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Specific short hairpin RNA-mediated inhibition of viral DNA packaging of human cytomegalovirus

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Abstract To clearly demonstrate the role of the human cytomegalovirus (HCMV) portal protein pUL104 RNA interference was applied. Expressing cell lines were constructed by transduction of shRNAs via the infection with retroviral vectors. After infection of these cells with HCMV AD169 the expression of pUL104 was reduced up to 80% for shRNA S1 and 54% for shRNA S2 at late times of infection compared to controls. In addition, the inhibitory effect was corresponding with a decrease in viral mRNA and plaque formations. Electron microscopic analysis demonstrated that infection of cells expressing pUL104-specific shRNAs resulted in the inhibition of formation of replicative particles.

Keywords: RNA interference; Short hairpin RNA; Portal protein; Packaging; Human cytomegalovirus

1. Introduction

Herpesviruses as well as double-stranded DNA bacteriophages express numerous proteins with analog functions in viral DNA packaging. One of those is the so-called portal protein, in bacteriophages also known as head-to-tail connector. Large macromolecular oligomers of portal proteins generate a central channel at a single vertex of the icosahedral capsid through which DNA translocation occurs [2,7,26–28]. All portal proteins form a dodecameric ring that serves as the gate for DNA entry and exit out of the capsid [20,39,41]. Although the mechanism of DNA packaging into the procapsid is still a mystery, this process requires an accessory terminase complex, thus providing ATP hydrolysis [3,13]. The resulting packaging motor consisting of the portal ring, the large and the small terminase subunits has the capacity to package approximately 2 bp of DNA per ATP [22,35]. A high-resolution structure ($\leqslant$10 Å) of portal proteins has been determined for dsDNA bacteriophage Φ29, T7 and SPP1 [1,20,21,31,34]. Recently, we identified and characterized the portal protein of human cytomegalovirus (HCMV), pUL104 [8,9]. HCMV pUL104 like other known portal proteins forms high molecular weight multimers.

In order to define the role of pUL104 for viral replication RNA interference (RNAi) was performed. In the last couple of years it has been demonstrated that short interfering RNAs (siRNAs) are able to silence genes *in vivo*. These siRNAs are generated by members of the Dicer family, that cleave double-stranded RNA into fragments with a length of 21–25 bp [12,24]. The resulting double-stranded siRNAs are then incorporated into a silencing complex called RISC which binds mRNA with a complementary sequence followed by cutting the homologous region [19,23]. The resulting mRNA is instable and fragile for degradation, leading to a reduction in expression of the target gene. Here we used the vector-mediated expression system RNAi-Ready leading to stable delivery of shRNA to cells. These short hairpin RNAs (shRNAs) were processed *in vivo* into siRNA molecules.

2. Materials and methods

2.1. Cells and viruses

Human foreskin fibroblasts (HFF) or 293T cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (5 U/ml), and streptomycin (50 μg/ml). HFF cells at passages 10–15 were used for infections and experiments were carried out with confluent cell monolayers (1.5×10^7 cells). Infection of HFF with HCMV AD169 at a MOI of 3 was carried out as described before [4].

2.2. Plasmid construction

For direct detection of cells containing the gene silcencing construct eGFP together with an IRES was inserted after the puromycin resistant gene into the vector pSIREN-RetroQ (BD Bioscience Clontech), yielding pSIREN-RetroQ-eGFP (provided by Frank Neipel). The RNAi-Ready pSIREN-RetroQ-eGFP vector was used to clone double stranded oligonucleotides encoding UL104-specific shRNA. The shRNA oligonucleotides were designed with 19 bases of sense and antisense strand (Table 1) separated by a hairpin loop (TTCAAGAGA), a 6T terminal sequence followed by the sequence for *Xho*I restriction according to the manufacturer (BD Bioscience Clontech). Single stranded sense and antisense shRNA oligonucleotides were synthesized (Biomers.net) and both strands were annealed prior to ligation into pSIREN-RetroQ-eGFP. As a negative control luciferase-specific shRNA oligonucleotide was inserted yielding the construct pSIREN-RetroQ-eGFP si luciferase (provided by Thomas Stamminger).

