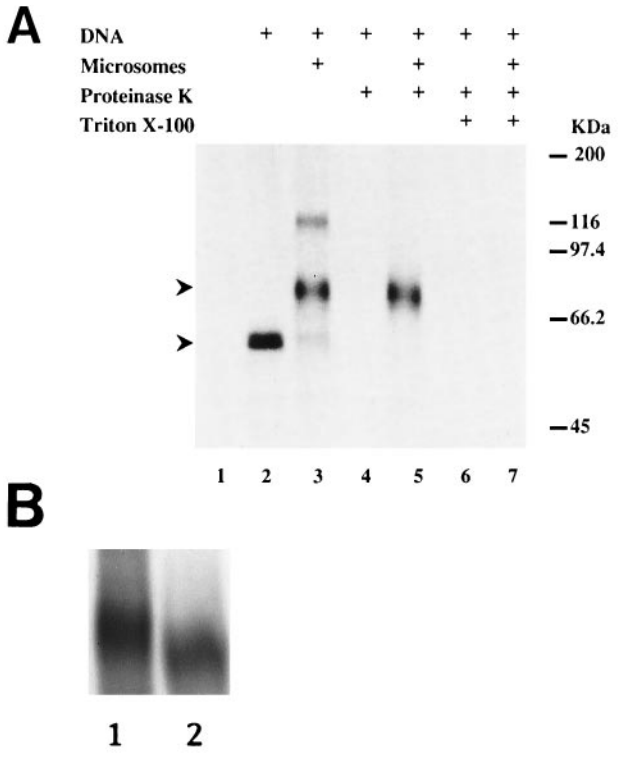

FIG. 3. Orientation of gpUL37 in microsomes. (A) pUL37 was synthesized and labeled with [^{35}S]Met in vitro in the presence (lanes 3, 5, and 7) or absence (lanes 2, 4, and 6) of microsomes. Control, unprogrammed lysates are shown in lane 1. Aliquots of each reaction mixture were digested with proteinase K in the presence (lanes 6 and 7) or absence (lanes 4 and 5) of the detergent Triton X-100 as indicated. Labeled proteins were resolved by SDS–10% PAGE and visualized by fluorography. The migration positions of molecular mass standards are indicated. The top and bottom arrowheads indicate the migration positions of gpUL37 and pUL37, respectively. (B) IVT/T reaction products analogous to those in panel A, lanes 3 and 5, were resolved further by SDS–10% PAGE in lanes 1 and 2, respectively. Only the 80- to 85-kDa region of the gel is shown.

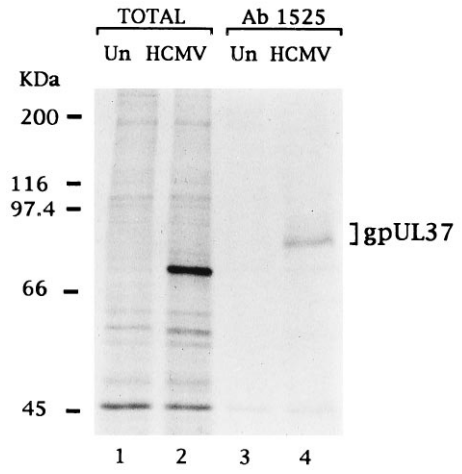

FIG. 4. Immunoprecipitation of gpUL37 from HCMV-infected HFF cells. Cycloheximide-treated HFF cells were uninfected (Un) or HCMV infected (HCMV). Proteins were metabolically labeled after block removal. Total lysates of uninfected (lane 1) or HCMV-infected (lane 2) cells or proteins immunoprecipitated by Ab1525 from uninfected (lane 3) or HCMV-infected (lane 4) cells were resolved by SDS–10% PAGE. The bracket indicates the migration position of gpUL37.

(Fig. 4, lane 2). The identity of this protein as IE1 was verified independently by immunoprecipitation with MAB810, a mouse monoclonal antibody which reacts with the epitope encoded by IE1 exon 3 sequences (1). We used immunoprecipitation to demonstrate the presence of gpUL37 in the total HCMV-infected cell extracts. gpUL37, with an apparent molecular mass of 83 to 85 kDa, was specifically immunoprecipitated by Ab1525 from HCMV-infected cells but not from uninfected cells (Fig. 4, lanes 3 and 4). The mass of this species is similar to the value obtained for gpUL37 synthesized and modified in vitro (Fig. 2 and 3). Neither the 60- nor the 120-kDa species of pUL37 observed in vitro was apparent in the HCMV-infected cells following the 2-h labeling period. These results verify the production of gpUL37 in HCMV-infected HFF cells and suggest its posttranslational modification.

To determine whether gpUL37 is modified posttranslationally by N-glycosylation in HCMV-infected cells, we examined the effects of the N-glycosylation inhibitor tunicamycin on posttranslational processing of gpUL37 (Fig. 5). The Ab1525 used in this experiment was not previously blocked with uninfected cellular proteins; consequently, an unrelated cellular protein was nonspecifically immunoprecipitated from both uninfected and HCMV-infected cells (lanes U and H). The UL37 protein specifically immunoprecipitated from HCMV-infected lysates (lane H) and not from uninfected cells (lane U) is indicated by the arrow. All of the other bands are cellular proteins which were visible in the uninfected-HFF lane upon longer exposure of the gel. The extracts used for immunoprecipitation were standardized by the amount of protein and not the amount of incorporated radioisotope. HCMV is known to stimulate the synthesis of cellular, as well as viral, proteins. Consequently, a longer exposure of the uninfected-cell lysates was required to show that the other bands are cellular proteins (1).

