Human Cytomegalovirus-Encoded pUL7 Is a Novel CEACAM1-Like Molecule Responsible for Promotion of Angiogenesis

MacManiman, JD

http://hdl.handle.net/10026.1/3437

10.1128/mbio.02035-14

mBio
American Society for Microbiology

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.
Human Cytomegalovirus-Encoded pUL7 Is a Novel CEACAM1-Like Molecule Responsible for Promotion of Angiogenesis

Jason D. MacManiman, Andrew Meuser, Sara Botto, Patricia P. Smith, Fenyong Liu, Michael A. Jarvis, Jay A. Nelson, Patrizia Caposio

Vaccine and Gene Therapy Institute, Oregon Health & Science University, Portland, Oregon, USA; Division of Infectious Diseases, School of Public Health, University of California, Berkeley, California, USA

ABSTRACT Persistent human cytomegalovirus (HCMV) infection has been linked to several diseases, including atherosclerosis, transplant vascular sclerosis (TVS), restenosis, and glioblastoma. We have previously shown that factors secreted from HCMV-infected cells induce angiogenesis and that this process is due, at least in part, to increased secretion of interleukin-6 (IL-6). In order to identify the HCMV gene(s) responsible for angiogenesis promotion, we constructed a large panel of replication-competent HCMV recombinants. One HCMV recombinant deleted for UL1 to UL10 was unable to induce secretion of factors necessary for angiogenesis. Fine mapping using additional HCMV recombinants identified UL7 as a viral gene required for production of angiogenic factors from HCMV-infected cells. Transient expression of pUL7 induced phosphorylation of STAT3 and was again associated with increased IL-6 expression. Analysis of the UL7 structure revealed a conservation domain similar to the immunoglobulin superfamily domain and related to the N-terminal V-like domain of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Our report therefore identifies UL7 as a novel HCMV-encoded molecule that is both structurally and functionally related to cellular CEACAM1, a proangiogenic factor highly expressed during vasculogenesis.

IMPORTANCE A hallmark of cytomegalovirus (CMV) infection is its ability to modulate the host cellular machinery, resulting in the secretion of factors associated with long-term diseases such as vascular disorders and cancer. We previously demonstrated that HCMV infection alters the types and quantities of bioactive proteins released from cells (designated the HCMV secretome) that are involved in the promotion of angiogenesis and wound healing. A key proangiogenic and antiapoptotic factor identified from a proteomic-based approach was IL-6. In the present report, we show for the first time that HCMV UL7 encodes a soluble molecule that is a structural and functional homologue of the CEACAM1 proangiogenic cellular factor. This report thereby identifies a critical component of the HCMV secretome that may be responsible, at least in part, for the vascular dysregulation associated with persistent HCMV infection.

Human cytomegalovirus (HCMV) is a β-herpesvirus that has been epidemiologically linked to several diseases that require angiogenesis (AG), including atherosclerosis, restenosis following angioplasty, transplant vascular sclerosis (TVS) associated with chronic allograft rejection, and glioblastoma (1–4). The best evidence that CMV is directly involved in these pathogenic processes comes from solid-organ transplantation models (5–7). Specifically, rat CMV (RCMV) was shown to decrease the mean time to allograft failure as well as increase the degree of TVS in the allograft vessels in a rat heart transplantation model (5–7). Treatment of allograft recipient rats with a CMV DNA polymerase inhibitor, ganciclovir, resulted in prolonged survival of the allograft, indicating that CMV replication was a requirement for acceleration of disease (8, 9). Microarray analysis of the RCMV-infected heart allograft transcriptome indicated that a significant number of RCMV-induced genes, including those encoding angiopeitin, vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), chemokines, members of the tumor necrosis factor receptor (TNFR) superfamily, and cytokines such as interleukin-6 (IL-6), were associated with AG and wound healing (WH) (10). Together, these results suggest that CMV-mediated acceleration of TVS occurs through direct or indirect alteration of AG and WH processes.

HCMV infection of cells in vitro alters the production of bio-
active proteins released from infected cells into the supernatant (HCMV secretome) that, when applied to endothelial cell cultures, induce an angiogenic phenotype (11). Many of these factors have important roles in vascular disease, and we have hypothesized that a major role of CMV infection in the acceleration of TVS occurs through the increased production of AG/WH factors within the allograft. Mass spectrometry analysis of HCMV-infected and mock-infected secretomes identified more than 1,200 soluble proteins, more than 1,000 of which were specific to or highly enriched in the HCMV secretome. Pathway analysis indicates that a large number of angiogenic molecules, i.e., proteins involved in TGF-β and other factor signaling pathways, cytokines, and chemokines, as well as factors involved in extracellular matrix remodeling, are present in the HCMV secretome. Importantly, we demonstrated that the HCMV secretome contains high levels of IL-6 that promote AG and long-term survival of endothelial cells (EC). In EC, IL-6 prevented apoptosis by blocking caspase 3 and 7 activation through the induction of survivin (12). Although the identity of the HCMV gene(s) responsible for AG is unknown, this function was shown to be due to an HCMV late gene, since a drug that blocks the viral polymerase function also inhibits the ability of viral supernatants to induce neovessel formation.

In the current study, mutational analysis of the HCMV genome identified UL7 as the viral gene responsible for induction of AG factors during HCMV infection. UL7 is a late gene encoding a transmembrane protein that is cleaved to produce a small soluble protein secreted from the cell (13). We show that UL7 is sufficient to induce the AG phenotype and is structurally and functionally related to carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a cellular protein that also induces AG.

