Human Cytomegalovirus UL130 Protein Promotes Endothelial Cell Infection through a Producer Cell Modification of the Virion†

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Human cytomegalovirus (HCMV) growth in endothelial cells (EC) requires the expression of the UL131A-128 locus proteins. In this study, the UL130 protein (pUL130), the product of the largest gene of the locus, is shown to be a luminal glycoprotein that is inefficiently secreted from infected cells but is incorporated into the virion envelope as a Golgi-matured form. To investigate the mechanism of the UL130-mediated promotion of viral growth in EC, we performed a complementation analysis of a UL130 mutant strain. To provide UL130 *in trans* to viral infections, we constructed human embryonic lung fibroblast (HELFL) and human umbilical vein endothelial cell (HUVEC) derivative cell lines that express UL130 via a retroviral vector. When the UL130-negative virus was grown in UL130-complementing HELFL, the infectivity of progeny virions for HUVEC was restored to the wild-type level. In contrast, the infectivity of the UL130-negative virus for UL130-complementing HUVEC was low and similar to that of the same virus infecting control noncomplementing HUVEC. The UL130-negative virus, regardless of whether or not it had been complemented in the prior cycle, could form plaques only on UL130-complementing HUVEC, not control HUVEC. Because (i) both wild-type and UL130-transcomplemented virions maintained their infectivity for HUVEC after purification, (ii) UL130 failed to complement *in trans* the UL130-negative virus when it was synthesized in a cell separate from the one that produced the virions, and (iii) pUL130 is a virion protein, models are favored in which pUL130 acquisition in the producer cell renders HCMV virions competent for a subsequent infection of EC.

Human cytomegalovirus (HCMV) is a betaherpesvirus that establishes life-long, subclinical infections ubiquitously in human populations (5). HCMV causes serious morbidity in settings of immune system immaturity or depression: it is the leading viral cause of defects at birth and generates a potentially life-threatening disease in immunocompromised patients. In patients with HCMV disease, the virus can be demonstrated in a variety of cells, including hematopoietic cells (monocytes-macrophages, dendritic cells, and neutrophils), endothelial cells (EC), epithelial cells, fibroblasts, neurons, smooth muscle cells, and hepatocytes (5, 38). Much recent work has focused on HCMV infections of EC, for several reasons: (i) arterial endothelia, along with CD34⁺ myeloid progenitors, have been proposed as sites of HCMV persistence and latency (20); (ii) a bidirectional transmission of HCMV between EC and leukocytes can be demonstrated *in vitro* and may reflect a mechanism of dissemination *in vivo* (11, 14, 34, 44); (iii) circulating giant EC may similarly contribute directly to dissemination (32); (iv) HCMV infections of the uterine microvasculature and cytotrophoblasts may underlie mother-to-fetus transmission and compromise the placental trophic function in affected pregnancies (25, 47); and (v) HCMV may play a role in atherogenesis, postangioplasty restenosis, post-

transplantation endothelialitis, and other vasculopathies, although this role is disputed and contrasting pathogenic mechanisms have been proposed (2, 19, 37, 46).

All HCMV clinical isolates are initially able to replicate in both EC and fibroblasts. However, passaging in fibroblasts consistently results in a loss of EC tropism. Studies of HCMV strains that have retained/lost their original EC-tropic phenotype support the conclusion that EC tropism relies on multiple viral genes (3, 40) and that non-EC-tropic strains are impaired in their ability to translocate the viral DNA to the nucleus (3, 4, 39, 41). Thus, the loss of EC tropism is typically due to a cytoplasmic blockade rather than to a failure to either bind to or penetrate into EC. Reports suggesting that the vascular bed of origin may also influence HCMV-EC interactions have provided conflicting findings. While lytic infections of cultured venous and microvascular EC are well established, a report about a noncytopathic persistence of HCMV in human arterial EC has raised the possibility that the continuous clearance of intracellular virions makes possible a long-term productive infection in that EC type (9). However, work by others (22, 23) supported the opposite view, that interstrain differences in the cytopathic potential, rather than the EC source, account for different outcomes of infections. This again indicated that virus-encoded products may fine-tune EC infection mechanisms as well as the potential to cause direct endothelial injury.

The search for the genetic determinants of HCMV EC tropism commenced with the advent of bacterial artificial chromosome (BAC) cloning and bacterial manipulation of cytomegalovirus genomes. A first insight was gained with the related murine cytomegalovirus (MCMV) model. A random

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the virions in order to affect the EC phenotype, suggests that pUL130 functions as a viral envelope component which is needed in the initial stages of EC infection.

MATERIALS AND METHODS

Cells. Human embryonic lung fibroblasts (HELFL) were cultured in minimum essential medium (MEM; Gibco) containing 10% fetal bovine serum (FBS). HeLa and HEK-293gagpol cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% FBS. Human umbilical vein endothelial cells (HUVEC) were maintained in EGM-2 medium (Clonetics) and used at passage 2 to 5 after isolation.