Tunicamycin treatment of HCMV-infected cells resulted in an increased rate of migration of the UL37 protein to an apparent molecular mass of 68 kDa (lane H). This new molecular species migrated more rapidly than the processed gpUL37 obtained in HCMV-infected cells or by modification

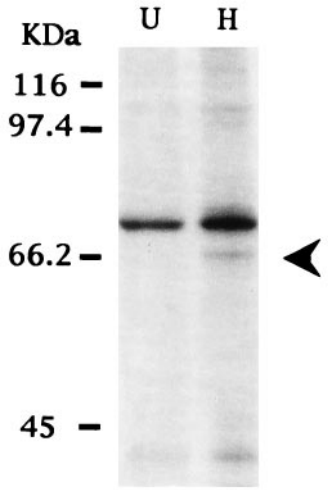

FIG. 5. gpUL37 is N glycosylated in HCMV-infected HFF cells. Cycloheximide-treated HFF cells were uninfected (U) or HCMV infected (H) for 18 h, the block was reversed, tunicamycin was added, and after 1 h, proteins were metabolically labeled for 2 h. Proteins were immunoprecipitated with Ab1525 following preclearing with preimmune Ab1525. The arrowhead indicates the UL37 protein.

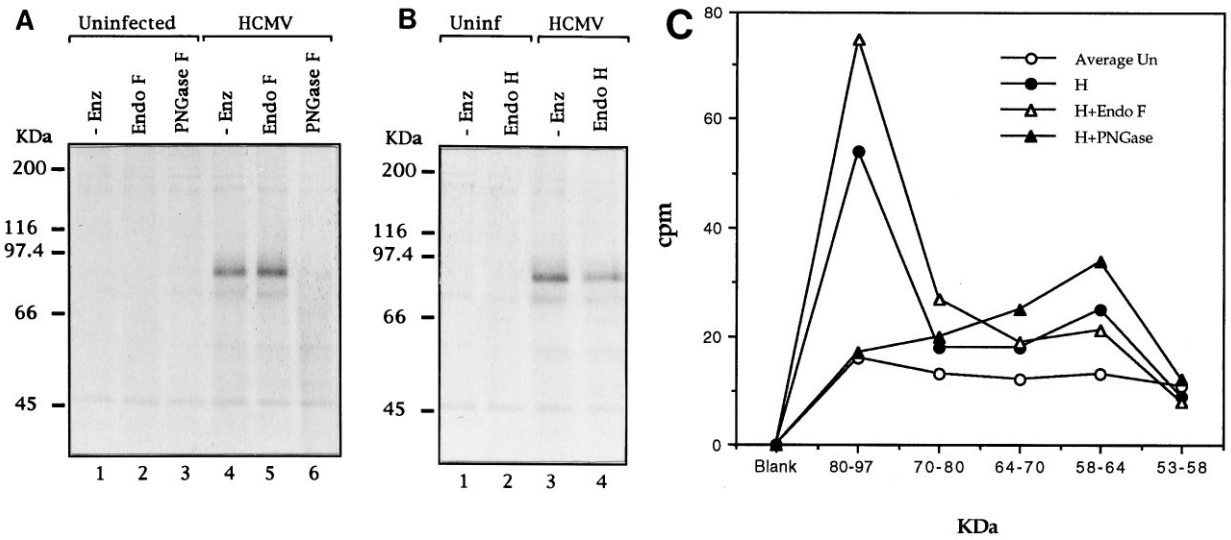

FIG. 6. Enzymatic removal of N-linked oligosaccharides from gpUL37. (A) endo F and PNGase digestion. Cycloheximide-treated HFF cells were uninfected or HCMV infected for 18 h, the block was reversed for 1 h, and proteins were metabolically labeled for 2 h. The lysates were immunoprecipitated with Ab1525 following preclearing with preimmune Ab1525. The immune complexes from uninfected (lanes 1 to 3) or HCMV-infected (lanes 4 to 6) cells were either not treated (−Enz), treated with endo F, or treated with PNGase before analysis by SDS-PAGE. (B) endo H digestion. Immune complexes generated as for panel A were either untreated or treated with endo H. The reactions were analyzed by SDS-PAGE. Uninf, uninfected. (C) Quantitation of ^{35}S-labeled digestion products. Immunoprecipitated lysates from uninfected (Un) or HCMV-infected (H) HFF cells were separated as for panel A, and each lane of the gel was cut into five 0.5-cm-long slices corresponding to proteins of 80 to 97, 70 to 80, 64 to 70, 58 to 64, and 53 to 58 kDa. The counts per minute for each slice with the blank subtracted were plotted against the corresponding molecular size range. To simplify the graph, the control uninfected values shown are the averages of the three control lanes.

in vitro. It did, however, have a slightly higher molecular mass than the primary 60-kDa pUL37 species obtained in vitro (Fig. 2 and 3). This experiment indicates that the gpUL37 synthesized in HCMV-infected cells is modified specifically by N-glycosylation.