2.3. Transfection of shRNA expression plasmid

For transient expression 293T cells (5×10^6) were seeded on 10 cm diameter dishes. 293 T cells at 90% confluency were transfected with the pSIREN-RetroQ-eGFP, pSIREN-RetroQ-eGFP-shRNA1, -shRNA2, -shRNA3, and -luciferase together with pcDNA-UL104 [8] by the lipofectamine™ 2000 method (Invitrogen). As a control pcDNA-UL104 was co-transfected with pHM829 (expressing eGFP). The transfection efficiency was monitored by GFP fluorescence. Cells were harvested 40 h post transfection and subjected to Western blot analysis.

2.4. Western blot

Extracts from mock-infected, infected or transfected cells were separated on 8% (w/v) polyacrylamide gel and transferred to nitrocellulose sheets and subjected to Western blot analysis as described previously

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Table 1
Oligonucleotides encoding pUL104 target or luciferase sequence

Name	Nucleotide sequence	Nucleotide location
shRNA-1	5′-CGA CGA CTA TGG TCT CTA C-3′ 5′-G TAG AGA CCA TAG TCG TCG-3′	503–522
shRNA-2	5′-CGT CTC GGA CAG CAA ATC G-3′ 5′-C GAT TTG CTG TCC GAG ACG-3′	1061–1080
shRNA-3	5′-TTA CAC GGT AGA GCG CGT T-3′ 5′-A ACG CGC TCT ACC GTG TAA-3′	1709–1726
shRNA-lu	5′-TGC GTT GCT AGT ACC AAC-3′ 5′-GTT GGT ACT AGC AAC GCA-3′	1311–1328

[15]. The pAbUL104 antibody (1:10; [8]) specific for pUL104 was used as the primary antibody prior to incubation with horseradish peroxidase-conjugated anti-human F(ab′)2 fragments (1:5000 in 3% BSA). The membranes were reprobed with an antibody against β-actin (1:5000) and horseradish peroxidase-conjugated anti-mouse F(ab′)2 fragments (1:5000) to verify equal loading.

2.5. Lentivirus production and construction of expression cell lines

For construction of retroviral virions the plasmids pHIT60 encoding for the MLV gag- and pol-proteins (provided by Prof. Überla, University Bochum) and pVSV-G encoding the vesicular stomatitis virus G-protein [6] were used. 4.5 μg of pHIT60, 4.5 μg pVSV-G and 3 μg pSIREN-RetroQ-eGFP-shRNA1, -shRNA2, -luciferase or vector alone were cotransfected into 293T cells (5×10^6 cells). The medium was replaced 24 h after transfection with fresh one containing 10% FCS. The supernatant containing retroviruses was harvested 48 h after transfection.

HFF cells (8×10^4 cells) were infected with supernatant of retroviruses to introduce shRNAs of either UL104 shRNA1, shRNA2, luciferase shRNA or the vector alone. The cells were grown in the presence of puromycin (5 μg/ml). Stable clones were obtained after one week of selection.

2.6. Plaque reduction assay

Expressing shRNA1, shRNA2, luciferase or vector cell lines were seeded in 24-well plates and infected with HCMV AD169 with a MOI of 1 in DMEM. After 1 h of infection the inoculum was replaced with medium containing 10% FCS and 0.3% agarose. After incubation for eight days at 37 °C the cells were stained with crystal violet and plaques were counted by using a microscope. Effects of the shRNAs were calculated by comparing shRNA-expressing cells with control wells.