RESULTS
HCMV-induced angiogenesis is mediated by the UL7 late gene. In order to identify the HCMV gene involved in AG, we generated a library of 6 recombinant viruses with large-scale deletions of nonessential open reading frames (ORFs) that include HCMV TR-bacterial artificial chromosome (BAC) \( \Delta U L 1 \) to \( U L 1 0 \) (\( \Delta U L 1 \)–10), \( \Delta U L 1 1 \)–20, \( \Delta U L 2 2 \)–25, \( \Delta U L 1 8 \)–121, \( \Delta U S 7 \)–12, and \( \Delta U S 1 5 \)–22. Analysis of secretomes from these HCMV deletion mutants in a Matrigel angiogenesis assay indicated that only deletion of UL1–10 significantly decreased tubulogenesis in human aortic endothelial cells (HAEC) (70%) (Fig. 1A). The microscopy pictures (Fig. 1B) show some differences between the mock secretome and the UL1–10 mutant; however, the structures present in the TR\( \Delta U L 1 \)–10 secretome are mainly branching cells rather than lumens delimited by intact capillary-like tubule structures. As secretomes do not contain infectious virus, this finding suggests a direct or indirect role of HCMV protein(s) from the UL1–10 region in release of soluble proangiogenic factors.

In order to determine which of the HCMV genes encoded in the TR \( \Delta U L 1 \)–10 mutant were responsible for HCMV-induced AG, a library of HCMV Towne mutants with transposon mutations in UL3, UL4, UL5, UL6, UL7, UL8, UL9, or UL10 was used to generate secretomes in fibroblasts (14). The UL2 mutant was not included in these studies since this virus exhibits a growth defect (14). The role of UL1 was also not examined since the parental HCMV Towne strain contains a spontaneous deletion in this gene that does not affect the ability of the virus to induce a secretome that stimulates AG (15). Analysis of HCMV mutant secretomes for induction of AG indicated that mutation of UL7 resulted in a 50% reduction of tubulogenesis (Fig. 1C). As further confirmation that UL7 was important for HCMV-induced AG, an HCMV UL7 mutant was generated in the clinical strain TR (TR \( s t o p U L 7 H A \)) by insertion of two stop codons as well as an influenza virus hemagglutinin (HA) epitope tag in frame at the 3′ end of the UL7 ORF. A correlate HCMV TR revertant virus (TR wild-type UL7HA [wtUL7HA]) was also constructed by replacing the \( s t o p U L 7 - H A \) cassette with a functional UL7-HA insertion (see Fig. S1 in the supplemental material). The replication kinetics of TR wtUL7HA and TR \( s t o p U L 7 - H A \) were similar to those seen with the TR wt (see Fig. S2). Expression of UL7 in TR wtUL7HA- or TR \( s t o p U L 7 - H A \)-infected cells was determined by immunofluorescence using antibodies to the HA epitope, with results indicating that only the revertant virus expressed UL7 (see Fig. S3). Subsequently, analysis of secretomes obtained from TR \( s t o p U L 7 - H A \)- and TR wtUL7HA-infected HAEC indicated that only the revertant virus and not the UL7 mutant induced AG (Fig. 1D and E). These results demonstrate that UL7 is the gene within the UL1–10 region required for HCMV-induced AG.

UL7 is a novel CEACAM-like protein and is sufficient to induce angiogenesis. UL7 is a member of the HCMV RL11 gene family (15,16). Previous studies indicated that UL7 is expressed as an early-late gene and encodes a 222-amino-acid type I glycoprotein, which is proteolytically cleaved to release a heavily glycosylated ectodomain (13). The late gene expression of UL7 was confirmed by Northern blotting (see Fig. S4 in the supplemental material) and is consistent with the previous observation that HCMV-induced AG is due to expression of a viral late gene (11). Although pUL7 was previously reported to have homology to the CD229 lymphocyte-activating molecule receptor (13), a query of the HCMV TR UL7 protein against NCBI GenBank conserved domains revealed a similarity to the first Ig-like domain of the carcinoembryonic antigen (CEA)-related cell adhesion molecule (CEACAM) protein subfamily (cd, 05741; E value, 3.90e-04). A BLASTp alignment performed with the four members of the CEACAM family (CEACAM1, CEACAM3, CEACAM5, and CEACAM6) displayed 39% similarity and 29% amino acid identity between the UL7 Ig-like domain and the N-terminal V-like domain of CEACAM1 (Fig. 2). Interestingly, the CEACAM1 molecule is highly expressed during vasculogenesis (17), and soluble recombinant CEACAM1 added to endothelial cell cultures has been shown to stimulate significant tubule formation (18).