We next used endoglycosidase digestion to confirm this finding and to determine the complexity of the oligosaccharide modifications on gpUL37 from HCMV-infected cells (Fig. 6). endo F preferentially cleaves biantennary chains but can cleave high-mannose oligosaccharides (15). PNGase cleaves simple, as well as complex, oligosaccharide chains linked to the peptide backbone at asparagine residues. Again, untreated gpUL37 immunoprecipitated from HCMV-infected cells had an apparent molecular mass of 83 to 85 kDa (Fig. 6A, lane 4). endo F digestion of gpUL37 (Fig. 6A, lane 5) did not alter the mobility of gpUL37 compared with that of undigested gpUL37 (lane 4). In contrast, digestion with PNGase resulted in loss of the gpUL37 83 to 85-kDa band, presumably because of its conversion to multiple partially deglycosylated species. Close examination of the original film indicated accumulation of radioactive species in the 60- to 70-kDa molecular mass species. To detect these signals efficiently, the lanes were cut into 0.5-cm-long slices and the radioactivity in each slice was quantified. The molecular size range of each fraction was graphically plotted against the counts per minute obtained (Fig. 6C). As expected, the peak counts per minute for untreated and endo F-treated gpUL37 coincided with the molecular mass range of processed gpUL37 (80 to 97 kDa). A much smaller peak, corresponding to the size of primary pUL37, was found in the 58- to 64-kDa range. In contrast, PNGase treatment of gpUL37 shifted the sharp peak at 80 to 97 kDa down to molecular mass ranges of 64 to 70 and 58 to 64 kDa. This result is consistent with the conversion of gpUL37 by PNGase to multiple partially deglycosylated species. Taken together, our data corroborate our previous finding that gpUL37 is indeed glycosylated in HCMV-infected cells, specifically, via N linkage, and are consistent with passage of gpUL37 into the Golgi apparatus.

To determine if gpUL37 immunoprecipitated from HCMV-infected cells retained core glycosylations, it was treated with endo H (Fig. 6B), which cleaves high-mannose and certain hybrid, but not complex-type, oligosaccharide chains (15). gpUL37 did not show significant sensitivity to endo H digestion; however, some sensitivity was observed (lanes 3 and 4). These results indicate that within 2 h of synthesis, gpUL37 acquires both core glycosylation in the ER and more-complex-type sugar modifications in the Golgi network.

Specificity of Ab1601 for the carboxy terminus of gpUL37. To determine the location of gpUL37 in cells, we made use of Ab1601 (Fig. 1). This antibody is specific for the putative cytoplasmic tail of gpUL37 (aa 473 to 487). Its specificity for this domain was demonstrated by its selective reactivity with in vitro-synthesized pUL37 (Fig. 7). Primary wt pUL37 (Fig. 7A, lane 1), pUL37 lacking its carboxy terminus (lane 2), a mutant lacking aa 200 to 487 (lane 3), and mutant pUL37 lacking aa 200 to 326 but retaining the UL37 carboxy terminus (lane 4) were synthesized in vitro and then reacted with Ab1601 (Fig. 7B). Ab1601 specifically immunoprecipitated wt UL37 protein and mutant UL37 protein Δaa200-326, which retains the pUL37 carboxy-terminal sequences (lanes 1 and 4, respectively). However, it did not immunoprecipitate the product of the *Nru*I-cleaved DNA template or mutant pUL37, both lacking the pUL37 carboxy terminus (lanes 2 and 3, respectively). Traces of immunoprecipitated protein were observed in the lane containing the *Nru*I template and were demonstrated, by migration, to be wt pUL37 encoded by residual uncleaved wt UL37 template DNA. These results establish the specificity of Ab1601 for its epitope (aa 473 to 487), against which it was generated and which is the carboxy terminus of gpUL37.

Subcellular trafficking of gpUL37. The biochemical data shown above suggest that gpUL37 traffics through the Golgi network. To demonstrate this translocation directly, we exam-

