Role of human cytomegalovirus UL131A in cell type-specific virus entry and release

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The human cytomegalovirus (HCMV) genes UL128, UL130 and UL131A are essential for endothelial cell infection. Complementation of the defective UL131A gene of the non-endotheliotropic HCMV strain AD169 with wild-type UL131A in cis in an ectopic position restored endothelial cell tropism. The UL131A protein was found in virions in a complex with gH. Coinfection of fibroblasts with UL131A-negative and -positive viruses restored the endothelial cell tropism of UL131A-negative virions by complementing the virions with UL131A protein. Virus entry into endothelial cells, but not into fibroblasts, was blocked by an antipeptide antiserum to UL131A. AD169, cis-complemented with wild-type UL131A, showed an impaired release of infectious particles from fibroblasts. A comparable defect in virus release was observed when UL131A was expressed ectopically in a virus background already expressing an intact copy of UL131A. In contrast, virus release from infected endothelial cells was not affected by UL131A. These data suggest a dual role for pUL131A in virus entry and virus exit from infected cells.

INTRODUCTION

Human cytomegalovirus (HCMV) is the major cause of morbidity in immunocompromised patients and allogeneic bone-marrow or organ-transplant recipients. It is also the leading agent of birth defects among congenitally transplanted infections (Pass, 2001). In vivo, HCMV can infect a broad range of cell types, including endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, macrophages and monocytes (Plachter et al., 1996). However, the adaptation of clinical isolates to fibroblasts results in the inability to replicate efficiently in endothelial cells or monocytes (Waldman et al., 1989; MacCormac & Grundy, 1999; Sinzger et al., 1999; Kahl et al., 2000; Grazia Revello et al., 2001) and to transfer virus from virus-infected cells to leukocytes (Grazia Revello et al., 2001; Hahn et al., 2004). The three open reading frames of the UL128–131A locus are indispensable for virus growth in endothelial, epithelial and dendritic cells (Grazia Revello et al., 2001; Hahn et al., 2004; Gerna et al., 2005; Wang & Shenk, 2005a). All non-endotheliotropic laboratory strains tested show mutations in the UL128–131A locus affecting at least one of the genes (Akter et al., 2003; Hahn et al., 2004). Thus, an intact/wild-type UL128–131A locus is essential for infection of most of the host cells of HCMV, the major exception being fibroblasts.

It has been claimed that HCMV enters fibroblasts by fusion at the cell membrane (Compton et al., 1992), and epithelial and endothelial cells by endocytosis (Bodaghi et al., 1999; Ryckman et al., 2006). Endothelial cell entry of HCMV laboratory strains is apparently blocked at a post-entry step before transfer of the DNA to the nucleus (Sinzger et al., 2000). As the entry block can be released by reversion of mutations in the UL128–131A locus, a role of these proteins in the entry process can be assumed (Hahn et al., 2004; Wang & Shenk, 2005a). UL128–131A genes are transcribed with late kinetics (Akter et al., 2003). As all three proteins are necessary for the capacity of HCMV to infect endothelial cells, it is likely that they interact functionally or even physically. pUL128 and pUL130 have recently been reported to be virion constituents (Patrone et al., 2005; Wang & Shenk, 2005b) and to form a complex with the viral envelope proteins gH and gL (Wang & Shenk, 2005b). The UL131A gene product has not been studied so far.

Here, we studied the role of pUL131A in infection of fibroblasts and endothelial cells. UL131A encodes a 129 aa long protein with no obvious sequence similarity to known proteins. The non-endotheliotropic HCMV strain AD169 encodes a truncated pUL131A. Repair of the defective UL131A gene in AD169 reconstitutes endothelial cell tropism (Hahn et al., 2004; Wang & Shenk, 2005a). To study pUL131A, we cis-complemented the bacterial artificial chromosome BAC-AD169 genome by insertion of the wild-type UL131A gene or a haemagglutinin (HA)-tagged
construct at an ectopic position. The complementation reconstituted the endothelial cell tropism, but negatively influenced virus spread in fibroblast cultures. pUL131A was detected in lysates of purified virions and was found to form a complex with gH. Endothelial cell infection was blocked selectively by pUL131A antiserum. Taken together, our data imply that pUL131A plays a dual role in HCMV infection, namely in cell-specific entry and in exit from infected cells.