To confirm that UL7 is cleaved from the cell surface, HEK293 cells were transfected with a plasmid expressing pUL7 tagged at the C terminus with a HA epitope. After 48 h, immunoblot analyses of cell lysates by the use of an antibody specific for the HA epitope and of supernatants by the use of a specific polyclonal UL7 antibody were performed. Two prominent bands of 75 and 12 kDa, corresponding to the UL7 precursor and the cytoplasmic tail, were detected in the pUL7HA-expressing cells. PNGaseF treatment led to a reduction of the UL7 precursor to the predicted molecular mass of 25 kDa (see the left panel of Fig. S5 in the supplemental material). In the supernatant of the transfected cells, only the 75-kDa ectodomain was present, demonstrating that pUL7 is partially cleaved in HEK293 cells (see Fig. S5, right panel). To allow us to express detectable levels of pUL7 in HAEC, we constructed an adenoviral vector expressing pUL7HA (designated AdUL7). Western blotting of cell lysate from HAEC transfected with AdUL7 showed the 12-kDa protein, corresponding to cytoplasmic tail regions of UL7, and a faint band around 75 kDa.
FIG 1   Deletion of UL7 within the UL1–10 region impaired tubulogenesis. (A) Quantitative measures of angiogenesis consisting of the numbers of lumens and branch points observed in the presence of secretomes generated from mock infections (Mock scr), TR secretome (TR scr), TRΔUL1–10 secretome (TR ΔUL1–10 scr), TRΔUL11–20 secretome (TR ΔUL11–20 scr), TRΔUL22–25 secretome (TR ΔUL22–25 scr), TRΔUL118–121 secretome (TR ΔUL118–121 scr), TRΔUS7–12 secretome (TR ΔUS7–12 scr), and TRΔUL15–12 secretome (TR ΔUS5–12 scr). Three independent experiments were performed. Data are expressed as means ± SEM of the results of three independent biological replicates for each viral construct assayed in triplicate. Statistical significance is represented with three asterisks (***, P < 0.001). (B) Representative examples of mock secretome (Mock scr), HCMV TR secretome (TR scr), and HCMV TRΔUL1–10 secretome (TR ΔUL1–10 scr) are shown as a low-power image (magnification, ×10). (C) HFFF cells were infected with wild-type Towne (wt Towne), Towne ΔUL3, Towne ΔUL4, Towne ΔUL5, Towne ΔUL6, Towne ΔUL7, Towne ΔUL8, Towne ΔUL9, and Towne ΔUL10 or were mock infected. Secretomes were collected at 96 hpi (Continued)
Our previous studies showed that pUL7 is a secreted protein with an Ig-like domain with structural and functional homology to CEACAM1, a molecule highly expressed during vasculogenesis. We found that UL7 expression stimulates an inflammatory response in EC, inducing the secretion of IL-6 and the activation of STATs and of ERK1/2 (Fig. 5C and D). Treatment with neutralizing antibodies against pUL7 or IL-6 resulted in a 70% decrease in IL-6 secretion and a 40% reduction in STAT3 phosphorylation (Fig. 6D).

Together, these observations indicate that binding of secreted pUL7 to an unknown cellular receptor on HAEC induces IL-6 through the activation of STATs and of ERK1/2/mitogen-activated protein kinase (MAPK) pathways.

DISCUSSION

In the present study, we demonstrated that the presence of HCMV pUL7 is sufficient to induce endothelial cell angiogenesis. Interestingly, pUL7 is a secreted viral protein and contains an Ig-like domain with structural and functional homology to CEACAM1, a molecule highly expressed during vasculogenesis. We found that UL7 expression stimulates an inflammatory response in EC, inducing the secretion of IL-6 and the activation of STATs and of MAPK pathways. Importantly, these findings identify a mechanism by which HCMV can influence and manipulate the vascular environment despite limited infection of the vessel wall.

The CMV genome encodes 12 multigene families (16, 19), including the RL11 family, which contains the RL11–13, UL1, and UL7 genes (20). All members of the RL11 family are located adjacent to each other near the left terminus of the genome and are dispensable for virus growth in vitro (14). Among the RL11D

**Figure Legend Continued**

and tested in a Matrigel assay using HAEC. Three independent experiments were performed. Data are expressed as means ± SEM of the results of three independent biological replicates for each viral construct assayed in triplicate. Statistical significance is represented with two asterisks (**; **P < 0.01). (D) HAEC were infected with TR wtUL7HA or TR stopUL7HA and secretomes collected at 96 hpi. Statistical significance is represented with three asterisks (**; **P < 0.001).

(E) Representative examples of mock secretome (Mock scr), TR wtUL7HA secretome (TR wtUL7HA scr), and TR stopUL7HA secretome (TR stopUL7HA scr) are shown as a high-power image (magnification, ×20).

### TABLE 1

| Gene   | Accession | Description |
|--------|-----------|-------------|
| CEACAM1| 1         | Coiled-coil domain |
| TR UL7 | 36        | Significant role in virus replication |
FIG 3  pUL7 is secreted and sufficient to induce AG. HAEC were transduced with AdGFP and AdUL7, and the secretomes were collected at 48 h. (A) Cell lysates were analyzed by Western blotting with antibodies specific for HA and actin. Right margin, the arrowhead indicates the pUL7 high-molecular-weight form. (B) Quantitative measures of angiogenesis consisted of the numbers of lumens and branch points. Three independent experiments were performed. Data are expressed as means ± SEM of the results of three independent biological replicates for each adenoviral construct assayed in triplicate. Statistical significance of the results of comparisons with the AdGFP secretome is indicated with two asterisks (**; P < 0.01). (C) Representative examples of AdGFP and AdUL7 secretomes are shown as a high-power image (magnification, ×20).