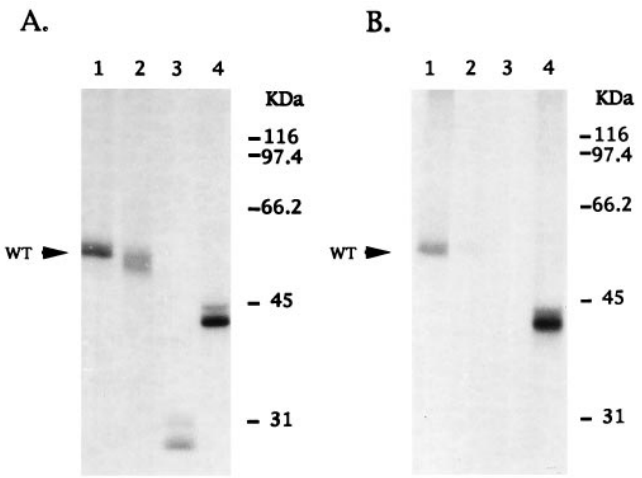

FIG. 7. Specificity of Ab1601 for the aa 473 to 487 epitope. (A) Intact p591 DNA containing the wt UL37 cDNA (lane 1) or *Nru*I-cleaved p591 DNA (lane 2) was transcribed, and the RNA was translated in vitro. Alternatively, mutant pU37 protein was produced in vitro from p590 (lane 3) or p589 (lane 4). An aliquot of each reaction mixture was analyzed by SDS-PAGE. (B) Aliquots of the reaction mixtures shown in panel A were immunoprecipitated with Ab1601. Lanes: 1, wt pUL37; 2, pUL37 lacking aa 461 to 487; 3, mutant pUL37 (aa1-199); 4, mutant gpUL37 (Δaa200-326). The arrows indicate the migration position of wt pUL37.

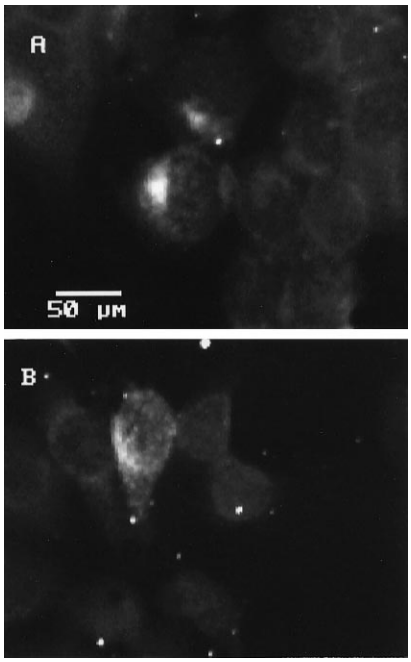

FIG. 8. Comparison of subcellular locations of two HCMV glycoproteins, gpUL37 and gB. HeLa cells were transfected with expression vectors for gpUL37 and gB and stained with Ab1601 (A) or CH28-2 (B). Each primary antibody was reacted with the corresponding secondary FITC-labeled antibody. The stained cells were analyzed by confocal microscopy.

ined gpUL37 localization in human cells by an indirect immunofluorescence assay with Ab1601. HeLa cells were transfected with an expression vector encoding wt gpUL37 or, as a control, with an expression vector which encodes the major IE1 protein and stained with either preimmune or immune Ab1601, followed by a secondary FITC-labeled goat anti-rabbit IgG antibody (1). Ab1601 specifically detected the production of gpUL37 in cells transfected with its expression vector. No signal was observed in HeLa cells expressing gpUL37 and stained with control preimmune serum. This result shows the specificity of the production of an antibody against gpUL37. Control cells expressing IE1 and reacted with Ab1601 also did not show any staining. These results demonstrate the specificity of Ab1601 and its lack of cross-reactivity with another HCMV IE protein in transfected human cells by indirect immunofluorescence assay.

The majority of the cells expressing gpUL37 did so at very high abundance, as judged by the intensity of the signal. As a result, resolution of the morphology of the cells with high levels of protein expression was compromised by the strong signal. This aspect is likely due to the high level of accumulation of the protein in the ER (see below), which is a common phenomenon during transient expression of proteins (24). Therefore, cells with moderate levels of expression and clear cellular morphology were selected for further, more refined analysis by confocal microscopy. The pattern of gpUL37 staining in transfected cells was a granular perinuclear type of staining (Fig. 8A) similar to the staining pattern of another HCMV glycoprotein, gB (Fig. 8B). gB is an HCMV structural glycoprotein and shares with gpUL37 the features of a type I integral membrane protein (12). gB is known to undergo slow folding, N-glycosylation, and oligomerization in the rough ER and the Golgi network (4, 5). These results suggest that gpUL37 traffics through the ER and the Golgi network, similarly to HCMV gB.

To determine directly whether gpUL37 traffics through the ER and the Golgi network as suggested by the previous results, double labeling for gpUL37 and either an ER-resident protein, PDI (22), or a Golgi marker was performed. The stained cells were analyzed by confocal microscopy (Fig. 9). HeLa cells transfected with a gpUL37 expression vector were stained with rabbit Ab1601 and with either a mouse anti-PDI (Fig. 9A to C) or a mouse anti-Golgi zone (Fig. 9D to F) antibody. The presence of each antigen was distinguished by secondary antibodies labeled with FITC–anti-rabbit IgG or with Texas Red–anti-mouse IgG. Colocalization was determined by image overlap where close proximity of the green signal to the red signal resulted in a yellow signal. As indicated by the yellow areas, gpUL37 significantly colocalized with an ER-resident protein, as well as with a Golgi antigen, in nonpermissive HeLa cells (Fig. 9). Similar results were obtained when gpUL37 was colocalized with ER and Golgi markers in permissive HFF cells transfected with a UL37 expression clone (1). These results are consistent with the previous biochemical data and with the prediction that gpUL37 traffics through the rough ER and the Golgi network.