2.7. Preparation of RNA and cDNA

For quantification of UL104-mRNA stable shRNA1, shRNA2, Luciferase or vector expressing HFF cells (1×10^6) were infected with HCMV at a MOI of 1. Four days post infection cells were harvested and RNA was extracted. After incubation in 1 ml peqGOLD TriFast™ (PEQLAB-Biotechnologie GmbH) for 5 min at RT 200 μl chloroform were added. Samples were mixed, incubated for 10 min and centrifuged ($12000 \times g$, 5 min, 4 °C). The upper, aqueous phase was removed, mixed with 500 μl isopropanol and sedimented ($12000 \times g$, 10 min, 4 °C). The pellet was washed with 70% ethanol, air-dried, and resuspended in 30 μl Aqua bidest. The RNA was immediately used for cDNA-Synthesis. 20 μl RNA and 2 μl Random Hexamer Primers (Fermentas) were incubated at 80 °C for 5 min to dissolve secondary structures and then immediately placed on ice for at least 2 min. RT reactions consisted of PCR buffer II (PE Biosystems), 3 mM $MgCl_2$, 1 mM dNTPs, 10 mM dithiothreitol, and 40 U MLV reverse transcriptase (Invitrogen) in a final volume of 40 μl. The reactions were incubated at 25 °C for 5 min and 37 °C for 50 min followed by inactivation at 70 °C for 15 min. Final cDNA products were diluted 1:5 in Aqua bidest.

2.8. Real-time quantification PCR

Amounts of mRNA of the shRNA-expressing cells were determined by quantification of real-time PCR.

Oligonucleotides for real-time PCR were synthesized by biomers.net (Ulm, Germany): UL104rtp-fwd 5′-GCTCGGAGCGGCTGGAGGCG-3′/UL104rtp-back 5′-GCTCGTGGCTGGCGGTC-3′. Real-time PCR was performed using 10 μl of cDNA, 0.3 μM ROX-K buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.1× SYBR green (Invitrogen), 10 pmol of each primer, 1× *Taq* PCR buffer and *Taq* DNA polymerase in a final volume of 50 μl. Fifty cycles of amplification (94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s) were performed in an ABI Prism 7700 spectrofluorometric thermal cycler (Applied Biosystems).

DNA standards were amplified from pcDNA-UL104 with primers UL104Mi-fwd (GCGAATTCGAGCGGGCGCAATTTGTACGC) and UL104Mi-back (GCGCGGCCGCGCCGGGCACGAACTGCGAGAA). The standards were prepared by serial dilution from 10^7 to 10^3 copies per reaction. Standard and cDNA products were tested in duplicate. The amount of genomic RNA in each probe was also determined by using primers against actin (provided by K. Metzner, Erlangen).

2.9. Biostatistical analysis

In order to determine the efficiency of the shRNAs on the decrease in mRNA, plaque formation and Western blot statistical analysis were performed. The results obtained from paired Student's *t*-test were used to calculate significance. A P value of $\leqslant 0.05$ was considered significant.

2.10. Growth characteristics

HFF cells (8.5×10^4 cells per well) expressing shRNA1, shRNA2, luciferase or vector were seeded in 24-well culture plates. Confluent cells were infected with HCMV AD169 at an MOI of 3. At 0, 24, 48, 72, 96 and 120 h p.i. the supernatants were removed from the well and frozen at −80 °C. After collection of all time points the supernatants were thawed, transferred to 24-well plates with HFF cells on cover slips and the titers were determined by detection of immediate-early protein 1 (IE1) expression with indirect immunofluorescence.

2.11. Thin sections

Stable shRNA1, shRNA2, Luciferase or vector expressing HFF cells were infected with HCMV at a MOI of 1. Four days post infection cells were fixed with harvesting buffer (20 mM HEPES, pH 7.4) containing 4% paraformaldehyde, 2.5% glutaraldehyde and 1% tannin according to Gelderblom et al. [14]. After dehydration and poststaining with 0.2% (w/v) uranyl acetate the specimen was embedded in glycid ether 100 (Carl Roth) with 1.5% (w/v) 1-methyl-5-norbornene-2,3-dicarboxylic acid anhydride, methylnadic anhydride (MNA; Carl Roth). Polymerization was performed at 60 °C for several days prior to sectioning with ultracut S (Reichert-Jung). The sections were transferred to slot grids coated with pioloform (Plano), stained for 10 min with 1% (w/v) uranyl acetate in 40% EtOH followed by lead citrate staining for additional 10 min prior to analysis by electron microscopy.