Viruses and infections. The HCMV VR1814 clinical isolate (35) and the Towne laboratory strain were used. All infections were performed in 2% FBS-containing medium by incubating confluent HELFL or HUVEC monolayers at 37°C for 1 h with the indicated HCMV strain and then replacing the incubation mixture with fresh medium. Virus stocks were produced by infecting confluent monolayers at a multiplicity of infection (MOI) of 0.1; 3 days after the cytopathic effect extended to >90% of the cells, viruses were harvested by sonication of the cells (cell-free virus) or by collecting the culture medium and clarifying it by centrifugation at $3,000 \times g$ for 1 h at 4°C (released virus). In some cases, released viral particles were sedimented by centrifugation at $23,500 \times g$ for 55 min at 4°C and then further purified on a sorbitol gradient as previously described (10), except that a 40–55–70% step gradient was used. For analyses of the virion composition, the virions were further centrifuged at $110,000 \times g$ for 2 h through a 10 to 50% Nycodenz (Sigma) gradient prepared in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA. The virion purity was confirmed by controlling in an immunoblot the absence of markers of the endoplasmic reticulum (ER) (GRP78-BiP) and the Golgi apparatus (p58-Golgi). Unless stated otherwise, VR1814 was cultured in HUVEC. In transcomplementation experiments, retrovirus-transduced HELFL (see below) were infected with HCMV strains, and the viruses were harvested and purified as described above. Virus stocks were stored at –70°C in 10% sorbitol. After the stocks were thawed, viral titers were measured in each aliquot as infectious units/ml in HELFL (HELFL IU) or HUVEC (HUVEC IU) by infecting HELFL/HUVEC seeded in 96-well plates with serial dilutions of virus and counting the pp72/86-positive cells at 24 h postinfection (hpi). For single-cycle output and plaque assays, confluent monolayers were infected as described above, and infection mixtures were replaced with fresh medium containing 160 µg/ml of human gamma globulin (Sigma).

To inhibit N-linked glycosylation, we added 5 µg/ml tunicamycin (Sigma) to the medium of infected cells at 4 days postinfection (dpi). After another day of infection, the cells were harvested for analysis. In deglycosylation experiments, infected cells were washed twice with phosphate-buffered saline (PBS) and lysed in *N*-glycosidase F (endo F) buffer (20 mM phosphate, pH 7.2, 10 mM EDTA, 0.1% sodium dodecyl sulfate [SDS]). Alternatively, the cells were washed with 150 mM NaCl and lysed in endoglycosidase H (endo H) buffer (50 mM citrate buffer, pH 5.5, 0.1% SDS). Cell lysates were heated for 3 min at 96°C and incubated for 3 h at 37°C after the addition of 0.2 U/µl endo F or 0.2 mU/µl endo H (Roche). Deglycosylation reaction mixtures were mixed with 2 volumes of 2× Laemmli sample buffer and analyzed by Western blotting (WB).

Plasmids. PCR products were generated with *Pfu* polymerase (Promega) and sequenced after cloning to rule out unwanted mutations. The UL130fs and UL130rev genes were amplified from Towne and Towne revertant DNAs, respectively, with primers 1 and 3 (the sequences of the oligonucleotides cited in this section are shown in Table S1 of the supplemental material). All other amplification reactions used VR1814 as a template. The wild-type UL130 gene was amplified by using primers 1 and 2, and the deletion mutant UL130Δ2–25 was amplified by using primers 4 and 2.

The UL130 mutants N85A and N201A were obtained by PCR-directed mutagenesis. Each mutant was created by amplifying contiguous tracts of the gene by using the primer sets 1–6 and 5–2 (N85A) and 1–10 and 9–2 (N201A). Each couple of amplimers was then combined into a single, full-length UL130 variant by a second PCR round using the external primers 1 and 2 and exploiting the central overlap created by the internal primers (5–6 and 9–10, respectively) in the former round. An N118A mutant was similarly produced by the separate amplification of two UL130 tracts (primer sets 1–8 and 7–2); in this case, the fusion of the amplimers into a single gene was obtained by direct ligation of a PstI site introduced close to the mutation by silent mutagenesis with the internal primers 7 and 8 (see Table S1 in the supplemental material).

All PCR products were cloned into the pCDNA3.1(+) expression vector (Invitrogen), which allows for both expression in transfected eukaryotic cells and T7 bacteriophage RNA polymerase-driven transcription in vitro. The UL130

amplimer was also cloned into the pLNCX2 retroviral vector (Clontech) to create the pLNUL130 plasmid.

In vitro expression, cell transfection, and retrovirus-mediated transduction. Coupled in vitro transcription and translation of the pCDNA3.1(+)-UL130 and pCDNA3.1(+)-UL130Δ2–25 plasmids in rabbit reticulocyte lysates were carried out in the presence or absence of canine pancreatic microsomal membranes (Promega), according to the manufacturer's instructions. For the separation of microsome-associated proteins from cytosolic proteins, 35 µl of the translation mixture was centrifuged at $10,000 \times g$ for 15 min at 4°C through a 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 250 mM sucrose cushion (0.5 ml). For enzymatic deglycosylation, the pellet was dissolved in 40 µl of endo F buffer and the mixture was treated with endo F as described above. Balanced amounts of crude or fractionated translation reactions were resolved by SDS-polyacrylamide gel electrophoresis, and radioactive protein bands were visualized by phosphorimaging.

HeLa cells were transfected with UL130 variants cloned into pCDNA3.1(+) by use of the Lipofectamine Plus reagent (Invitrogen) and were harvested at 48 h posttransfection. HUVEC were transfected by using Amaxa nucleofector and HUVEC-specific nucleofection medium according to the manufacturer's instructions. For proteolysis inhibition experiments, cells were treated/mock treated for 24 h with 10 µM lactacystin (Calbiochem) or 100 µM pepstatin plus 50 µM leupeptin (Sigma). For time-decay experiments, 50 µg/ml cycloheximide (Sigma) was added to the medium of the transfectants, and the cultures were incubated for 0.5 to 5 h. Cells were lysed in 2× Laemmli sample buffer (4,000 cells/µl) for immunoblot analysis.

Pseudotyped retroviral particles were produced by calcium phosphate transfection of the HEK-293gagpol cell line with the plasmid pLNUL130 (or a pLNCX2 void vector) along with pVSV-G, which encodes vesicular stomatitis virus glycoprotein G (42). At 48 h posttransfection, the culture medium was harvested and used to transduce subconfluent HELFL or HUVEC monolayers. Cells expressing the integrated provirus were selected with 400 µg/ml G418 (Sigma) in the appropriate culture medium.