FIG 4  Recombinant UL7 promotes HAEC tube formation. Increasing concentrations (50, 150, and 300 ng/ml) of pUL7Fc were directly added to serum-free basal media in the presence or absence of neutralizing antibodies against UL7 or the isotype control (iso ctl). Controls included HAEC stimulated with 300 ng/ml of Fc and 300 ng/ml of CEACAM1Fc. (A) Quantitative measures of angiogenesis consisted of the numbers of lumens and branch points. Three independent experiments were performed. Data are expressed as means ± SEM of the results of three independent biological replicates for each condition assayed in triplicate. Statistical significance is represented with three asterisks (***; P < 0.001) or with two asterisks (**; P < 0.01). (B) Representative examples of Fc, pUL7Fc, and pUL7Fc plus αUL7 (pUL7Fc+αUL7) are shown as a high-power image (magnification, ×20).
genes, UL7 exhibits high sequence conservation across different CMV genotypes (97% to 100% intra- and 83% to 93% intergenotype conservation), suggesting a critical role of UL7 for viral fitness (15). Consistent with the literature data, we found that pUL7 is shed from HAEC cells and that the transcript is expressed in the late phase of the viral replication cycle. Upon further sequence inspection of pUL7, however, we identified homology with the first Ig-like domain of the carcinoembryonic antigen (CEA)-related cell adhesion molecule (CEACAM) protein subfamily. Interestingly, another HCMV RL11 protein, UL1, which is virion associated and required for efficient replication in epithelial cells, had previously been reported to share sequence homology with CEACAM1 (21). Because RL11 members are more likely the descendants of a common cellular ancestor that was incorporated into the HCMV genome, duplicated, and subsequently subjected to functional divergence during coevolution with the host, pUL7 may have evolved to mimic the angiogenic properties of soluble CEACAM1 whereas pUL1 has retained the receptor function contributing to HCMV access to host cells by interacting with members of the CEA family.

CEACAM1 is a transmembrane cell adhesion molecule that is abundantly expressed in epithelia, vessel endothelia, and hematopoietic cells (22). In primary EC culture, recombinant human CEACAM1 enhances the sprouting and migration of EC, acting synergistically with VEGF (18). EC produces both secreted and membrane-bound forms of CEACAM1. The soluble CEACAM1 form may stimulate proliferation and chemotaxis of EC, whereas the membrane-bound form may be involved in morphogenetic events such as formation of capillary tubes. Moreover, lack of endothelial CEACAM1 expression in Ceacam1<sup>−/−</sup> mice leads to defective vascular remodeling in vivo, whereas endothelial overexpression of CEACAM1 in Ceacam1<sup>ende+</sup> mice induces extensive vascular growth (23). Membrane-bound CEACAM1 can have a long (L) or a short (s) cytoplasmic domain (24). Both isoforms are coexpressed in most CEACAM1-expressing tissues, and the ratio between the two isoforms determines the signaling outcome (25, 26). The structural and functional homology of UL7 with CEACAM1 and the inter-strain-specific conservation level reflect the existence of a selective pressure against mutations in the protein sequence, supporting a relevant role of this viral product. The short cytoplasmic tail of pUL7 resembles the “s” isoform of CEACAM1, suggesting that pUL7 does not function as a signal receptor but more likely evolved to mimic the angiogenic properties of soluble CEACAM1. However, our studies presented here do not rule out a possible function for the membrane-bound form, in addition to the AG functions of the soluble form.

We have previously shown that one of the key cytokines present in the HCMV secretome is IL-6, which promotes AG and blocks apoptosis through increased levels of survivin (12). The inflammatory effects of IL-6 have been implicated as a factor in the failure of organ and tissue grafts, and elevated levels of the cytokine have been reported to accompany HCMV replication in

FIG 5  UL7 upregulates IL-6 protein secretion. Data represent ELISA quantification of IL-6 levels in HAEC transduced with AdGFP and AdUL7 (A), infected with TR wtUL7HA or with TR stopUL7HA (B), and stimulated with recombinant pUL7Fc (C). IL-6 secretion is shown as the means ± SEM of the results of two independent experiments. ***, P < 0.001; *, P < 0.05.
FIG 6  UL7 induces STAT3 and ERK1/2 phosphorylation. Cell lysates from HAEC transduced with AdGFP or AdUL7 (A), infected with TR UL7HA or TR stopUL7HA for 96 h (B), and stimulated with pUL7Fc (C) were subjected to SDS-PAGE followed by immunoblotting. The membranes were decorated with antibodies specific for phospho-STAT3 and phospho-ERK1/2. Immunodetection was performed with total STAT3, ERK1, and actin serving as the internal controls. (D) Cells were treated with increasing concentrations of recombinant pUL7Fc in the presence or absence of UL7 and IL-6 neutralizing antibodies (Ab) for 24 h. ELISA was used to quantify the amount of IL-6 secreted from HAEC. Cell lysates were analyzed by Western blotting with specific antibodies for phospho-STAT3, total STAT3, and actin.
transplanted lungs and bone marrow during episodes of inflammation or rejection (27–29). Interestingly, we found that binding of pUL7 stimulates an inflammatory response in vascular endothelial cells that leads to IL-6 secretion. Our data are therefore in contrast with the observations by Engel et al., who reported that activated monocyte-derived immature dendritic cells (DCs) and
monocytic cell lines transiently expressing pUL7 secreted less IL-8, IL-6, and tumor necrosis factor (TNF) than the control cells (13). It is possible that the binding of pUL7 with its cellular receptor(s) triggers a pro- or anti-inflammatory response depending on the role played by different cell types in HCMV spread and persistence in the host. For example, CEACAM1-L, upon activation, can discriminate between binding of cytoplasmic protein tyrosine phosphatase SHP-1/SHP-2 and binding of Src family tyrosine kinases in a manner that is regulated by and dependent on CEACAM1-L supramolecular organization. The discriminatory binding of SHP-1/SHP-2 and of Src is the basis for the different effects of signal regulation by CEACAM1, which have found to be both inhibitory and stimulatory (30).