3. Results

3.1. Suppression of pUL104 gene expression

In order to identify the efficiency of gene silencing of chosen shRNA-sequences recombinant pSIREN-RetroQ-eGFP vectors containing either UL104 shRNA1 (pS-sh1), shRNA2 (pS-sh2), shRNA3 (pS-sh3), luciferase shRNA (pS-lu) or the vector alone (pS-v) were co-transfected with a pUL104 expression vector (pUL104). In the cells shRNAs were converted into siRNA-like molecules, initiating RNAi. The expression of pUL104 was knocked down to 30% in the presence of shRNA1 and shRNA2 against pUL104 (Fig. 1, lanes 5 and 6). In the presence of the control shRNAs as well as shRNA3 pUL104 expression was identical to cells transfected only with UL104 encoding vector (Fig. 1, lanes 2–4; 7). These observations demonstrated that both shRNAs at least partially silence pUL104 expression.

3.2. Reduction mRNA levels in shRNA-expressing cells

To determine the influence of the effective shRNA1 and shRNA2 on the amount of mRNA real-time PCR was per-

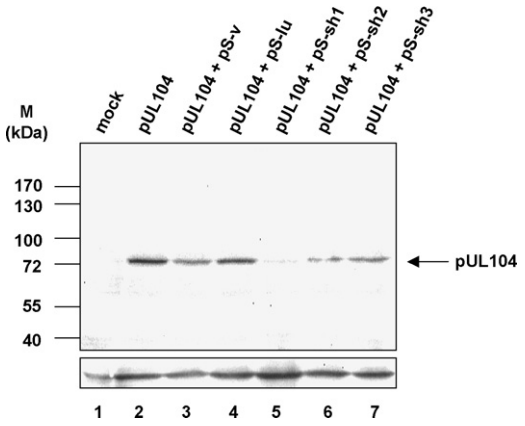

Fig. 1. Effect of shRNA on expression of pUL104. 293T cells (5×10^6) were at 90% confluency transfected with pSIREN-RetroQ-eGFP (pS-v), pSIREN-RetroQ-eGFP-shRNA1 (pS-sh1), -shRNA2 (pS-sh2), -shRNA3 (pS-sh3) and -luciferase (pS-lu) together with pcDNA-UL104 by the lipofectamine™ 2000 method (Invitrogen). Cells were harvested 40 h post transfection and subjected to Western blot analysis with the antibody pAbUL104. As a loading control the immunoblot was consecutively stained with an antibody against actin. The arrow indicates the positions of pUL104, the molecular weight markers (M) are shown on the left.

formed. Expressing shRNA human fibroblasts were infected with HCMV AD169. After harvesting the cells at 96 h p.i. the RNA was extracted and used for quantification of UL104 mRNA copy numbers. While infection of cells expressing shRNA1 resulted in a high decrease in UL104 copy numbers, the effect of shRNA2 was only twofold of UL104 copy numbers (Table 2). The significance of the results was provided by biostatistics analysis by comparison with shRNA luciferase and the vector control (Table 2). These results showed that both specific shRNAs have the ability to reduce the amount of mRNA.

3.3. Influence of shRNAs on virus yield

Plaque assays were carried out to analyze the ability of shRNAs to reduce virus yield. Both UL104-specific shRNAs were able to reduce the plaque formation. While the number of plaques in the presence of shRNA 1 decreased 70% the reduction with shRNA 2 was approximately 60% (Table 2). In cells infected in the presence of shRNA luciferase a reduction of 30% was observed (Table 2). Cells with the integrated vector sequence served as control (Table 2). These results showed that both specific shRNAs have the ability to reduce virus yield, but could not inhibit it.