Antibodies, immunoblots, and immunocytochemistry. Anti-pUL130 antisera were obtained by DNA immunization of 6-week-old female BALB/c mice. In preparation for intramuscular injection, the DNA was purified with a Midi extraction kit (Promega) and dissolved in endotoxin-free PBS (Sigma) at 1 mg/ml. Mouse tibialis anterior muscles were pretreated bilaterally with 100 µl of *Naja nigricollis* snake venom cardiotoxin (10 µM in PBS; Latoxan, France). Five days later, 50 µl of pCDNA3.1(+)-UL130 DNA was injected into the regenerating muscle. Sera were collected and tested at 4 weeks postimmunization. Other primary antibodies for immunostaining were the anti-HCMV pp72/86 monoclonal antibody (MAb) clone 5D2, the anti-HCMV pp65 MAb clone 4C1 (33), the anti-major capsid protein (MCP) MAb clone 28.4 (24), and the anti-cyclin B1 MAb clone GNS1 (Santa Cruz).

Immunoblot analysis was performed according to standard procedures. Balanced amounts of protein samples were separated by SDS-polyacrylamide gel electrophoresis, blotted onto Protran-83 nitrocellulose membranes (Schleicher & Schuell), and incubated with the indicated primary antibodies. Chemiluminescent signals were developed with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Bio-Rad) and the Super Signal West Dura substrate (Pierce).

For immunocytochemistry, cells in 24-well plates were fixed with methanol at –20°C for 10 min, incubated with 0.5 µg/ml MAb 5D2 in PBS, and stained by use of a Vectastain ABC kit (Vector Laboratories) and 3-amino-9-ethylcarbazole (Sigma). Representative fields of cytological images were acquired with an Olympus IX71 phase-contrast inverted microscope equipped with a charge-coupled device camera (Roper Scientific Photometrics) and operated with the MetaMorph imaging application (Universal Imaging Corporation).

Purification of secreted pUL130. HeLa cells (2.7×10^7) were transfected with pCDNA3.1(+)-UL130 and cultured for 48 h. The culture medium (30 ml) was clarified and dialyzed overnight at 4°C against 50 mM Na⁺-HEPES, pH 7.5, 0.1 mM EDTA, and complete EDTA-free protease inhibitor mix (Roche) (buffer A). Secreted pUL130 was captured from the dialyzed supernatant on a 0.4-ml P11 phosphocellulose column (Whatman) which was previously equilibrated in buffer A. After washing of the column with 120 ml of buffer A, 30 µl of column resin was boiled in 40 µl of 2× Laemmli buffer and analyzed by WB. Alternatively, the same volume of resin was resuspended in 30 µl of endo H or endo F buffer and deglycosylated with the corresponding enzyme, as described above, before immunoblot analysis.

RESULTS

The UL130 product is a triglycosylated protein and is largely retained intracellularly. UL130 is the central and the

FIG. 2. The UL130 protein is translocated into the ER lumen and N-glycosylated, and its signal sequence is shortened in vitro. The UL130 (VR1814) gene or a deletion mutant lacking amino acids 2 to 25 ($\Delta 2-25$) was subjected to coupled in vitro transcription-translation in rabbit reticulocyte lysates, supplemented or not with canine pancreatic microsomes for cotranslational translocation-glycosylation. Abbreviations: endo F, *N*-glycosidase F treatment; cfg, centrifugation of the translation mixture through a buffered sucrose cushion at $10,000 \times g$; P10, centrifugation pellet; S10, centrifugation supernatant. The cartoon colors and symbols are the same as those described in the legend to Fig. 1. *, N-linked glycosylation sites.

largest (214 codons) gene of the UL131A-128 locus, and it is the only one that is not interrupted by introns (Fig. 1A and B) (1, 16). A conceptual translation of the gene predicts a long (25 amino acids) signal sequence that precedes a hydrophilic protein containing two potential N-linked glycosylation sites (Asn85 and Asn118) within a putative chemokine domain (amino acids 46 to 120) (29) and an additional glycosylation site (Asn201) close to the end of a unique C-terminal region (Fig. 1B). A UL130 orthologue is found in all sequenced members of the primate (but not rodent) cytomegaloviruses.

The expression of UL130 in a rabbit reticulocyte system, with or without added canine pancreatic microsomes to mimic endoplasmic reticulum (ER)-associated translation, translocation, and glycosylation, was utilized to verify these predictions (Fig. 2). The translation of UL130 in microsome-free lysates produced a single polypeptide of the expected mass (25 kDa). In the presence of microsomes, this band was scattered into three bands, all of which had apparent masses above that of the unprocessed protein. These were easily interpreted as mono-, di-, and triglycosylated forms of an ER-translocated pUL130 protein. Treatment with *N*-glycosidase F (endo F) produced a single band that migrated faster than the unprocessed UL130 protein and comigrated with a pUL130 mutant ($\Delta 2-25$) devoid of the signal sequence. These results indicated that in vitro, pUL130 is translocated into the ER lumen by use of a cleaved signal sequence and is modified at all of the predicted N-linked glycosylation sites. The glycosylation of the distal site is unusual, since potential glycosylation sites located in the last 60 residues of a protein are rarely used.