**METHODS**

**Cells and viruses.** Human foreskin fibroblasts (HFFs) were used from passage 12 to 22 and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. TIME (telomerase-immortalized human microvascular endothelial) cells (Venetsanakos et al., 2002) were maintained in an EGM-2 MV BulletKit medium system (Cambrex).

The HCMV strain AD169 (Borst et al., 1999) and the endotheliotropic HCMV isolate VR1814 (Hahn et al., 2002) have been cloned as BACs (BAC-AD169 and BAC-VR1814). Viruses reconstituted from BACs (V-AD169 and V-VR1814) retain the parental cell tropism. Supernatants were cleared of (V-AD169 and V-VR1814) retain the parental cell tropism. Supernatants were cleared of virus titres were determined by TCID₅₀ assays on HFFs in 96-well plates. Green fluorescent protein (GFP)-expressing virus was titrated in the presence of 1 μg doxycycline ml⁻¹ and only green plaques were counted.

To infect cells, the medium was removed from 90% confluent cell monolayers and replaced by virus diluted in medium for 2 h. For some experiments, virus uptake was enhanced by a centrifugation step (30 min, 2000 g at room temperature). For co-culture infections, HCMV-infected HFFs or TIME cells showing complete CPE were mixed at 10-fold dilutions with uninfected HFFs or TIME cells. Infections were screened either by indirect immunofluorescence staining for HCMV immediate-early protein or by inspection for GFP-positive cells after induction with 1 μg doxycycline ml⁻¹.

To block infection, infected cells or virus stocks were preincubated with a 1:20 dilution of anti-pUL131A rabbit antiserum or preimmune serum. Preincubation was performed in a 100 μl volume for 1 h at 4°C. The antigen specificity of the block was tested by depletion of the antiserum with 15 μg KLH-coupled peptides, either one specific for pUL131A (CDFFQQRRGGTNKRR) or an irrelevant control peptide (VALRCPDGEVCYSPE).

**Gradient purification of HCMV virions.** For gradient purification of HCMV virions, infectious supernatants from infected HFF cultures showing approximately 100% late-stage CPE were cleared from cell debris by centrifugation for 10 min at 2800 g. Supernatants were then ultracentrifuged for 70 min at 80 000 g. Pellets containing virions were resuspended in 1 ml PBS and transferred onto a preformed, linear glycerol/tartrate gradient (15–35% sodium tartrate and 30–0% glycerol in 0.04% sodium phosphate), which was ultracentrifuged for 45 min at 80 000 g. The virion-containing band was harvested with a syringe and the virions were washed and pelleted by an additional ultracentrifugation for 70 min at 80 000 g. The pellet was resuspended in 0.04% sodium phosphate and stored at −80°C until used for experiments.

**Plasmids.** The UL131A reading frame was amplified by PCR from cDNA generated from total RNA of VR1814-infected HFFs by using oligo(dT)₁₈₋₂₂ primers (Invitrogen). UL131A was amplified as an untagged sequence (UL131Afor, 5′-GGCCGATTTCATGCGCCT-TGTGCGGTTGGCTGCTG-3′; UL131Arev, 5′-ATCTGACTCTGAC-TGGCAAAGACTGCGAC-3′) or as a C-terminally HA-tagged sequence (UL131Afor; UL131ArevHA, 5′-ATCTGACTCTCTAAG-CGATCTCGGAGCCTCGATAGTTGCGGAAAGATGGC-ACGC-3′). The PCR products were cloned into the EcoRI/BglII sites of pSG5 (Stratagene) to be expressed under the control of the simian virus 40 (SV40) promoter. For ectopic expression of UL131A proteins, SalI fragments of pSG5 containing the complete SV40–UL131A expression cassette were filled in by Klenow polymerase and cloned into the EcoRV site of pOriR6K-zeo (Bubeck et al., 2004). Inducible GFP was inserted into the pHBS/FRT BAC from pO6-SVT-gfp-TR (Rupp et al., 2005).