Membrane association of gpUL37 produced in HCMV-infected HFF cells. gpUL37 is predicted to be an integral membrane protein, a suggestion corroborated by our in vitro analysis of gpUL37. To determine if gpUL37 is associated with cellular membranes in HCMV-infected HFF cells, we fractionated labeled uninfected and infected cells into their nuclear, cytosolic, and membrane fractions. Each fraction of uninfected and HCMV-infected cells was immunoprecipitated with Ab1525 and analyzed by SDS-PAGE (Fig. 10A). The immune Ab1525 used for this experiment was reacted with purified fractions from unlabeled, uninfected HFF cell lysates prior to its use for immunoprecipitation. Processed gpUL37 (83 to 85 kDa) was immunoprecipitated from total HCMV-infected cell lysates (lane 2). The processed gpUL37 83- to 85-kDa species and a 65- to 68-kDa species, similar in size to the primary

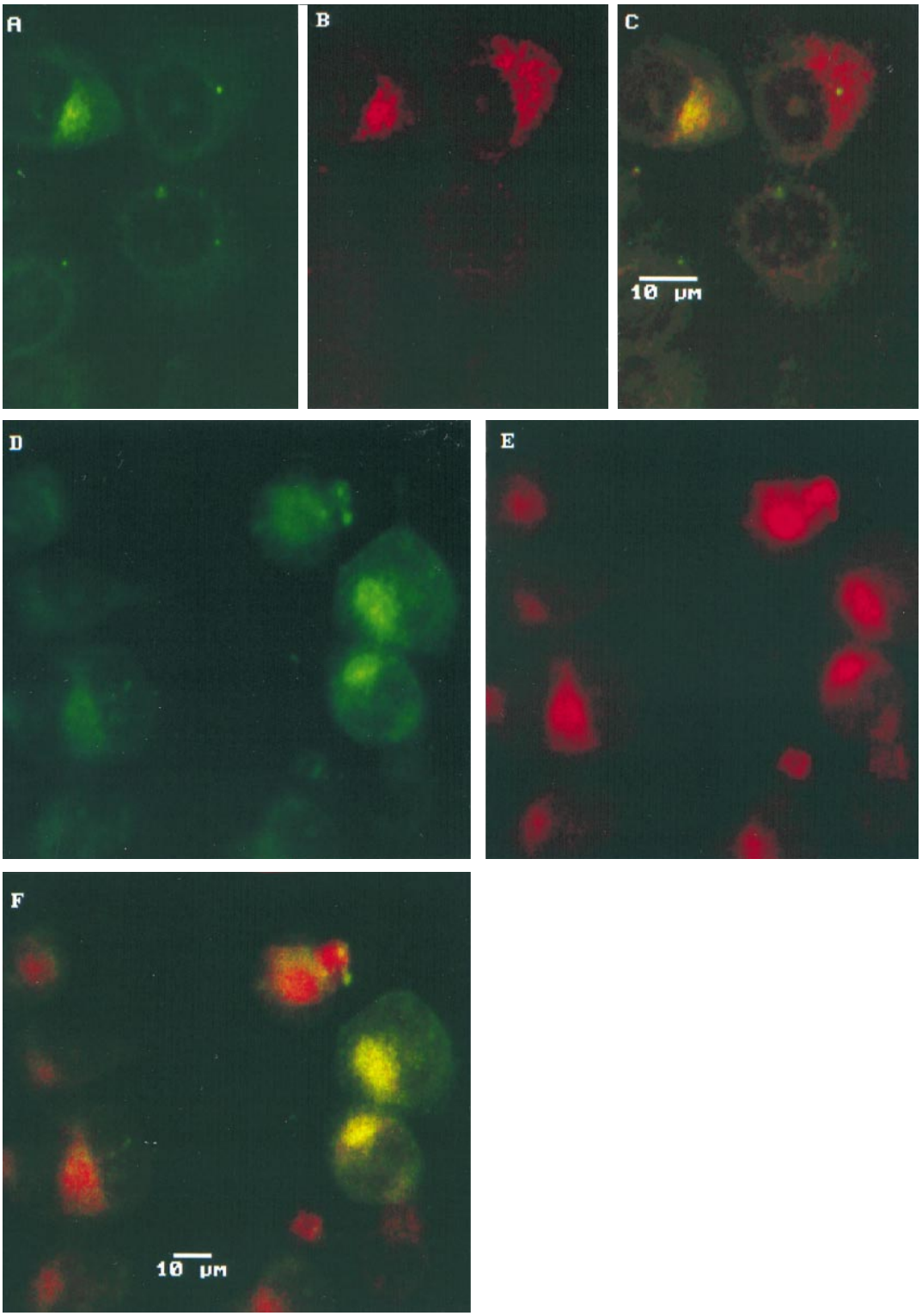

FIG. 9. gpUL37 traffics through the ER and the Golgi network. HeLa cells were transfected with a gpUL37 expression vector and reacted with rabbit Ab1601 (A to F) and either mouse anti-PDI (A to C) or mouse anti-Golgi zone (D to F) antibodies. The cells were then stained with secondary antibodies (FITC–anti-rabbit IgG and Texas Red–anti-mouse IgG) and examined by confocal microscopy for both signals simultaneously. Shown are FITC (A and D), Texas Red (B and E), and both (D and F) signals.