Notably, the concentration of IL-6 was increased after AdUL7 transduction or addition of recombinant pUL7Fc protein (Fig. 5A and C). IL-6 is a potent, pleiotropic, inflammatory cytokine that mediates multiple physiological functions, including development of lymphocytes, cell proliferation, cell survival, and inhibition of apoptotic signals (31–34). IL-6 mRNA has also been detected in human atherosclerotic lesions, indicating that this cytokine is expressed at the site of injury (35). Engagement of IL-6 with the IL-6 receptor activates three main signaling pathways, the JAK/STAT, ERK1/2-MAPK, and phosphoinositol 3-kinase (PI3K)/Akt pathways (36, 37). Activation of the JAK-STAT pathway was reported to have short-term cardioprotective effects as well as long-term inflammation-promoting effects (38). ERK1/2-MAPK activation has been implicated in hypertrophy and cell survival (39). IL-6 and the JAK/STAT and MAPK pathways are also linked to malignant glioblastoma through the expression of the US28 HCMV chemokine receptor (40). US28 activity might be responsible for the residual activation of STAT3 and ERK1/2 in TR stopUL7HA-infected cells compared to the mock-infected cell results (Fig. 6B).

In conclusion, we show for the first time that HCMV encodes a homologue structurally and functionally similar to CEACAM1. Ongoing and future work will be focused on the identification of the UL7 cellular receptor(s) as well as on the function of this viral gene in HCMV pathogenesis. Thus, alteration of UL7 binding or cell signaling may offer a novel strategy for the treatment of the angiogenic processes involved in HCMV-associated vascular diseases.

MATERIALS AND METHODS

Cells and virus preparation. Human aortic endothelial cells (HAEC) (Lonza) were cultured in endothelial growth medium (EGM-2; Lonza), as previously described (12). Human fetal foreskin fibroblasts (HFF) (purchased from Sigma) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro; Corning) supplemented with 10% fetal bovine serum (FBS) (HyClone; Thermo Scientific), 4.5 g/liter glucose, 1% sodium pyruvate, and antibiotics (penicillin [10 units/ml]-streptomycin [10 μg/ml]). The human embryonic kidney 293 cell line (HEK293) and HEK293/Cre cell line (Microbix Biosystems Inc.) were cultured in minimum essential medium (MEM) (Cellgro; Corning) supplemented with 10% FBS, penicillin, streptomycin, and l-glutamine. PD98058 was purchased from Cell Signaling Technology and STAT3 inhibitor XI (STX-0119) from Calbiochem. HCMV strain TR was derived from the titers of the bulk stocks were determined on 293 cells by limiting dilution. Gene expression was driven by coexpression with Ad-Trans expressing the Tet-off transactivator as previously described (46). The recombinant adenovector expressing green fluorescent protein (GFP) was a gift from D. Strebelow at Oregon Health & Science University (OHSU) (47).

Preparation of HCMV and adenoviral secretomes. HCMV TR secretomes were generated by infecting HAEC at a multiplicity of infection (MOI) of 3 PFU/cell for 96 h, as previously described (12). HCMV Towne secretomes were generated in fibroblasts infected at an MOI of 0.5 and harvested 96 hpi. For the adenovirus secretome, HAEC were starved for 12 h in serum-free endothelial basal medium-2 (SF-EBM2) and then coinfected with the appropriate adenovirus and transactivator (MOI of 250 PFU/cell). The supernatant from infections was harvested and clarified at 48 hpi.

Angiogenesis assay. The ability of secretomes to induce angiogenic activity was measured using a modified version of the standard Matrigel in vitro tube formation assay, as previously described (12). For experiments involving neutralization, the secretome containing anti-UL7 antibody or an anti-rabbit isotype control was incubated for 1 h at 37°C prior to stimulation of HAEC.

Fc fusion UL7 and antiserum production. To express UL7 in a glycosylated form comparable to that of the native protein, stable clones were

pUL7 Induces Angiogenesis
made by transfection of HEK293 with pCMV-UL7Fc and selection with neomycin (G418; Gibco-BRL). Stable clones with G418 resistance were obtained by trypsination of colonies, followed by plating at limiting dilutions onto a 96-well plate. Ten single clones were then expanded and analyzed for UL7 secretion by the use of mouse Fc and an IgG enzyme-linked immunosorbent assay (ELISA) kit (MedinaBio). The clone producing the largest amounts of fusion protein was cultured in EX-Cell 293 serum-free medium (Sigma-Aldrich). The supernatant containing the fusion protein was purified using an HiTrap protein A HP column (GE Healthcare) according to the manufacturer’s specifications.

To generate an anti-UL7 serum, the recombinant protein was used for rabbit immunization. The serum obtained by bleeding at 10 days after the fourth immunization was purified on an HiTrap protein A HP column (GE Healthcare) according to the manufacturer’s specifications. An isocontrol type was generated by purifying rabbit serum prior to immunization. Fc and CEACAM1 recombinant proteins were purchased at R&D Systems.