**BAC mutagenesis.** For insertion of a 48 bp FRT site into the UL45 locus, a linear PCR fragment containing a kanamycin-resistance gene flanked by two 48 bp FRT sites and sequences homologous to the HCMV UL45 coding region (Hahn et al., 2002) was generated with primers UL45-FRT-Kanfor (5′-GGCCAGTTTACACTGAGC-ATCTGTTGCCAAGAAGCAGTCGGCGCTATCCCCAGTACGATAG-TAGCGGGGTTCTGCGGTTCCC-3′) and UL45-FRT-Kanrev (5′-ACATCTGAGACAGATTTTAAACCGTATGTTCGCGG-CCATCTAGACTCTACTTTTTAGGAAACTCCTCGCTGATGTGTTGT- GTG3′) and pCP15 as template (Cherepanov & Wackernagel, 1995). The resulting fragment was inserted into BAC-AD169 and BAC-VR1814 by homologous recombination in Escherichia coli, thereby deleting 2-7 kb of the UL45 locus as described previously (Hahn et al., 2002; Wagner et al., 2002). The kanamycin-resistance gene was subsequently excised by FLP-mediated site-directed recombination (Bubici et al., 2004).

SV40-driven UL131A with or without a HA tag cloned in pOriR6K-zeo was inserted into the FRT sites by using the pOriR6K-zeo vector and the temperature-sensitive FLP expression plasmid pCP20 (Bubeck et al., 2004). To introduce an inducible GFP, the construct pO6-SVT-gfp-TR was used (Rupp et al., 2005).

Insertions/deletions were controlled by restriction-pattern analysis, followed by Southern blot analysis using a UL45 PCR product amplified from a pHBS BAC plasmid (UL45for, 5′-GGCCGATCAC-GACGCGCCGAG-3′; UL45rev, 5′-TGCTCGAGATCCGATTGGCGG-3′) to detect restriction fragments with UL45 sequences. The UL131A SalI fragment from the pSG5 construct was used to detect restriction fragments containing the UL131A expression cassette.

**Reconstitution of virus from recombinant BACs.** BAC plasmids were reconstituted to virus by transfection of BAC DNA into HFFs with an MBS transfection kit (Stratagene) following the instructions of the manufacturer. Circular viral DNA was isolated by the method of Hirt (1967) as described previously (Messerle et al., 1997).

**Antibodies and antisera.** HCMV-specific mAbs used were mouse anti-pp65 antibody (MAB8870; Chemicon), mouse anti-immediate-early protein 1 antibody (anti-ie1; Perkin Elmer), mouse anti-gB antibody (2F12; Abcam) and mouse anti-gH antibody (AP86, kindly provided by W. Britt, University of Alabama, Birmingham, AL, USA). HA-tagged protein was detected by using a rat anti-HA antibody (3F10; Roche Diagnostics). Fc receptors were blocked by preincubation of the cells with Fc-blocking reagent (1:20) (Miltenyi Biotec).

To generate a pUL131A-specific antisera, rabbits were immunized with two pUL131A peptides (CDFFQQRRGGTNKRR and
CSRALPDQTRYKVEQ (Eurogentec). Antibodies were purified by affinity columns coupled with the peptides used for immunization (Eurogentec).

**Immunoprecipitation and Western blot analysis.** Cells or purified virions were lysed in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0-1% SDS, 0-5% deoxycholate] containing a protease inhibitor cocktail (Roche Diagnostics). Comparable protein amounts were subjected to immunoprecipitation by using the rabbit anti-pUL131A antiserum and protein G-Sepharose (Amersham Biosciences) for precipitation of protein–antibody complexes. For Western blot analysis, precipitates, cell pellets or purified virions were lysed in sample buffer [8-13 M Tris/HCl (pH 6-8), 6% SDS, 10% 2-thioglycerol], separated on 10 or 15% polyacrylamide gels and transferred onto nylon Hyb-Bind-P membranes (Amersham Biosciences). Membranes were blocked with 5% low-fat milk in PBS, incubated with the respective primary antibodies and detected by using peroxidase-coupled secondary antibodies and enhanced chemiluminescence (ECL system; Amersham Biosciences).

**Indirect immunofluorescence staining.** HCMV-infected cells were detected by staining for immediate-early protein 1 (ie1) of HCMV. Cells were fixed in 50% acetone/50% methanol, incubated with an anti-ie1 antibody for 45 min and detected with Cy3-coupled goat anti-mouse antibody (Dianova).