3.4. Reduction of viral growth

In order to analyze the effect of UL104-specific shRNAs on viral replication growth curves were performed after infection of expressing cell lines with HCMV AD169. At 0, 24, 48, 72, 96, 120 h p.i. the supernatants were harvested and transferred to uninfected HFF. The amount of released virus was measured by detection of immediate-early protein 1 (IE1) using immunofluorescence. Both shRNAs had the capacity to reduce virus release, whereas shRNA1 inhibits virus formation by approximately 83% while the inhibitory effect of shRNA2 was 67% at 96 h p.i. (Table 3). At 120 h p.i. the reduction of shRNA1 and 2 were both approximately 60% (Table 3). Taken together the data presented suggested that the UL104-specific shRNAs were able to reduce particle release by over 60%.

3.5. Inhibition of pUL104 expression by shRNAs after infection

To examine whether UL104-specific shRNA can inhibit pUL104 expression, stable shRNA 1 (pS-sh1), shRNA2 (pS-sh2), shRNA-luciferase (pS-lu) or vector (pS-v) expression cell lines were infected with HCMV AD169 at an MOI of 3 and Western blot analysis were performed. The amount of detected pUL104 dimer decreased during infection. At 72 h p.i. the suppression of pUL104 was approximately 35–40% (Fig. 2A and D), at 96 h p.i. 70% for shRNA1 and 45 for shRNA2 (Fig. 2B and E) and at 120 h p.i. approximately 80% for shRNA1 and 55% for shRNA2 (Fig. 2C and F). In the presence of the control shRNA (luciferase) or empty vector no obvious effect on the pUL104 gene expression was detected. The significance of the results was determined by using paired Student's *t*-time test (Table 4). We could demonstrate that the effects of the shRNA were sequence specific, because control cell lines expressing luciferase shRNA or vector alone showed no detectable effect.

3.6. Effect of shRNAs on viral maturation

To determine whether the UL104-specific shRNAs have an effect on viral maturation electron microscopy of thin section was performed. Stable expressing cell lines were infected with HCMV AD169 with an MOI of 3. Five days after infection the cells were embedded and subjected to sectioning. Three different forms of capsids are formed in the nucleus: B-capsids containing the scaffolding protein (as an electron dense ring), C-capsids with the packaged DNA and A-capsids representing abortive products of DNA packaging (empty capsids). The

Table 3
Infectious units per ml during infection

shRNA	Time post infection (h)				
	24	48	72	96	120
pS-v	0	720	1450	24000	740000
pS-lu	0	800	1640	26000	885000
pS-sh1	0	120	480	4000	275000
pS-sh2	0	300	470	8000	292500

Table 2
Effects of shRNAs on UL104-mRNA (copies per μl) and viral replication (percentage of vector control)

shRNA	Real -time PCR		Plaque-assay	
	Copy number ($\times 10^4$)	*P* value pS-v/pS-lu	%	*P* value pS-v/pS-lu
pS-v	5.08		100	
pS-lu	4.57		43	
pS-sh1	1.32	0.002/0.004	19.6	0.001/0.006
pS-sh2	2.68	<0.0001/<0.0001	28.7	0.006/0.01