**Immunoblotting.** Extracts were run on an 8% to 12% SDS-PAGE gel, transferred to Immobilon-P transfer membranes (Milipore Corp.), and visualized with antibodies specific for phospho-STAT3 (Tyr705 and 32E; Cell Signaling), STAT3 (79D7; Cell Signaling), phospho-ERK1/2 (Thr202 and Tyr204; Cell Signaling Technology), ERK1 (C16; Santa Cruz), mouse monoclonal UL99 (pp28) and HA (HA-7; Sigma), rabbit UL7, and actin (C4; Millipore).

**IL-6 immunoassay.** A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of human IL-6 levels in tissue culture media was performed on the stored supernatants according to the manufacturer’s recommended procedure (R&D Systems).

**Sequence analysis.** Conserved domains in the UL7 sequence were identified using the NCBI Conserved Domain program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Protein sequence alignment was performed using the NCBI Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Statistical analysis.** Data are expressed as means ± standard errors of the means (SEM). Statistical significance of differences was determined by Student’s t-test and considered significant at P values of ≤0.05.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02035-14/-/DCSupplemental.

**REFERENCES**

1. Almond PS, Matas A, Gillingham K, Dunn DL, Payne WD, Gores P, Grossner R, Nejarian JS. 1993. Risk factors for chronic rejection in renal allograft recipients. Transplantation 55:752–756. http://dx.doi.org/10.1097/00007890-199304000-00013.

2. Melnick JL, Adam E, DeBakey ME. 1998. The link between CMV and atherosclerosis. Infect. Med. 15:479–486.

3. Spear E, Modali R, Huang ES, Leon MB, Shawl F, Finkel T, Epstein SE. 1994. Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 265:391–394. http://dx.doi.org/10.1126/science.8023160.

4. Cobbs CS, Harkins L, Samanta M, Gillespie GY, Bharara S, King PH, Nabors LB, Cobbs CG, Britt WJ. 2002. Human cytomegalovirus infection and expression in human malignant glioma. Cancer Res. 62: 3347–3350.

5. Orloff SL, Yin Q, Corless CL, Orloff MS, Rabbkin JM, Wagner CB. 2000. Tolerance induced by bone marrow chimerism prevents transplant vascular sclerosis in a rat model of small bowel transplant chronic rejection. Transplantation 69:1295–1303. http://dx.doi.org/10.1097/00007890-200004150-00005.

6. Orloff SL, Streblow DN, Soderberg-Naucler C, Yin Q, Kreklywich C, Corless CL, Smith PA, Loomis CB, Mills LK, Cook JW, Bruggeman CA, Nelson JA, Wagner GB. 2002. Elimination of donor-specific allograft rejection prevents cytomegalovirus-accelerated chronic rejection in rat small bowel and heart transplants. Transplantation 73:679–688. http://dx.doi.org/10.1097/00007890-200203150-00005.

7. Streblow DN, Kreklywich C, Yin Q, De La Melena VT, Corless CL, Smith PA, Brakebill C, Cook JW, Vink C, Bruggeman CA, Nelson JA, Orloff SL. 2003. Cytomegalovirus-mediated upregulation of chemokine expression correlates with the acceleration of chronic rejection in rat heart transplants. J. Virol. 77:2182–2194. http://dx.doi.org/10.1128/JVI.77.3.2182-2194.2003.

8. Merigan TC, Renlund DG, Keay S, Bristow MR, Starnes V, O’Connell JB, Resta S, Dunn D, Gamberg P, Ratkovc VM, Richenbacher WE, Millar RC, Dumond C, DeAmont B, Sullivan V, Cheney T, Buhles W, Stinson EB. 1992. A controlled trial of ganciclovir to prevent cytomegalovirus disease after heart transplantation. N. Engl. J. Med. 326:113–119. http://dx.doi.org/10.1056/NEJM199204023261803.

9. Valantine HA, Gao SZ, Menon SG, Renlund DG, Hunt SA, Oyer P, Stinson EB, Brown BW, Jr, Merigan TC, Schroeder JS. 1999. Impact of prothrombotic immediate post-transplant ganciclovir on development of transplant atherosclerosis: a post hoc analysis of a randomized, placebo-controlled study. Circulation 100:61–66. http://dx.doi.org/10.1161/01.CIR.100.1.61.

10. Streblow DN, Kreklywich CN, Andoh T, Moses AV, Dumortier J, Smith PP, DeFilippis V, Fruh K, Nelson JA, Orloff SL. 2008. The role of angiogenic and wound repair factors during CMV-accelerated transplant vascular sclerosis in rat cardiac transplants. Am. J. Transplant. 8:277–287. http://dx.doi.org/10.1111/j.1600-6143.2007.02062.x.

11. Dumortier J, Streblow DN, Moses AV, Jacobs JM, Kreklywich CN, Camp D, Smith RD, Orloff SL, Nelson JA. 2008. Human cytomegalovirus secretomes contain factors that induce angiogenesis and wound healing. J. Virol. 82:6524–6535. http://dx.doi.org/10.1128/JVI.00502-08.