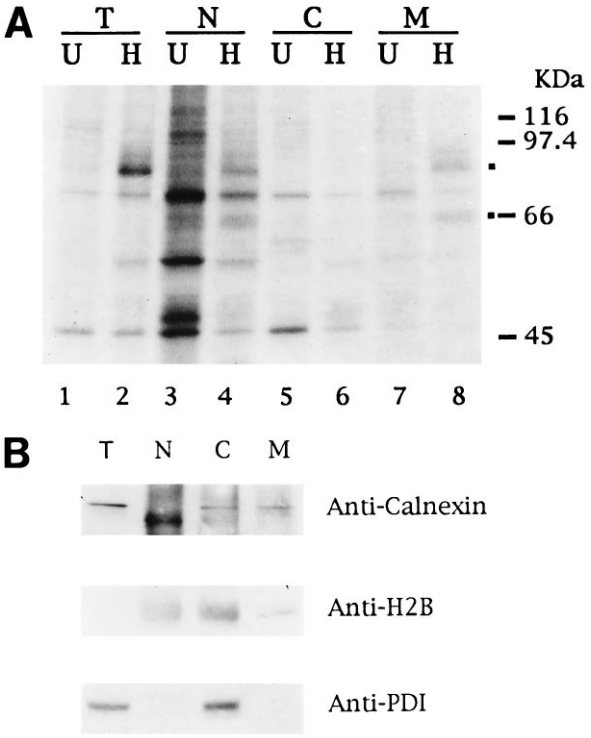

FIG. 10. gpUL37 is an integral membrane protein in HCMV-infected HFF cells. (A) Proteins from uninfected (U) (lanes 1, 3, 5, and 7) and HCMV-infected (H) (lanes 2, 4, 5, and 8) cells were metabolically labeled and were unfractionated (T) (lanes 1 and 2) or fractionated into nuclear (N) (lanes 3 and 4), cytosolic (C) (lanes 5 and 6), and membrane (M) (lanes 7 and 8) fractions. Proteins in the samples were immunoprecipitated with Ab1525 after the antibody was blocked with unlabeled cellular fractions. The immune complexes were analyzed by SDS-PAGE. The migration positions of molecular mass standards are indicated. The gpUL37 species are indicated by small squares. (B) Total (T) and fractionated proteins from uninfected cells used for panel A were analyzed for the presence of cellular marker proteins. Total lysates (T, 25 μg) and nuclear (N), cytosolic (C), and membrane (M) (10 μg) fractions were separated by SDS–15% PAGE and transferred to a membrane. The blotted proteins were reacted with rabbit anti-calnexin (1:700), rabbit anti-histone H2B (1:500), or mouse anti-PDI (1:10,000) antibodies. The proteins were reacted with the corresponding secondary antibodies (peroxidase-labeled anti-rabbit IgG or anti-mouse IgG) and a chemiluminescence substrate. Different film exposure times were used to produce an optimal signal.

translation product and to the deglycosylated pUL37 species (Fig. 2 and 5), were present primarily in the membrane fraction of HCMV-infected cells (lane 8). None of the UL37 species were detected in the cytosolic fraction (lanes 5 and 6). The presence or absence of gpUL37 in the nuclear fraction (lane 4) was unclear because of the high background level present in the uninfected nuclear fraction (lane 3), despite our attempts to decrease the background by blocking with the unlabeled, uninfected nuclear fraction. This fact and the possibility that the nuclear fraction might have residual outer membrane proteins (from the ER membrane) dissuaded us from making a conclusive statement about the presence of gpUL37 in the nuclear fraction. Nevertheless, this experiment conclusively established that the gpUL37 synthesized in HCMV-infected cells is an integral membrane protein.

The effectiveness of our fractionation protocol was determined by Western blot analysis (Fig. 10B). Aliquots of total uninfected-cell lysates and each uninfected-cell fraction used in Fig. 10A were blotted and reacted with an antibody against a protein resident in each fraction. The marker proteins selected for this analysis were calnexin, an integral ER membrane protein (51); histone H2B, a nuclear protein; and PDI, a soluble ER-resident protein (22). The major calnexin species (104 kDa) seen in the total cellular lysate was present in both cytosolic and membrane fractions but was absent from the nuclear fraction. A smaller reactive species (91 kDa), possibly an alternatively processed form of calnexin, was found in the nuclear fraction. The nuclear protein histone H2B was present, as expected, in both the nuclear and cytosolic fractions, its site of synthesis. A small amount of histone H2B was observed in the membrane fraction. Nevertheless, the soluble protein PDI was present exclusively in the cytosolic fraction and absent from the nuclear and membrane fractions. The results indicate the preponderance of marker proteins in their correct fractions. Significantly for the localization of gpUL37, soluble cytosolic proteins were not found in the membrane and nuclear fractions used in these experiments.