For confocal laser-scanning microscopy, cells were seeded on glass coverslips, fixed with 4% paraformaldehyde and permeabilized with 0-1% Triton X-100. To block Fc receptors expressed in HCMV-infected cells, cells were preincubated with an FcR block for 30 min and then stained with affinity-purified anti-pUL131A and anti-pp65 antibodies and detected by Fluor488-coupled goat anti-rabbit antibody (Molecular Probes) and Texas red-coupled goat anti-mouse antibody (Dianova), respectively. Stained cells were mounted in Vectashield (Vector Laboratories) and analysed by using a Zeiss Axiovert 200M microscope and the Zeiss LSM510 Meta laser system.

**RESULTS**

**Wild-type UL131A reconstitutes the endothelial cell tropism of HCMV strain AD169**

The laboratory strain AD169, a prototypic non-endotheliotropic strain, carries a frame-shift mutation in the UL131A gene that results in a putative truncated protein with an aberrant C-terminal amino acid sequence. We complemented the defect UL131A in cis by ectopic expression of a wild-type UL131A reading frame. An FRT site cloned in the UL45 locus (Hahn et al., 2002) served for the integration of the wild-type UL131A gene. The gene cassette contained the 129 aa long UL131A reading frame from the HCMV isolate VR1814 (Grazia Revello et al., 2001) or a C-terminally HA-tagged version under the control of the SV40 promoter (Fig. 1a). The gene cassettes were inserted into BAC-AD169 and also into BAC-VR1814, which carries a functional UL131A. The latter BAC thus carries two copies of UL131A, which permit controls for phenotypic changes due to positional effects or due to SV40 promoter control. Insertion at the UL45 locus was confirmed by restriction analysis of the BACs followed by Southern blots. In EcoRI digests, the 3.4-kbp EcoRI fragment specific for UL45 in BAC-AD169 and BAC-VR1814 was lost in the FRT BACs and the BACs carrying the ectopic UL131A (Fig. 1b) and a new fragment of 0.7 kbp, representing a UL45 residual sequence, appeared. A fragment of 1.4 kbp revealed the UL131A expression cassette in addition to the EcoRI fragments carrying the endogenous UL131A sequence (3.3 kbp for BAC-AD169 and 12.7 kbp for BAC-VR1814) (Fig. 1c). The genotype of viruses reconstituted from BACs was confirmed by restriction analysis of viral DNA and Southern blot (Fig. 1d).

The reconstituted viruses were tested for their capacity to infect endothelial cells. VR1814- and AD169-derived viruses are poor at infecting endothelial cells as free virus (Grazia Revello et al., 2001; Gerna et al., 2002; Hahn et al., 2004). Endothelial cell tropism is revealed by co-culture experiments in which infected fibroblasts are mixed with uninfected endothelial cells and the spread of infection from fibroblasts to the neighbouring uninfected cells is
monitored. Non-endotheliotropic strains fail to infect endothelial cells in co-culture experiments. We used the microvascular cell line TIME as endothelial host cells (Venetsanakos et al., 2002). TIME cells offer two advantages. First, they can be cultured continuously and are thus more convenient to handle than primary human umbilical cord-derived HUVECs, and second, due to the small size, the microvascular cells can be distinguished easily by optical inspection from fibroblasts in co-culture experiments (data not shown). *cis*-Complementation with wild-type UL131A restored the endothelial cell tropism of AD169. In co-culture experiments, V-AD169/UL131A or V-AD169/UL131A/HA spread from infected HFFs to TIME cells, whereas the parental virus V-AD169/FRT did not spread (Fig. 2a). The endotheliotropic control viruses V-VR1814/FRT and V-VR1814/UL131A showed a comparable pattern of spreading (Fig. 2a). The presence of pUL131A in V-AD169/UL131A or V-AD169/UL131A/HA led to an only about fivefold increase in the efficiency of infection of TIME cells by free virus (Table 1). Even an m.o.i. of 5 was insufficient to infect all TIME cells in a culture.

The UL131A protein was detected in HCMV-infected cells by using a rabbit antiserum raised against a pUL131A peptide. HFFs were stained 96 h after infection (Fig. 2b). Infected foci were detected by staining for HCMV pp65 tegument protein, which showed the typical nuclear staining early after infection and a cytoplasmic staining at later times of infection (Sanchez et al., 2000). There was no pUL131A-specific staining in foci of V-AD169/FRT-infected cells, whereas foci of V-AD169/UL131A- and V-AD169/UL131A/HA-infected cells were strongly positive for pUL131A. Expression of endogenous pUL131A in V-VR1814/FRT-infected cells was very weak, whereas V-VR1814/UL131A-infected cells showed a pattern comparable to that of V-AD169/UL131A-infected cells. Endogenous pUL131A in V-VR1814/FRT-infected cells was detectable only at late time points after infection, which is in accordance with the kinetics of UL131A RNA expression (Akter et al., 2003). Remarkably, expression of the SV40-driven pUL131A also followed late kinetics (data not shown).