To characterize pUL130 in cells, we raised mouse polyclonal antibodies by DNA immunization with a eukaryotic vector driving the expression of UL130. For HeLa (Fig. 3A) and HEK-293 cells (data not shown) transiently expressing UL130, an immunoblot analysis showed a band with the electrophoretic mobility predicted for an N-terminally processed,

fully glycosylated pUL130 protein, based on in vitro translation data. A less abundant, fast-migrating counterpart and a ladder of intermediate bands in between were also observed in some, but not all, replicates (compare the two "nt" lanes in Fig. 3A). Due to the oligosaccharide trimming that takes place in the secretory pathway, a correlation of the electrophoretic mobility with the number of N-glycosylation sites utilized can be unreliable. To definitively determine the number of sites modified by carbohydrates, we eliminated each of the three predicted N-linked sites in pUL130 by Asn-to-Ala mutation and transfected the mutated forms into HeLa cells. All of the mutants were found to express glycosylated pUL130 variants that migrated faster than the wild type and roughly comigrated with each other (Fig. 3B). This indicated that all of the sites are indeed exploited, as anticipated by the in vitro translation results.

The assignment of carbohydrate modifications on the UL130 protein was further confirmed by adding a glycosylation inhibitor (tunicamycin) 16 h prior to the harvest of cell transfectants. Tunicamycin treatment shifted the UL130 signal to the fast-migrating form (Fig. 3A). A concordant result was obtained by subjecting lysate proteins to enzymatic deglycosylation prior to immunoblot analysis. Both endoglycosidase H (endo H) and endo F treatments shifted the UL130 signal to fast forms. A difference in the migration of endo H- and endo F-treated samples was noted: the pUL130 band observed after endo H treatment migrated slightly slower than the endo F-treated sample because endo H leaves a residual sugar moiety attached to proteins (Fig. 3A). More importantly, the sensitivity to endo H indicated that the bulk of pUL130 accumulating in HeLa cells bears sugars that have not undergone the maturation to low-mannose forms that takes place in the Golgi apparatus, since endo H does not digest low-mannose oligosaccharide trees.

The expression and posttranslational modifications of UL130 were next investigated in human embryonic lung fibroblasts (HELFI) and HUVEC infected with the reference endotheliotropic virus VR1814 and harvested at late infection times. For cells harvested at 5 days postinfection (dpi), WB revealed a pUL130 ladder similar to the one observed for transfected cells (Fig. 3C and D). However, for cells harvested at 10 dpi, when a 100% cytopathic effect was visible, the appearance of heavier forms that migrated slower than the fully glycosylated pUL130 protein detected in both transfectants and cells infected for shorter times was noticed (shown for HUVEC in Fig. 3D). This finding suggested that there were additional modifications of pUL130 oligosaccharides. To further investigate this point, we treated proteins in cell lysates with deglycosylating enzymes as described above. While the UL130 signal for 5-dpi lysates was shifted to the fast-migrating form by endo H, the most slowly migrating forms of pUL130 at 10 dpi were selectively resistant to endo H (Fig. 3C and D) and represented >10% of the pUL130 (Fig. 3D).

An important remaining question was whether pUL130 is secreted. The calculated isoelectric point for pUL130 is basic (9.3 to 9.9, depending on the algorithm). We verified that intracellular pUL130 can be quantitatively retained on a weak anion-exchange resin (see Materials and Methods) and used this procedure to concentrate soluble pUL130 in culture supernatants. We failed to detect free pUL130 in infected cell

FIG. 3. Western blot (WB) analysis of human cells expressing UL130. (A) pUL130 glycosylation state in UL130-transfected HeLa cells. nt, not treated; tun, overnight treatment with 5 μ g/ml tunicamycin. (B) pUL130 glycosylation mutants in HeLa cells. wt, wild-type UL130 product. (C and D) pUL130 glycosylation in HCMV-infected HELF (5 dpi) (C) and HUVEC (D) lysates; in panel D, the lane profile plots and percentages of areas under the peaks refer to the right-most lane. For untreated samples in panels A (left), B, and D, the underglycosylated forms of pUL130 are not observed.

supernatants (data not shown). Minute amounts of bona fide secreted pUL130 (containing endo H-resistant glycoforms) could be detected in the medium from UL130-transfected HeLa cells (see Fig. S1 in the supplemental material). However, the quantification of signals present on the immunoblot indicated that extracellular pUL130 makes up <1% of the total pUL130 and may represent a small fraction that escapes retention under overexpression conditions.

Taken together, these results indicate that the UL130 product is a luminal protein of the secretory pathway which is largely retained intracellularly both in transfected cells, in which it is expressed in the absence of other viral proteins, and in infected cells. Our experiments did not establish the site of pUL130 retention, but it is presumably a pre-Golgi compartment, as most intracellular pUL130 does not undergo carbohydrate rearrangements typical of the Golgi. However, a proportion of pUL130 did mature to a Golgi-type, endo

H-resistant form that was coincident with prolonged infections of both HELF and HUVEC.

Towne pUL130 is a labile protein. The HCMV vaccine strain Towne bears an inactivating point mutation (a double T-nucleotide insertion) at the 3' end of the UL130 open reading frame (7, 16), whose reversion or transcomplementation is absolutely required for a rescue of strain spread in endothelial cells (12, 16). The mutation generates a frameshift, replacing the 12 carboxy-terminal amino acids of wild-type pUL130 with a 26-amino-acid stretch translated from the -1 phase (Fig. 1A and B). Since Towne was adopted as a nonendotheliotropic strain for our experiments (see below), the expression of the mutant gene was characterized initially. Lysates of HELF infected with the Towne strain were analyzed by WB as described above. A pattern reminiscent of that produced by wild-type pUL130, but much weaker, was detected (Fig. 4A). A similar result was observed when the Towne UL130fs gene was