DISCUSSION

These studies demonstrate that the regulatory product of the HCMV UL37 IE gene is an integral membrane N-glycoprotein. These features are uncommon for regulatory proteins of herpesviruses. Nevertheless, several of the gpUL37 structural motifs are conserved in another human betaherpesvirus, HHV-6 (18, 36). In addition to the conservation of UL37 exon 3 motifs, the UL36 and IE2 protein motifs are also conserved in HHV-6 (18, 33, 36). Importantly, the regulatory activities of the HCMV IE proteins are conserved in the HHV-6 homologs as well (33, 36). These conserved IE products and functions appear to be betaherpesvirus specific and may help determine important biological properties of these human herpesviruses.

The structure and processing of gpUL37 were predicted from its ORF (7, 26). The predicted UL37 structure is similar to that of the HCMV structural glycoprotein gB, which is also a type I protein and also has 17 N-linked glycosylation sites (12). Indeed, its hydrophobic signal sequence is recognized, permitting translocation of the UL37 protein into membrane vesicles. Such translocation is the major step through which the pathway taken by membrane and secretory proteins diverges from that taken by soluble proteins. Once translocated into microsomes in vitro, the UL37 protein remains a type I integral membrane protein, as the majority of its sequences are protected within the vesicular lumen. The UL37 transmembrane domain and cytoplasmic tail have clear physical demarcations, as 27 hydrophobic amino acids are followed immediately by a series of strongly charged amino acids (RRDLLED. . .). It is therefore unlikely that the length of the cytoplasmic tail could change much from that predicted.

gpUL37 was found to be an integral membrane protein in HCMV-infected cells. Low concentrations of Triton X-100 did not remove gpUL37 from the membrane fraction. This treatment is known to remove peripherally associated proteins from the membrane (45). The use of PDI as a marker for the cytosolic fraction verified the efficacy of Triton X-100 treatment for removal of nonintegral membrane proteins from the membrane fraction. PDI, which is loosely associated with the luminal side of the ER membrane (22), was present exclusively in the soluble cytosolic fraction. In addition, IE1, which is known to be membrane associated (37) but is not an integral membrane protein, was present almost completely in the soluble cytosolic fraction and partially in the nuclear fraction (1).

Similar to the synthetic pathway of other HCMV glycoproteins (4–6), the initial steps in the synthesis of gpUL37 in vitro predictably involve addition of carbohydrate side chains to the N-linked sites of pUL37. This conclusion was experimentally verified by treatment of in vitro-synthesized gpUL37 with PNGase

(1). This enzyme cleaves between *N*-acetylglycosamine and asparagine of the polypeptide chain (15). These results indicate that the N-linked glycosylation signals of pUL37 are recognized; that the posttranslationally modified UL37 protein is derived from the primary translation product, pUL37; and that the in vitro-glycosylated UL37 product is cotranslationally and posttranslationally modified by the N-glycosylation processing pathway.

gpUL37 immunoprecipitated from HCMV-infected cells consistently migrated as a diffuse band in SDS-PAGE, a feature characteristic of glycosylated proteins. In HCMV-infected HFF cells, N-glycosylation of gpUL37 was verified by tunicamycin treatment and by enzymatic digestion. Tunicamycin treatment of HCMV-infected cells increased its rate of migration. Tunicamycin inhibits the attachment of *N*-acetylglucosamine to a lipid carrier (dolichol phosphate), and this inhibitor, in turn, blocks core N-glycosylation in eukaryotic cells (50). Consistent with the tunicamycin results, gpUL37 produced in HCMV-infected HFF cells is sensitive to PNGase digestion. The positive controls for the glycosidase digestion were α1 acid glycoprotein and the antibody molecules used for immunoprecipitation. These proteins were deglycosylated as anticipated (1). However, gpUL37 has 17 N-linked glycosylation sites. Partial digestion of gpUL37 by glycosidases would predictably result in a shift of the mature form to multiple forms having one or various sites deglycosylated. In fact, we observed a displacement of radioactivity from the sharp peak of processed gpUL37 at 80 to 97 kDa to a broad peak of intermediate forms (58 to 70 kDa). In vitro-synthesized gpUL37 is deglycosylated by PNGase to the single primary species (1); however, in the cell lysate context, the deglycosylation reaction did not go to completion, resulting in multiple migrating forms. Since the detection level of film is low, we used a quantitative measurement to verify this shift in peaks of labeled protein. Degradation was not responsible for this result, as several other minor background bands and antibody species in the same reaction mixture did not show any apparent degradation following PNGase treatment (1).

N-linked oligosaccharides in gpUL37 appear to make up about 18% of its molecular mass. In contrast, N-linked oligosaccharides make up about 33% of the molecular mass of gB (5). This difference may reflect differences in the further processing of the oligosaccharide moieties or in the number of N-glycosylation sites modified in each protein.

The size of the presumptive unglycosylated UL37 protein obtained by tunicamycin treatment did not correspond precisely to that of the primary pUL37 synthesized in vitro. This result suggests either that the presumptive unglycosylated pUL37 from tunicamycin-treated cells was underglycosylated (rather than unglycosylated) or, alternatively, that gpUL37 has an additional modification, possibly O-glycosylation or phosphorylation. In the UL37 ORF (7, 26), there are multiple potential sites for either O-glycosylation or phosphorylation. Consistent with the possibility of O-glycosylation are the modification of gB by O-glycosylation (5) and the presence of low levels of N-linked residues and high levels of O-linked oligosaccharides in the HCMV glycoprotein cII complex (25).