**UL131A expression impairs virus release from fibroblasts**

After infection of HFFs with V-AD169/UL131A and V-AD169/FRT at a low m.o.i., the titres of cell-associated virus were comparable (Fig. 3a). However, V-AD169/UL131A showed a significant delay in the release of free virus into the supernatant when compared with V-AD169/FRT (Fig. 3a). V-VR1814/UL131A showed the same phenotype. This difference was most apparent 5 days after infection when

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**Fig. 2.** Reconstitution of the endothelial cell tropism of V-AD169 by UL131A. (a) HFFs infected with V-AD169/FRT, V-AD169/UL131A, V-AD169/UL131A/HA, V-VR1814/FRT or V-VR1814/UL131A were co-cultured with non-infected TIME cells on 96-well plates for 5 days. Spread of infection was detected by indirect immunofluorescence staining for HCMV ie1. (b) Indirect immunofluorescence staining of HFFs infected with the indicated BAC-derived viruses. Cells were stained for pUL131A (green) and pp65 (red). Bar, 20 μm.
cell monolayers were still intact. The number of HCMV immediate-early protein-positive nuclei was comparable 24 h after infection, indicating that the infection rate of fibroblasts was not affected by the UL131A copy (data not shown). We explain the slightly higher titres of cell-associated virus of V-AD169/FRT compared with V-AD169/UL131A by virus spread via the supernatant. In contrast, virus production in endothelial cells was not affected by ectopic expression of UL131A (Fig. 3a). We also compared virus productivity of the endotheliotropic viruses V-VR1814/FRT and V-VR1814/UL131A in HFFs and TIME cells. After infection with V-VR1814/UL131A at a low m.o.i., a significant reduction of supernatant virus was observed in fibroblasts (Fig. 3b). No difference in virus production was

<table>
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<tr>
<th>Virus</th>
<th>No. ie-positive cells*</th>
<th>No. GFP-positive cells†</th>
<th>Fold increase in ie-positive cells compared with V-AD169/gfp</th>
<th>Fold increase in GFP-positive cells compared with V-AD169/gfp</th>
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<tr>
<td>V-AD169/gfp</td>
<td>12.6 ± 2.9</td>
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<tr>
<td>V-AD169/UL131A</td>
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<td>5.1 ± 1.4</td>
<td>NA</td>
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<tr>
<td>V-AD169/gfp + V-AD169/UL131A</td>
<td>NA</td>
<td>59.7 ± 2.9</td>
<td>4.1 ± 0.6</td>
<td>NA</td>
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</table>

*TIME cells were infected with V-AD169/gfp or V-AD169/UL131A (m.o.i. of 0.3). HCMV immediate-early protein 1 (ie) was monitored by indirect immunofluorescence 48 h after infection.
†TIME cells were infected with free virus derived from HFFs infected with V-AD169/gfp or coinfected with V-AD169/gfp + V-AD169/UL131A (m.o.i. of 0.3 GFP-positive virus). After infection, 1 μg doxycycline ml⁻¹ was added to the fresh medium and GFP expression was documented after 4 days.

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Table 1. UL131A-dependent complementation of endothelial cell tropism: transfer of endothelial cell tropism to GFP-positive virus

Data shown are means ± SD of three independent experiments. In each experiment, cells in four different microscopic fields were counted. NA, Not applicable; ND, not done.