FIG. 4. The Towne UL130 protein is inherently unstable. (A, top) The lanes under the “HELF” label correspond to HCMV-infected (5 dpi) HELF lysates, whereas “HeLa” indicates UL130-transfected HeLa lysates. mi, mock infected. (Bottom) Reprobing with antibodies against the HCMV tegument protein pp65. (B) HeLa transfectants accumulate low levels of the Towne mutant UL130 protein (pUL130fs) relative to VR1814 pUL130 and the equivalent protein of the endotheliotropic Towne revertant (Towne rev), in which the frameshift was corrected by an exact back mutation (12, 16). Two replicate lanes each are shown for Towne rev and Towne. The Towne rev lanes were underloaded (1/10 the total protein used in the adjacent Towne and VR1814 lanes) for a better appreciation of the pUL130fs upshift. (C, D, and G) pUL130fs decays rapidly in infected HELF (C), transfected HeLa cells (D), and nucleofected HUVEC (G). Protein synthesis in the experiments shown in panels C, D, and G was arrested with 50 μ g/ml cycloheximide (CHX) for increasing times prior to WB analysis. (E) pUL130fs is stabilized in HeLa cells by the lysosomal protease inhibitors pepstatin and leupeptin (lysos inh), but not by the proteasome inhibitor lactacystin, which effectively protects cyclin B1 (bottom) in the same cells.

expressed in HeLa cells: transfectants accumulated pUL130fs to much lower levels (approximately 1/50) than that of the wild-type form, and highly resolved gels confirmed that the gene exhibited the predicted modest increment in mass (Fig. 4B). An analysis of protein decay in the presence of a translation inhibitor (cycloheximide [CHX]) showed that in infected HELF, pUL130fs disappeared very quickly, in contrast with wild-type pUL130, which was stable inside the cell for many hours (Fig. 4C). In CHX-treated HeLa transfectants, pUL130fs also underwent an accelerated decay, with a half-life of approximately 1.5 h (Fig. 4D); in these cells, the degradation

of pUL130fs was not due to the ER-associated protein degradation pathway (26) (the protein was not stabilized by a proteasome inhibitor that stabilized cyclin B in the same cells) but depended at least in part on routing to lysosomes (the protein was attenuated by inhibitors of lysosomal proteases) (Fig. 4E). Finally, a similar accelerated decay was observed when pUL130fs was compared to wild-type pUL130 in transiently nucleofected HUVEC (Fig. 4G).

Therefore, the UL130fs mutation that renders the Towne strain nonendotheliotropic likely involves posttranslation degradation of the gene product in this virus. It is quite possible,

but not formally shown here, that the unstable UL130fs product is also nonfunctional, as its rapid degradation is indicative of misfolding.

Transient complementation of viral infection by UL130 expression in producer cells. We have shown previously that HUVEC expressing UL130 (via a retroviral vector) support the growth of genetically UL130-negative strains, specifically the Towne strain (16). This format of complementation assay did not distinguish between the possible mechanisms of action of pUL130. These may be categorized a priori on the basis of (i) which cell is targeted by pUL130 (the cell producing the virion or the endothelial cell target of infection) and (ii) how pUL130 reaches the target cell (is it an intracellular factor, a diffusible extracellular ligand, an autocrine ligand, or a virion protein?).

To determine the mechanism of action of pUL130, we changed the conditions of the transcomplementation assay in order to separate the complementing cell host from the one to be infected. We aimed at determining the effect of the provision of pUL130 within producer cells on virion infectivity for EC and on viral output during the subsequent cycle of replication in EC. Because the UL130-complemented, genetically UL130-negative virus thus generated had to be compared with a noncomplemented counterpart, HELF were chosen as the complementing host in order to enable similar viral growth in the presence or absence of complementation (HUVEC without complementation are nonpermissive for a UL130-negative strain). HELF were transduced with either a retroviral vector to express UL130 or the empty vector as a control. UL130-complementing HELF, in contrast to the controls, expressed detectable amounts of pUL130 (data not shown).

These cell lines were used for replication of the Towne strain. The viral progeny was harvested, with separate collection of the intracellular virions (cell-free virus) and the infectious medium at 7 dpi, and the virion infectivity on HUVEC monolayers was measured by determining the number of HUVEC infectious units (number of nuclei positive for IE1-IE2 gene expression at 24 h), normalized to the titer in HELF (which fell in all cases in the 10^6 to 10^7 range). As Fig. 5A shows, while the virus grown in control cells produced the expected low background level of HUVEC positive for IE1-IE2 (just above the 10^{-4} HUVEC/HELF infection ratio), both cell-free and released viruses from the UL130-complementing HELF generated much higher rates of infection in HUVEC (above the 10^{-2} HUVEC/HELF infection ratio), equaling that of the reference endotheliotropic strain VR1814 passaged once in mock-transduced HELF (Fig. 5A).

Did Towne phenotypic complementation in HELF result from a pUL130-activated modification of virions, or was it secondary to the presence of a soluble factor, possibly pUL130 itself, in the virion suspensions? To investigate this point, we subjected the UL130-complemented Towne virions, along with controls, to a two-step purification procedure consisting of sedimentation followed by isopycnic banding through a discontinuous sorbitol gradient; the supernatant from the first step was collected for subsequent reconstitution experiments. When the infectivities for HUVEC of the purified virions, the virus-free supernatant, and the reconstituted supernatant (purified virions plus virus-free supernatant) were compared, the result was clear-cut: all infectivity present in the unprocessed

sample was recovered with the purified virions (Fig. 5A and B), with no additional contribution by soluble components.