In addition to processed gpUL37 (83 to 85 kDa), we observed unprocessed pUL37 and a larger UL37 species in the in vitro translation reaction mixtures. The presence of remaining unprocessed pUL37 probably resulted from saturation of the microsomal enzyme machinery. The 120-kDa species observed following in vitro synthesis contains pUL37, as it is synthesized following addition of the UL37 ORF to the in vitro lysates and is specifically immunoprecipitated by UL37-specific antibodies (1). The persistence of the 120-kDa species in experiments with microsomes pretreated with Nikkol indicates that this is an unglycosylated species. Consistent with this is the finding that the 120-kDa species is resistant to PNGase treatment (1) and that this species is degraded by protease digestion. The former result indicates that it is unmodified by N-glycosylation, and the latter indicates that the 120-kDa species is not membrane associated and is located outside of the microsomal vesicles, as expected for an unglycosylated species. Its migration is consistent with its being a homodimer of pUL37 whose monomeric form has an apparent molecular mass of 60 kDa. Specific interactions between transmembrane helices are major determinants in the oligomerization of integral membrane proteins (31). The 120-kDa species is, however, unlikely to be the native form of gpUL37, as it is not membrane associated and is unglycosylated. gpUL37 produced in HCMV-infected cells is membrane associated and efficiently glycosylated within 2 h of its synthesis.

We did not detect the larger (120-kDa) species observed in vitro in HCMV-infected cells. This finding may result from the following. Unglycosylated pUL37 is present in much smaller amounts in total HCMV-infected cell extracts than in in vitro lysates. Unglycosylated pUL37 is rapidly processed in HCMV-infected cells and is detectable only in the purified membrane fraction of HCMV-infected cells. The low abundance and membrane association of pUL37 in HCMV-infected cells are distinguishing features from the in vitro-synthesized 60- and 120-kDa species. With these differences, it is not surprising that the 120-kDa species was not detected in HCMV-infected cells. Moreover, the 120-kDa species was found in denaturing gels, that is, in SDS-PAGE gels following stringent reducing and denaturation conditions. Although IVT/T reactions are done under reducing conditions, SDS-resistant aggregates and oligomer have been shown to occur with a variety of viral proteins (13, 14, 20, 34, 43). Taken together, our results suggest that the larger UL37 species observed in vitro may result from the artificially high concentration of soluble pUL37 present in the in vitro lysates.

The primary UL37 translation product was not detected in total lysates of HCMV-infected cells after a 2-h labeling period. However, under similar conditions, a 65- to 68-kDa UL37 species was detected in the membrane fraction from HCMV-infected cells. This detection probably resulted from the increased concentration of membrane proteins in this fraction and the consequent increased sensitivity for gpUL37 detection. The migration of this smaller species is consistent with the migration of UL37 products following tunicamycin and glycosidase treatments, suggesting that this species is an incompletely processed form of gpUL37. Nevertheless, within the 2-h labeling period, gpUL37 acquired N-linked oligosaccharides which were further processed, making them resistant to endo F and endo H digestion. These results indicate the movement of gpUL37 into the Golgi apparatus. In contrast, gB is retained in the ER for an unusually prolonged time (half-maximal time of approximately 160 min [6]). Moreover, the precursor form of gB is not depleted even following a prolonged chase period of 24 h (4). Taken together, our results suggest that the posttranslational processing of pUL37 is more rapid than that of gB.

The trafficking of gpUL37 through the ER and Golgi was directly demonstrated by confocal microscopy. This trafficking is consistent with the modification by N-glycosylation which results in gpUL37's resistance to endo F and endo H. The same pattern of subcellular location for gpUL37 is optically similar to that of gB and nonoverlapping with that of another HCMV IE regulatory protein, IE1 (54). Although gpUL37 processing and location are unusual for herpesvirus regulatory proteins, it appears that these events are intimately associated with

gpUL37 function. The N-glycosylated domain is required for gpUL37's regulatory activity; a mutant gpUL37 which lacks the first 11 N-glycosylation sites lacks the ability to transactivate the human *hsp70* promoter (55).

Although they share multiple features, such as location, modification, and structural features, gpUL37 and gB are readily distinguishable. First, gpUL37 is the product of an IE gene, whereas gB is synthesized at later times of HCMV infection (19, 42). Second, gpUL37 has nuclear regulatory activity, whereas gB does not (11, 54). Finally, gB is an abundant protein present on virions and the cell surface (19), whereas the abundance of gpUL37 is exceedingly low. To detect radiolabeled gpUL37 in HCMV-infected cells, it was necessary to immunoprecipitate large quantities of radiolabeled lysates even in cells pretreated with protein synthesis inhibitors. Under these conditions, the abundance of gpUL37 is even lower than that of one of the products of the major IE locus (IE1), which was readily detected in total HCMV-infected cell lysates. The abundance of gpUL37 in untreated, HCMV-infected cells was, predictably, lower yet. Thus, similar to its encoding RNA, which is tightly regulated (46–48), gpUL37 is a relatively low-abundance IE product. We are currently examining the structural requirements of gpUL37 for its regulatory activity.

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