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Fig. 3. Effects of pUL131A on virus spread and release. (a) Multistep growth curves of the indicated viruses (m.o.i. of 0.2) on HFFs or TIME cells. Cells were infected in 24-well plates. Supernatants and infected cells were harvested at the indicated time points post-infection. A representative experiment is shown. (b) Virus titres (day 10) in supernatants of HFFs or TIME cells infected with V-VR1814/FRT (empty bars) and V-VR1814/UL131A (filled bars). Shown are means ± SD of at least three independent experiments. Titres in supernatants of HFFs infected with V-VR1814/UL131A compared with HFFs infected with V-VR1814/FRT were significantly different, as indicated by an asterisk (Student’s t-test; P = 0.0026). (c) Virus spread in HFFs and TIME cells. Cells were infected with an m.o.i. of 0.2. Six days after infection, cells were stained for HCMV ie1 by indirect immunofluorescence labelling. A centrifugation step was included to enhance the infection rates in endothelial cells.
seen in TIME cells and when HFFs were infected with a high m.o.i. (Fig. 3b). The time point 10 days after infection was chosen because virus titres in supernatants of TIME cells were very low at earlier time points of infection (Fig. 3a). Altogether, ectopic expression of pUL131A delayed and reduced virus production in HFFs and supported cell-associated virus spread, leading to the formation of small foci rather than to an even distribution of the infection throughout the cell culture (Fig. 3c).

**pUL131A is a virion constituent and complements the endothelial cell tropism of pUL131A-negative virions**

In Western blots from lysates of cells infected with viruses expressing UL131A ectopically, the antiserum to pUL131A detected three pUL131A-specific bands with a molecular mass of about 15 kDa (Fig. 4a). As expected, the addition of the HA tag shifted the size of the bands to higher molecular masses. The UL131A reading frame encodes a 129 aa long protein with an amino-terminal signal peptide of 18 aa. The three bands may reflect pUL131A proteins with and without signal peptide and/or other protein modifications. Remarkably, the fastest-migrating band of pUL131A/HA was also present in extracts from purified virions. The anti-pUL131A antiserum was less sensitive than the anti-HA antibody; therefore, it was not possible to detect the untagged protein in lysates of virions. The purity of the virion preparation was documented by the presence of the virion envelope protein gB and the absence of the abundant, but non-structural, 72 kDa immediate-early protein of HCMV (Fig. 4a). The anti-pUL131A antiserum precipitated gH from lysates of HFFs infected with V-AD169/UL131A or V-AD169/UL131A/HA (Fig. 4b) or from lysates of purified virions (Fig. 4c). Lysates of V-AD169/FRT-infected cells, which are negative for pUL131A, or lysates of virions derived from these cells showed no gH precipitation with the anti-pUL131A antiserum. We concluded that pUL131A forms a complex with the envelope glycoprotein gH and that this complex is incorporated into virions.

![Western blot analysis of pUL131A expression in infected cells and virions](image)

**Fig. 4.** pUL131A is present in lysates of infected cells and in virions and forms a complex with gH. (a) Lysates of infected HFFs and lysates of purified virions from supernatants were analysed by Western blot (WB) using antibodies directed against pUL131A, HA tag, HCMV gB and HCMV ie1. The specific protein bands are indicated by brackets (three pUL131A-specific bands) or by arrows. C, Cell lysates; V, virions. (b) Immunoprecipitation of gH by pUL131A antiserum from lysates of HFFs infected with different viruses. (c) Immunoprecipitation of gH by pUL131A antiserum from lysates of the respective purified virions. The amount of gH (indicated by an arrow) in total lysates is shown as a control.
The direct contribution of pUL131A to endothelial cell infection was also tested by pseudotyping UL131A-negative virus during mixed infections of fibroblasts with AD169 expressing ectopic UL131A and AD169 lacking pUL131A. V-AD169/gfp served as acceptor virus; it expresses GFP from the UL45 locus and cannot infect endothelial cells. The donor virus, V-AD169/UL131A, expresses pUL131A but lacks GFP. After complementation with the UL131A protein, V-AD169/gfp should be able to infect endothelial cells and, at the same time, express GFP. HFFs were infected at an m.o.i. of 3 with V-AD169/gfp only or with a mixture of V-AD169/gfp and V-AD169/UL131A. After 5 days, the number of viruses giving rise to green plaques was determined in the supernatant and supernatants were used to number of viruses giving rise to green plaques was determined in the supernatant and supernatants were used to infect TIME cells at an m.o.i. of 0.3. Supernatants from mixed infections showed a fourfold increase in the number of GFP-positive TIME cells over supernatants from HFFs infected with V-AD169/gfp only (Table 1). A comparison of the infection efficiency of the individual viruses V-AD169/gfp and V-AD169/UL131A showed a fivefold-better infection efficiency for the virus expressing pUL131A (Table 1). Thus, both genetic complementation by ectopic expression and pUL131A protein pseudotyping after mixed infections result in a comparable rescue of endothelial cell infection. No increase in the number of GFP-positive TIME cells was observed when V-AD169/gfp and V-AD169/UL131A virus stocks were mixed and given directly to TIME cells (data not shown). Recombination could result in stably GFP- and UL131A-positive viruses. One thousand GFP-positive TIME cells were screened microscopically over 10 days. Only single GFP-positive cells, but no GFP-positive plaques, could be detected. Thus, the phenotype only lasted for a single round of infection and was due to protein complementation (data not shown). Notably, when supernatants from mixed infections were preincubated with antisera to pUL131A, the infection of endothelial cells was blocked (Table 2). There was no inhibition after preincubation with preimmune serum and, also, the basal infection capacity of pUL131A-negative virions (V-AD169/gfp) was not affected (Table 2). We concluded from these data that pUL131A is a virion constituent and contributes directly to endothelial cell infection.