Initial viral output from infected HUVEC is independent of the strain. To compare the single-cycle viral outputs from HUVEC infected with UL130-complemented Towne and mock-complemented VR1814, we measured de novo virus production by HUVEC infected with purified virions by determining (i) the output of infectious virus from HELF at 3 dpi and (ii) the amount of sedimentable viral antigen (pp65) released per infected cell. Strikingly, cells infected with the different viruses released similar quantities of HELF IUs (Fig. 6A) and pp65 (Fig. 6B).

However, the viral progeny from HUVEC infected with UL130-complemented Towne virions proved to be both phenotypically and genotypically indistinguishable from those of the parental Towne strain, showing a very low infectivity for HUVEC (mean HUVEC/HELF infection ratio, 2.6×10^{-4}) and the continued presence of the diagnostic TT insertion within UL130, as confirmed by PCR (data not shown). Thus, the infectivity characteristics of the *trans*-complemented UL130-negative virus were restored for only a single viral replicative cycle in recipient HUVEC; thereafter, the virus reverted phenotypically to match its UL130-negative genotype.

UL130-conditioned medium does not complement viral infectivity in producer cells. Our data required further clarification: did effective complementation require UL130 expression in the same cell that assembled the virion or could pUL130 be supplied from without to the producer cell? The UL130 protein has the potential to function as a secreted, diffusible factor and could be effective even at subdetectable concentrations in the medium. To explore this possibility, we exposed HELF infected with the Towne strain to one of the following diffusible pUL130 sources: (i) conditioned culture supernatants from VR1814-infected HELF, UL130-transduced HELF, or UL130-transfected HeLa cells or (ii) the cells mentioned above, cultured in the upper chamber of a 0.02- μ m-pore-size Transwell to allow the free diffusion of secreted proteins to the Towne-infected HELF kept in the lower chamber, while preventing virus circulation between the two chambers. The infectivities of the released virions were tested on HUVEC, and none of the conditions was found to rescue Towne endotheliotropism (data not shown).

Complementation of infection spread by UL130 expression in recipient HUVEC. In our view, the above findings compellingly demonstrate the following: (i) a Towne UL130 mutation can be complemented in the cell that produces the virus; (ii) the producer cell needs not be an endothelial cell; (iii) complementation works through a virion modification; and (iv) HUVEC release comparable amounts of virus, regardless of whether wild-type or UL130-transcomplemented, genetically UL130-negative virions have initially infected them, that is, pUL130 is necessary for the initial infection of HUVEC but not for the assembly and egress of new virions from HUVEC.

These results are consistent with the interpretation that UL130 mediates a virion modification in producer cells, and they suggest several predictions. Expressing UL130 in recipient HUVEC should not raise the rate of primary infections produced by the Towne strain, as these are sustained by the same minority of cells that are, for unknown reasons, permissive in the absence of complementation; complementation, however,

FIG. 5. UL130 transcomplementation in HELF. Virus strains were grown in mock-complementing HELF (VR1814 and Towne) or UL130-complementing HELF (Towne 130) for 10 days, starting from an MOI of 0.1, in replicate experiments. (A) The infectivities of both crude and purified virus preparations for HUVEC and HELF were determined in parallel by assaying IE-positive cells at 24 hpi. Abbreviations: cell, cell-free virus from cell sonication; rel, released virus; pur, purified virus; cen, centrifuged medium; rec (reconstituted), purified virions reassociated with the corresponding medium. The data are shown as HUVEC/HELF relative infectivities (mean HUVEC IU ml⁻¹ + 95% confidence interval/mean HELF IU ml⁻¹; $n = 3$). (B) Microscope fields of HUVEC infected with a multiplicity of 5 HELF IU/HUVEC and then immunohistochemically stained for IE1-pp72 at 24 hpi. The Towne images (middle panels) were purposely chosen to include positive nuclei to attest to the virus addition; a random field would have been empty, on average. Magnification, $\times 25$.

should allow the formation of plaques. Conversely, Towne virions produced by UL130-complementing HELF should not form plaques in noncomplementing HUVEC, since the complementation effective during the primary infection event does not act in the next replication cycle, preventing virus spread to bystander cells. This was confirmed experimentally (Fig. 6). HUVEC were transduced with the retroviral vector to synthesize pUL130 or were mock transduced with the empty vector, as previously described (16). The cells were infected with VR1814, Towne, or Towne complemented with UL130 in HELF. Regardless of whether UL130 was expressed or mock expressed in the recipient cells, Towne produced background

levels of infection and UL130-complemented Towne had high infection rates at 24 hpi (Fig. 7); these results matched numerically those obtained with parental HUVEC (data not shown). Both Towne and UL130-complemented Towne could form plaques in UL130-complementing HUVEC only, with the number of plaques reflecting that for primary infections at 1 day, as expected (Fig. 7; see Fig. S2 in the supplemental material).

Detection of UL130 protein in virions. Genetic and biochemical data suggest that the UL130 protein may be incorporated into HCMV particles. In fact, (i) pUL130 is a late product (1, 16), (ii) it starts exhibiting Golgi-processed oligo-

FIG. 6. Single-cycle viral output by VR1814, Towne, and Towne 130 produced in the presence of pUL130, which were used to infect HUVEC. HUVEC monolayers were infected with each virus at 10 HELFIU/cell as described in Materials and Methods. After exposure of the cells to the viruses, pooled human sera were added to limit the infection to a single cycle of replication. After 2 days, the medium was changed to remove the blocking antibody. At 3 dpi, the medium was collected and HUVEC monolayers were stained for an IE antigen. The titers of HELFIU in the collected media were determined in parallel and plotted as HELFIU per infected HUVEC (A). Alternatively, the blocking antibody was left throughout the experiment and the virion tegument pp65 antigen released into the medium was measured by the quantification of WB lanes (B). The data are expressed as means $\pm$ 95% confidence intervals ($n = 5$).