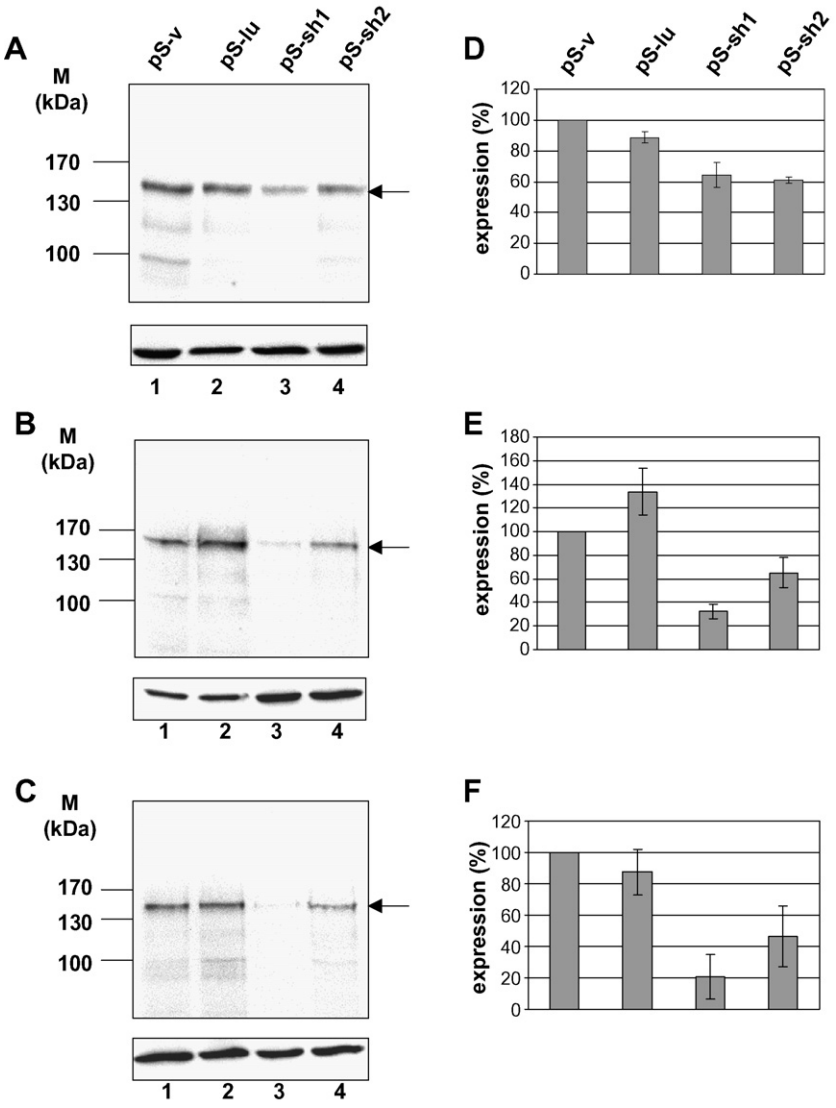

Fig. 2. Suppression of pUL104 in specific shRNA-expressing cell lines infected by HCMV. ShRNA-expressing shRNA1 (pS-sh1), shRNA2 (pS-sh2), luciferase (pS-lu) or vector (pS-v) HFF cells (1.5×10^6) were infected with HCMV AD169 at an MOI of 3. At 72 (A), 96 (B) and 120 h p.i. (C) cell extracts were subjected to 8% SDS–PAGE. The separated probes were transferred onto nitrocellulose prior to immunoblot analysis using the antibody pAbUL104. As a loading control the immunoblot was consecutively stained with an antibody against actin. Specifically the detected proteins were quantified using bioimaging analysis. The relative amounts of expression are indicated by percent values in the diagram. Error bars on the histogram are ±S.D. from three independent experiments. The arrows indicate the position of pUL104, the molecular weight markers (M) are shown on the left.

Table 4
Expression -levels of pUL104 as percentage of vector control

Time p.i. shRNA	72 h		96 h		120 h	
	%	*P* value pS-v/pS-lu	%	*P* value pS-v/pS-lu	%	*P* value pS-v/pS-lu
pS-v	100		100		100	
pS-lu	88.78		133.58		87.45	
pS-sh1	64.10	0.005/0.005	32.45	0.004/0.01	20.68	0.015/ 0.03
pS-sh2	60.93	0.001/0.003	65.35	n.s./n.s.	46.39	n.s./0.027

n.s., not significant.

resulting enveloped particles are non-infectious particles (NIEPS) arising from A-capsids and virions from C-capsids. Only NIEPs (Fig. 3B and D) and mainly B-capsids (Fig. 3A and C) were found in cells expressing shRNA1 and shRNA2. The amount of replicative structures (virions, dense bodies; Fig. 3F) in the control, shRNAs luciferase expressing cells, is comparable to wild-type HCMV. The number of C-capsids versus B-capsids was quantified and shown in Table 5. These