pUL131A antiserum specifically blocks infection of endothelial cells

To study the role of pUL131A for fibroblast and endothelial cell infection, virus-containing supernatants or virus-infected HFFs or TIME cells were preincubated with rabbit antiserum to pUL131A or with the preimmune serum for control. The antiserum had no effect on the infection of HFFs, independent of the virus source (Fig. 5a, b). In contrast, the infection of TIME cells by supernatant virus or by co-culture with infected HFFs was blocked (Fig. 5a). Preincubation of the immune serum with the KLH-coupled pUL131A peptide used for immunization lifted the inhibition, whereas a control peptide had no effect (Fig. 5c). This inhibition was not restricted to TIME cells, as infection of HUVECs was also blocked (data not shown). Preincubation of cells with anti-pUL131A rabbit antiserum had no effect, which excluded an effect on putative cellular receptors for HCMV (data not shown). Therefore, pUL131A contributes selectively and directly to infection of endothelial cells.

### DISCUSSION

The results reported here show the following. (i) Both protein complementation of the defective gene UL131A in HCMV strain AD169 and genetic complementation by a wild-type UL131A gene copy rescue endothelial cell infection. (ii) The UL131A protein is part of the virion and forms a complex with the envelope glycoprotein gH. (iii) Antibodies against the UL131A protein selectively block infection of endothelial cells. (iv) Increased expression of pUL131A impaired virus release in a cell type-dependent manner. High expression of pUL131A in fibroblasts delays virus release, whereas in endothelial cells, the high pUL131A expression level has no such effect. Collectively, the data suggest strongly that pUL131A contributes directly to endothelial cell entry and control of virus exit from fibroblasts.

HCMV infection of endothelial, dendritic and epithelial cells depends on three viral genes, UL128, UL130 and UL131A (Hahn et al., 2004; Gerna et al., 2005). Deletion or mutation of one, two or all three genes is found consistently.

### Table 2. UL131A-dependent complementation of endothelial cell tropism: transfer of pUL131A to GFP-positive virus

See legend to Table 1.

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<tr>
<th>Virus</th>
<th>GFP-positive cells*</th>
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<th>GFP-positive cells†</th>
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<tbody>
<tr>
<td></td>
<td>no serum</td>
<td>+preimmune serum</td>
<td>+anti-pUL131A</td>
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<tr>
<td>V-AD169/gfp</td>
<td>4.7±1.6</td>
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<td>V-AD169/gfp + V-AD169/UL131A</td>
<td>46.3±7.5</td>
<td>32.7±11.4</td>
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*TIME cells were infected with free virus derived from HFFs infected with V-AD169/gfp or coinfected with V-AD169/gfp + V-AD169/UL131A (m.o.i. of 0.3 GFP-positive virus). After infection, 1 µg doxycycline ml⁻¹ was added to the fresh medium and GFP expression was documented after 4 days.
†Before infection, virus-containing supernatants were preincubated with preimmune serum or anti-pUL131A antiserum for 1 h.
in fibroblast-adapted strains. Recently, the functions of the three individual genes were studied. The endothelial cell tropism of a UL130-negative HCMV strain could be trans-complemented in pUL130-expressing cells, which produced a genotypically UL130-negative, phenotypically endotheliotropic virus. The UL130 protein was found in virions of the endotheliotropic isolate VR1814 (Patrone et al., 2005).