12. Botto S, Streblow DN, DeFilippis V, White L, Kreklywich CN, Smith PP, Caposio P. 2011. IL-6 in human cytomegalovirus secretome promotes angiogenesis and survival of endothelial cells through the stimulation of survivin. Blood 117:352–361. http://dx.doi.org/10.1182/blood-2010-06-291245.

13. Engel P, Pérez-Carmona N, Albà MM, Robertson K, Ghazal P, Angulo JH, Horst A, Weil J, Fernando M, Wagener C. 2008. The role of angiogenic and wound repair factors during CMV-accelerated transplant vascular sclerosis in rat cardiac transplants. Am. J. Transplant. 8:277–287. http://dx.doi.org/10.1111/j.1600-6143.2007.02062.x.

14. Botto S, Streblow DN, Moses AV, Jacobs JM, Kreklywich CN, Smith PP, Caposio P. 2011. IL-6 in human cytomegalovirus secretome promotes angiogenesis and survival of endothelial cells through the stimulation of survivin. Blood 117:352–361. http://dx.doi.org/10.1182/blood-2010-06-291245.

15. Engel P, Pérez-Carmona N, Albà MM, Robertson K, Ghazal P, Angulo JH, Horst A, Weil J, Fernando M, Wagener C. 2008. The role of angiogenic and wound repair factors during CMV-accelerated transplant vascular sclerosis in rat cardiac transplants. Am. J. Transplant. 8:277–287. http://dx.doi.org/10.1111/j.1600-6143.2007.02062.x.

16. Dumortier J, Streblow DN, Moses AV, Jacobs JM, Kreklywich CN, Camp D, Smith RD, Orloff SL, Nelson JA. 2008. Human cytomegalovirus secretomes contain factors that induce angiogenesis and wound healing. J. Virol. 82:6524–6535. http://dx.doi.org/10.1128/JVI.00502-08.

17. Botto S, Streblow DN, DeFilippis V, White L, Kreklywich CN, Smith PP, Caposio P. 2011. IL-6 in human cytomegalovirus secretome promotes angiogenesis and survival of endothelial cells through the stimulation of survivin. Blood 117:352–361. http://dx.doi.org/10.1182/blood-2010-06-291245.

18. Ergün S, Kilik N, Ziegeler G, Hansen A, Nollau P, Götte J, Wurmback JH, Horst A, Weil J, Fernando M, Wagener C. 2000. CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effecter of
vascular endothelial growth factor. Mol. Cell 5:311–320. http://dx.doi.org/10.1016/S0092-8674(00)00426-8.

19. Chee MS, Bankier AT, Beck S, Bohni R, Brown CM, Cerny R, Horsnell T, Hutchison CA, Illi, Koziersides T, Martignetti JA, et al. 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. 154:125–169.

20. Dolan A, Cunningham C, Hector RD, Hassan-Walker AF, Lee L, Addison C, Dargan DJ, McGeoch DJ, Gatherer D, Emery VC, Griffiths PD, Sinzger C, McSharry BP, Wilkinson GW, Davison AJ. 2004. Genetic content of wild-type human cytomegalovirus. J. Gen. Virol. 85:1301–1312. http://dx.doi.org/10.1099/vir.0.79888-0.

21. Shikhaliev M, Mercè-Maldonado E, Isern E, Muntassell A, Albà MM, López-Botet M, Hengel H, Angeló A. 2012. The HCMV-specific UL1 gene encodes a late phase glycoprotein incorporated in the virion envelope. J. Virol. 86:4091–4101. http://dx.doi.org/10.1128/JVI.02629-11.

22. Gray-Owen SD, Blumberg RS. 2006. CEACAM1: contact-dependent control of immunity. Nat. Rev. Immunol. 6:433–446. http://dx.doi.org/10.1038/nri1864.

23. Horst AK, Ito WD, Dabelstein J, Schumacher U, Sander H, Turbide C, Brümmer J, Meinertz T, Beauchemin N, Wagener C. 2006. Carcinoembryonic antigen-related cell adhesion molecule 1 modulates vascular remodeling in vitro and in vivo. J. Clin. Invest. 116:1596–1605. http://dx.doi.org/10.1172/JCI24340.

24. Kuespert K, Pilis S, Haack CR. 2006. CEACAMs: their role in physiology and pathophysiology. Curr. Opin. Cell Biol. 18:565–571. http://dx.doi.org/10.1016/jceb.2006.08.008.

25. Turbide C, Kunath T, Daniels E, Beauchemin N. 1997. Optimal ratios of biliary glycoprotein isoforms required for inhibition of colonic tumor cell growth. Cancer Res. 57:2781–2788.

26. Singer BB, Scheffrath I, Obrink B. 2000. The tumor growth-inhibiting cell adhesion molecule CEACAM1 (C-CAM) is differently expressed in proliferating and quiescent epithelial cells and regulates cell proliferation. Cancer Res. 60:1226–1244.

27. Duncan SR, Paradis IL, Yousem SA, Similo SL, Gergurich WF, Williams PA, Dauber JH, Griffith BF. 1992. Sequelae of cytomegalovirus pulmonary infections in lung allograft recipients. Am. Rev. Respir. Dis. 146:1419–1425. http://dx.doi.org/10.1164/ajrccm.146.6.1419.

28. Humbert M, Delattre RM, Fattal S, Cerrina J, Davezelle P, Simonneau G, Douroux P, Galanau P, Emilius D. 1993. In situ production of interleukin-6 within human lung allografts displaying rejection or cytomegalovirus pneumonia. Transplantation 56:623–627. http://dx.doi.org/10.1097/00007890-199309000-00024.