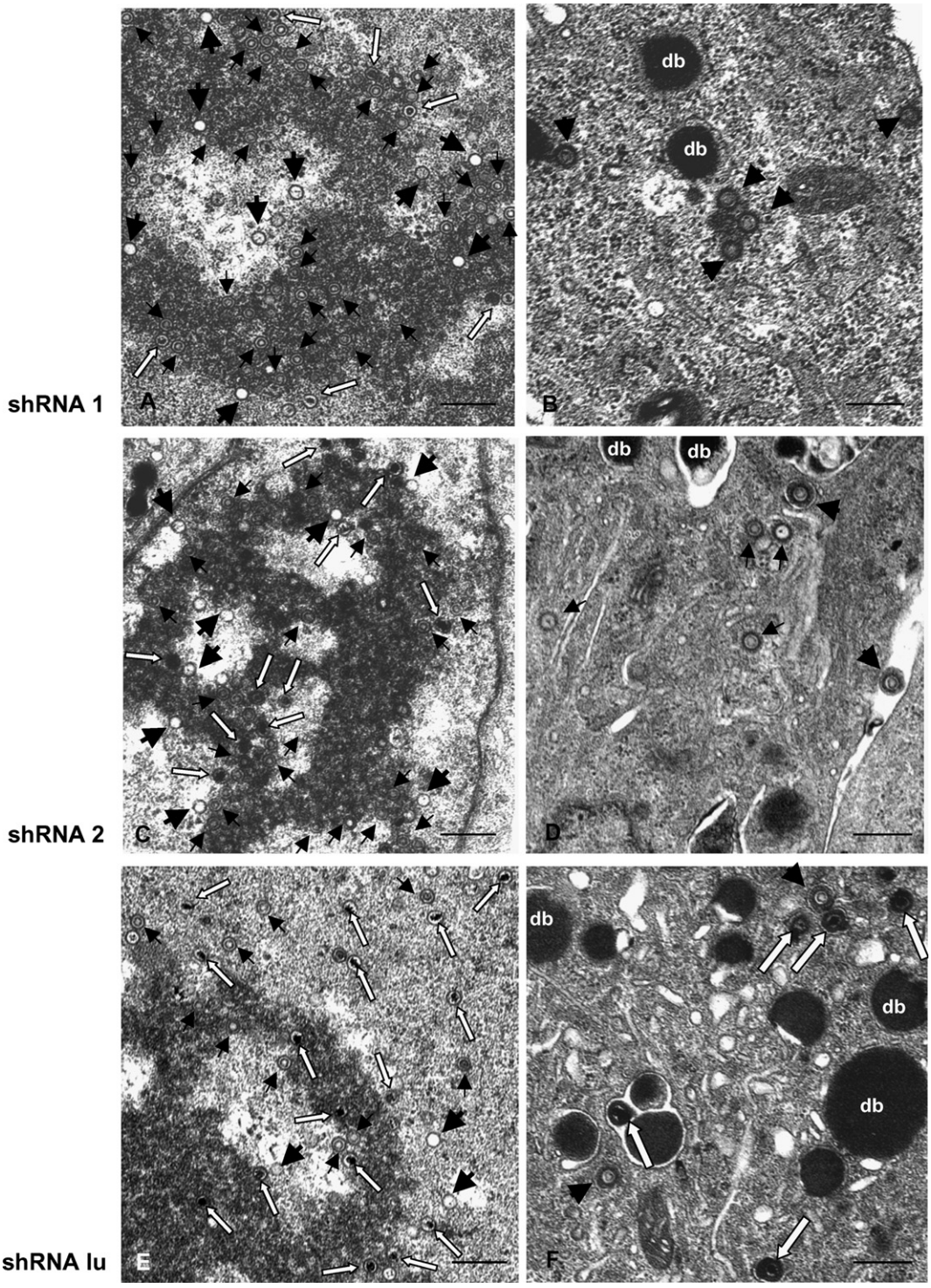

Fig. 3. Electron microscopy analysis of thin sections of shRNA1 (A,B), shRNA2 (C,D) or luciferase (E,F) expressing cells. After five days cells were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde with 1% tannin and prepared for electron microscopy. A, C and E represents an area of the nucleus while B, D and F one of the cytoplasm. B capsids are indicated by black arrows, A capsids by bold black arrows, C capsids by white arrows, noninfected particles by black arrows and virions by bold white arrows. db indicates dense bodies. The scale bars correspond to 50 nm.

results demonstrated that UL104-specific RNAi resulted in a block of DNA packaging and therefore in the formation of replicative particles. This is in line with the assumed function of pUL104 as a portal protein and demonstrates that pUL104 is required for DNA-packaging.