saccharides when the infected cell becomes replete with virions, and (iii) the UL130-mediated enhancement of EC tropism depends on UL130 expression in the cell producing virions. To examine this possibility, we subjected VR1814 virions to an additional purification step, by rate-zonal centrifugation through a Nycodenz gradient. When the fractionated gradient was analyzed by WB for pUL130, the protein species that was immunoreactive with the UL130 antibody was detected in the fractions containing peak concentrations of the capsid protein MCP (Fig. 8A). This suggested that pUL130 is indeed a virion protein. Interestingly, when the peak of pUL130 was analyzed for its oligosaccharide composition, it was shown to be resistant to endo H, but not endo F, digestion (Fig. 8B). To further define the efficacy of this enzymatic procedure, we performed an add-back experiment in which a HeLa lysate containing pUL130 was mixed with virions prior to analysis as described above. In this case, the pUL130 fraction derived from cells was selectively deglycosylated by endo H, demonstrating the absence of endo H inhibitors in virion preparations (Fig. 8C). In addition, virion-associated pUL130 was sensitive to a mild trypsin treatment in the absence of detergents (which suggests that it is exposed at the particle surface) (Fig. 8D) and was missing from particles stripped off the envelope by exposure to a nonionic detergent and subsequent centrifugation (Fig. 8D). For comparison, the tegument protein pp65 was resistant to trypsin and sedimented with the stripped particles while being sensitized to proteolysis after exposure to detergent (Fig. 8D). To obtain an estimate of the pUL130 abundance, we analyzed purified VR1814 particles titrated for their HELFIU content by WB along with serial dilutions of *in vitro*-translated pUL130 (which enabled the quantification of WB sensitivity by radioactivity counting). Assuming a virion-to-IU ratio of 50 to 200, approximately 50 to 200 pUL130 molecules per particle were present (see Fig. S3 in the supplemental material).

Taken together, these results make a strong case for pUL130

incorporation into the HCMV virion envelope. Virion-associated pUL130 is highly unlikely to be derived from contamination with cellular debris, because it consists exclusively of the form carrying Golgi-matured sugars, which amounts to a minor fraction of the intracellular pUL130, even in cells showing a 100% cytopathic effect.

The presence and abundance of pUL130s in purified Towne virions were similarly verified. When the peak of Towne particles recovered from a Nycodenz gradient was analyzed by WB, no pUL130s signal was detected (Fig. 8E). We ignored whether Towne pUL130s failed to be incorporated into viral particles or was included at levels reflecting its low intracellular concentration and falling below the WB detection limit.

DISCUSSION

HCMV's broad tropism can be explained in part by the promiscuous entry of this virus into many cell types. This in turn may depend on HCMV's ability to adapt its attachment and penetration strategy, since the epidermal growth factor receptor that functions as the HCMV docking receptor in fibroblasts, and possibly other adherent cell types (45), is not expressed on HCMV-permissive hematopoietic cells. Dendritic cell infections require HCMV binding to the C-type lectin DC-SIGN (17), a pathogen recognition receptor whose function is also subverted by human immunodeficiency virus type 1 (HIV-1) and Ebola virus. Indeed, cells that are restrictive for HCMV growth seem to hinder viral replication at a postentry stage (a notable exception is polarized epithelial cells, which prevent HCMV entry at the apical membrane by receptor segregation at the basolateral membrane [21]). In the conditionally permissive NTERA-T2 teratocarcinoma cell line, for instance, immediate-early (IE) gene expression is inhibited by a transcriptional block; a treatment with an inhibitor of

FIG. 7. UL130 transcomplementation in HUVEC. HUVEC harboring either retrovirally transduced UL130 (HUVEC 130) or a void retroviral vector (HUVEC mock) were infected with VR1814, Towne, or Towne 130. (A) Cells were infected with 5 HELF IU/HUVEC, fixed at 24 hpi, and stained for IE1-pp72. (B) Cells were infected with 0.5 HELF IU/HUVEC, fixed at 168 hpi, and stained as described above. Magnification, $\times 10$.

histone deacetylases removes the block and allows the progression of infection (28).

In EC, on the other hand, the inhibition point precedes IE gene expression, as the manifestation of the non-EC-tropic phenotype strictly correlates with a failure in the nuclear deposition of viral DNA (3, 4, 39, 41). The central questions regarding the UL131A-128 locus are thus as follows: (i) what precisely is the cytoplasmic mechanism arresting the replication of UL131A-128-defective strains in EC and (ii) how do the products of the locus remove it?

In the present study, we have started to address the second issue, focusing on the role of pUL130. A biochemical charac-

terization of the protein substantiated the main predictions regarding its intracellular processing: the UL130 product was found to be translocated into the ER lumen in vitro, and its signal sequence was removed by cleavage after amino acid 25. This also held true in HCMV-infected cells and in UL130-transfected human cell lines. Both in vitro and in cells, all three pUL130 N-linked glycosylation sites were quantitatively exploited, as confirmed by their elimination by site-directed mutagenesis.