29. Simmons P, Kaushansky K, Torok-Storb B. 1990. Mechanisms of cyto- megalovirus mediated myelosuppression: perturbation of stromal cell function versus direct infection of myeloid cells. Proc. Natl. Acad. Sci. U. S. A. 87:1386–1390. http://dx.doi.org/10.1073/pnas.87.4.1386.

30. Müller MM, Kläeke E, Vorontsova O, Singer BB, Obrink B. 2009. Homophilic adhesion and CEACAM1-S regulate dimerization of CEACAM1-L and recruitment of SHP-2 and c-Src. J. Cell Biol. 187:569–581. http://dx.doi.org/10.1083/jcb.200904150.

31. Kamimura D, Ishihara K, Hirano T. 2003. IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev. Physiol. Biochem. Pharmacol. 149:1–38. http://dx.doi.org/10.1007/s00224-003-0012-2.

32. Heinrich PC, Behrmann I, Müller-Newen G, Simons P, Emery VC, Griffiths PD, Sinzger C, McSharry BP, Wilkinson GW, Davison AJ. 2004. Genetic content of wild-type human cytomegalovirus. J. Gen. Virol. 85:1301–1312. http://dx.doi.org/10.1099/vir.0.79888-0.

33. Seino Y, Ikeda U, Ikeda M, Yamamoto K, Misawa Y, Hasegawa T, Kano S, Shimada K. 1994. Interleukin 6 gene transcripts are expressed in human atherosclerotic lesions. Cytokine 6:87–91. http://dx.doi.org/10.1016/1043-6666(94)90013-2.

34. Fischer P, Hilfiker-Kleiner D. 2007. Survival pathways in hypertrophy and heart failure: the gp130-STAT axis. Basic Res. Cardiol. 102:393–411. http://dx.doi.org/10.1007/s00395-007-0560-0.

35. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F. 2003. Principles of interleukin (IL)-6-type cytokine signaling and its regulation. Biochem. J. 374:1–20. http://dx.doi.org/10.1042/BJ20030407.

36. Kurdi M, Booz GW. 2009. JAK redux: a second look at the regulation and role of JAKs in the heart. Am. J. Physiol. Heart Circ. Physiol. 297:11545–11556. http://dx.doi.org/10.1152/ajpheart.00322.2009.

37. Lorenz K, Schmitt JP, Vidal M, Lohse MJ. 2009. Cardiac hypertrophy: targeting Raf/MEK/ERK1/2-signaling. Int. J. Biochem. Cell Biol. 41:2351–2355. http://dx.doi.org/10.1016/j.biocel.2009.08.002.

38. Slenger E, Maussang D, Schreiber A, Siderius M, Rahbar A, Fraile-Ramos A, Lira SA, Söderberg-Nauclér C, Smit MJ. 2010. HCMV-encoded chemokine receptor US28 mediates proliferative signaling through the IL-6-STAT3 axis. Sci. Signal. 3:r45a8. http://dx.doi.org/10.1126/scisignal.2001180.

39. Smith IL, Taskittuna I, Rahhal FM, Powell HC, Ai E, Mueller AJ, Spector SA, Freeman WR. 1998. Clinical failure of CMV retinitis with intravitreal cidofovir is associated with anti viral resistance. Arch. Ophthalmol. 116:178–185.

40. Murphy E, Yu D, Grimwood J, Schmutz J, Dickson M, Jarvis MA, Hahn U. S. A. G, Nelson JA, Myers RM, Shenk TE. 2003. Coding potential of laboratory and clinical strains of human cytomegalovirus. Proc. Natl. Acad. Sci. U. S. A. 100:14976–14981. http://dx.doi.org/10.1073/pnas.216652100.

41. Britt WJ, Jarvis M, Seo JY, Drummond D, Nelson J. 2004. Rapid genetic engineering of human cytomegalovirus using a lambda phage linear recombination system: demonstration that pp28 (UL90) is essential for production of infectious virus. J. Virol. 78:539–543. http://dx.doi.org/10.1128/JVI.78.1.539-543.2004.

42. Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG. 2005. Simple and highly efficient BAC recombineering using galk selection. Nuclear Acids Res. 33:e36. http://dx.doi.org/10.1093/nar/gni035.

43. Hisa DA, Mitra SK, Hauck CR, Streblow DN, Nelson JA, Ilic D, Huang S, Li E, Nemerov GR, Leng J, Spencer KS, Cheres S, Schlaepfer DD. 2003. Differential regulation of cell motility and invasion by FAK. J. Cell Biol. 160:753–767. http://dx.doi.org/10.1083/jcb.20021114.

44. Streblow DN, Söderberg-Nauclér C, Vieira J, Smith P, Wakabayashi E, Ruchti F, Mattison K, Altschuler Y, Nelson JA. 1999. The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. Cell 99:511–520. http://dx.doi.org/10.1016/S0092-8674(00)81539-1.

45. Murphy EA, Streblow DN, Nelson JA, Stinski MF. 2000. The human cytomegalovirus IE86 protein can block cell cycle progression after inducing transition into the S phase of permissive cells. J. Virol. 74:7108–7118. http://dx.doi.org/10.1128/JVI.74.15.7108-7118.2000.