Wang & Shenk (2005a) corrected the single nucleotide insertion in UL131A of AD169. The phenotype of the corrected virus was compromised virus replication in fibroblasts and the rescue of virus growth in endothelial and epithelial cells. In another study, Wang & Shenk (2005b) analysed the virion composition and detected the UL128 and UL130 gene products in a complex with gH and gL. Antibodies to pUL128 and pUL130 blocked the infection of endothelial cells, but not of fibroblasts. Viruses lacking a functional UL131A did not contain pUL128 or pUL130 as virion components. As pUL131A was not detected in the virions of endotheliotropic viruses, pUL131A could contribute directly or indirectly to the formation of the complex between pUL128, pUL130 and gH–gL.

Here, we show that the UL131A gene product contributes by direct complex formation with gH. The UL131A protein is a virion component and the antiserum to pUL131A prevents endothelial cell infection. Most likely, all three gene products of the UL128–131A locus are involved in the entry process and it will be the subject of future studies to clarify whether entry into endothelial and epithelial cells by endocytosis is determined by these three proteins (Ryckman et al., 2006). As cell-membrane penetration by virions has also been observed in HCMV strains that have a defective UL128–131A locus (Sinzger et al., 2000), these virions are probably misdirected to an abortive entry pathway.

Associated with a UL131A-complemented gain of entry into endothelial cells is compromised growth in fibroblasts after infection with a low m.o.i. cis-Complementation under the control of the SV40 promoter resulted in an about 100-fold reduction of virus release. A similar reduction of virus release into the supernatant was also observed when the UL131A gene was repaired (Wang & Shenk, 2005a). As both

**Fig. 5.** Antibodies to pUL131A block infection of TIME cells. (a) HFFs or TIME cells were either infected by co-cultivation with VR1814-infected HFFs or infected with VR1814 from supernatants of infected cells. VR1814-infected cells or virus stocks were preincubated for 1 h with the indicated antisera as described in Methods. Infection (m.o.i. of 1) with free virus was carried out by centrifugation. Cells were fixed 5 days after co-culture or 48 h after infection with supernatant virus and stained for HCMV ie1 by indirect immunofluorescence. (b) VR1814-infected TIME cells were co-cultured with HFFs to test the spread from TIME cells to HFFs. (c) TIME cells were infected with free virus pretreated with rabbit antiserum to pUL131A. The antiserum was preincubated with the pUL131A peptide or an irrelevant peptide. After infections with free virus, nuclei positive for HCMV immediate-early protein from three randomly chosen microscopic fields were counted. The mean ± SD of these three counts is depicted at the bottom of a representative field.
repairs and cis-complementation led to a virus-release deficit in fibroblasts, this is probably an aberrant property of the wild-type UL131A gene product and not an artefact of the genomic construction. The V-VR184/FRT / V-VR184/UL131A pair, which differs in the levels of pUL131A, provides a tool to compare infections in fibroblasts and endothelial cells. A higher level of pUL131A resulted in a reduced virus production specifically in fibroblasts. We could show that the reduced virus production was not due to a reduction in the number of infected cells, but rather to a change in the virus-release phenotype. The UL131A protein apparently redirects the virion to a more cell-associated, and therefore slower, spread. This is the reverse of what is selected for when clinical isolates are adapted for productivity in fibroblast cultures (Sinzer et al., 1999).

Virus-encoded proteins in the virion envelope define the cellular tropism of herpesviruses. At least three glycoprotein homologues, gB, gH and gL, shared by all herpesviruses, are essential for cell attachment and penetration. The gH–gL complex is complemented by additional proteins to mediate specific receptor binding (reviewed by Spear & Longnecker, 2003). Whereas herpes simplex virus requires in addition the glycoprotein gD, in Epstein–Barr virus, the presence or absence of gp42 in the gH–gL complex determines the tropism for B cells or epithelial cells, respectively (Borza & Hutt-Fletcher, 2002; Borza et al., 2004). For Human herpesvirus 6, two tripartite complexes, gH–gL–gO and gH–gL–gQ, have been described. The complex with gQ is a ligand for human CD46 (Mori et al., 2003, 2004). In HCMV, the gH–gL complex is associated with gO and, alternatively, with pUL128, pUL130 and pUL131A. Thus, the basic entry machinery is conserved among herpesviruses, but additional viral ligands modulate cell type-specific entry.

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