This analysis, however, brought to light an unanticipated aspect of pUL130 transport, that is, its intracellular accumulation, both in the context of natural infection and when ex-

FIG. 8. The UL130 protein is found in virions. (A) The UL130 protein cofractionates with MCP in VR1814 particles sedimented through a Nycodenz gradient. Fraction 1 is the lightest fraction. (B to D) Particles from pooled fractions 11 to 13 were exposed to the indicated treatments. For panel C, as a control of endo H's effectiveness, HeLa lysates containing pUL130 were treated alone or after being mixed with VR1814 particles (lanes HeLa and VR1814+HeLa, respectively). In panel D, pUL130 exhibits a quantitative, though small, mobility shift after trypsin treatment. This suggests that it was cut at a basic residue close to either extremity; lysine-43 was the most probable cleavage site, as arginine-196 is upstream of the most C-terminal glycosylation site, whose removal should cause a larger mobility change. (E) Towne virions from a gradient similar to that used for panel A were analyzed for pUL130 alongside equal amounts of VR1814 virions and HeLa UL130 transfectants as controls. Abbreviations: nt, not treated; TX-100+ucfg, incubation with 0.5% Triton X-100, followed by ultracentrifugation and pellet analysis.

pressed alone in transfectants. Three frequent reasons for a secretory protein to be retained in the cell are as follows: (i) it is a misfolded mutant which is retained by ER quality control and most often degraded (43), (ii) it includes a specific retention signal, or (iii) it is part of a complex and must undergo an obliged oligomerization step with other subunits to progress in the secretory pathway (43). The first setting does not apply to pUL130, as retention affected the wild-type protein, which is stable. A well-defined ER retention signal is the consensus sequence KDEL (Lys-Asp-Glu-Leu) found at the C termini of luminal ER proteins, which are retrieved from the *cis* Golgi by interaction with a transmembrane KDEL receptor and retrograde transport to the ER (31). Because pUL130 lacks a C-terminal KDEL sequence (Fig. 1B), it might exploit a distinct,

unknown signal. However, we propose that in accord with mechanism iii described above, pUL130 has to interact with the virion envelope and can be released from the cell as a peripheral virion component only after that association. Indeed, pUL130 was detected in association with purified HCMV particles in amounts roughly estimated as 50 to 200 molecules per particle. Virion-associated pUL130 has the hallmarks of an authentic envelope peplomer, i.e., it bears Golgi-matured (endo H-resistant) sugars, is sensitive to protease treatment, and is removed by detergents.

Previously, we had shown that the chief virologic phenotypes of UL131A-128 locus mutants (the inability to productively grow in HUVEC and the inability to pass from adherent cells to leukocytes) can be effectively *trans* complemented by the

provision of the relevant gene in HUVEC or HELF (16). For this study, by manipulating the original complementation assay, we defined the pUL130 mode of action in EC infection. The UL130-negative virus exploited was the Towne strain, whose UL130 product was shown to be rapidly degraded. Our results compellingly demonstrate that the positive effect of pUL130 on HCMV EC tropism requires its expression in the producer cell, although that effect is manifested in the subsequent target cell of infection. In other words, pUL130 must be synthesized in the cell that assembles new virions to modify them in some way. In fact, (i) UL130-complementing HELF produced a genotypically UL130-negative, phenotypically EC-tropic Towne strain; (ii) EC tropism was maintained in purified UL130-complemented Towne virions, but not in the virus progeny released from EC infected by them; (iii) the provision of UL130 to HUVEC did not raise their primary infection rate by either Towne or UL130-complemented Towne; (iv) Towne or UL130-complemented Towne formed plaques only in complementing HUVEC; (v) once the complementation in the previous replicative round had permitted the infection of HUVEC, the cells released Towne virions as efficiently as the virions of a reference EC-tropic strain; (vi) a Towne virus grown in HELF that were exposed to external sources of diffusible pUL130 was not made phenotypically EC tropic.

What, then, is the virion modification induced by pUL130? The simplest hypothesis is that pUL130 is a virion protein. The data presented here indicate that pUL130 is indeed a virion protein. We hypothesize, therefore, that virion-associated pUL130 acts in the recipient EC to permit a successful infection. As mentioned previously, the impact of UL131-128 products in the recipient cell is manifested as viral DNA nuclear targeting following viral entry. Accordingly, pUL130 exposed on virions may facilitate the targeting of viral DNA in recipient cells. We further speculate that pUL130 has two ways to do so: (i) as (part of) a ligand for a signaling receptor, conveying signals into EC that make possible the intracellular transport mechanism or that disable an innate antiviral immunity mechanism inside the EC; and (ii) as a component of the attachment and/or entry machinery, permitting infection-proficient penetration through a pathway different from the one used in fibroblasts, e.g., through a specific EC membrane microdomain.

The above interpretation must be made cautiously, though, as a strict demonstration of pUL130's role as an envelope constituent will require blocking its function in the purified virion, for instance, by a neutralizing EC infection through virion incubation with anti-UL130 antibodies. (Antisera used for pUL130 detection in this study failed to inhibit EC infection when added to VR1814 virions, but this is inconclusive since they were also unable to immunoprecipitate native pUL130 [M. Patrone, M. Secchi, A. Gallina, and G. Milanesi, unpublished results].) Currently, therefore, an alternative scenario, wherein pUL130 would operate only indirectly in recipient EC infection by promoting a virion alteration in the producer cell, such as the inclusion, exclusion, or posttranslational modification of a virion protein(s), cannot be discounted; in this case, the presence of pUL130 in the virion would represent a by-product. Indirect producer-cell modifications of virions are well known for other virus groups. The HIV-1 Nef and Vif accessory proteins, for example, are believed to enhance infec-

tivity in part by indirectly affecting the virion composition, even though both can be detected in HIV-1 particles (13, 36).

In the near future it will be important to disprove either model of pUL130 action, as well as to define how the companion products of the UL131A-128 locus can synergize with pUL130. Resolving these points would, in turn, provide clues to the permissivity mechanism regulated by UL131A-128 in EC.

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ADDENDUM

At the stage of revision of this work, a cyclooxygenase-2 homologue encoded by rhesus cytomegalovirus was reported as a novel EC tropism determinant (35a) and must be added to the list of nonhuman cytomegalovirus genes essential for EC infection cited in the introduction.

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