4. Discussion

For analyzing the function of viral proteins *in vivo* the construction of deletion mutants is often the method of choice. In the case of essential gene products this can not be used,

Table 5
Nuclear capsids of infected cells expressing shRNA

shRNA	No. of nuclei counted	No. of capsid form per nucleus (%)		
		A	B	C
pS-sh1	3	10.8	70.4	18.8
pS-sh2	3	18.1	52.5	29.4
pS-lu	3	16.5	38.4	45.3

because the virus with the deletion would in almost every case produce a non-replicating or even lethal phenotype. In the case of human cytomegalovirus it has been shown by random mutagenesis of the genome that 41 open reading frames including UL104 are essential for viral replication [10,11]. A new promising approach is the use of siRNAs that allow the analysis of the gene function [24,25]. In this study, we used plasmid-based UL104-specific short hairpin (sh) RNAs as specific inducers of RNA silencing [5,36,37,42]. This system allows a continuous intracellular synthesis of shRNAs resulting in constant siRNAs levels over a long period of time. The shRNAs were synthesized in HFF cells which were stable transfected with the U6-expression vector pSIREN-RetroQ-eGFP.

Infection of stable shRNA-expressing HFF with HCMV AD169 leads to the suppression of pUL104. This effect increased during time of infection, whereas after five days the maximum reduction of more than 40% for shRNA2 and even a remarkable reduction in pUL104 expression of 89% for shRNA1 were observed. We could demonstrate that the effects of the shRNA were sequence specific, because control cell lines expressing luciferase shRNA or vector alone showed no detectable effect. Interestingly, the shRNA-mediated block of pUL104 affects the release of virus both in plaque reduction and in virus yield assays up to threefold as well as a decrease in the amount of mRNA of up to fourfold.

Since a phenotype can be observed by using shRNA-mediated gene knockdown we performed electron microscopy analysis of the shRNA-stable expressing cells. While in the infected cells expressing shRNA 1 almost no replicative particles could be observed, in shRNA 2 cells some cytoplasmic virions were detected. However, the amount of replicative particles in the control, shRNA luciferase expressing cells, is comparable to wild-type HCMV. Thus demonstrated that UL104-specific RNAi resulted in a block of DNA packaging and therefore in the formation of replicative particles.

Furthermore, previous investigators have shown for HSV-1 and the dsDNA bacteriophages Φ29 and P22 that in contrast to other bacteriophages capsid formation can take place in the absence of a portal protein both *in vitro*/*in vivo* [29,30,32–34,38,40]. Given that the overall number of capsids in both UL104 and luciferase specific shRNA expressing cells is similar, it can be speculated that this is also the case for HCMV. Since there is no *in vitro* assembly system for the HCMV capsid, RNAi may provide a tool to study the effects that the lack of single components has on capsid formation and the packaging process.

Recently, Godfrey et al. [18] demonstrated that primary effusion lymphoma can be inhibited by lentiviral vectors encoding shRNA of the oncogenic cluster of KSHV *in vitro*/*in vivo* in a mice model. The usage of lentiviral-based vectors with incorporated sequence-specific shRNA enable the efficient targeting of RNA *in vivo*. However, it has been reported that extensive treatment of enterovirus with RNAi leads to the development of escape mutants [16,17]. The authors have shown that it might be prevented by targeting multiple gene products [17]. Our observations lead us to the hypothesis that lentivirus-delivered RNAi can be used as a potential therapy against HCMV.

In conclusion, expression of UL104-specific hairpin shRNAs (i) can silence the target gene and (ii) furthermore inhibit the formation of replicative particles confirming the role of pUL104 in viral DNA packaging. Given the need of new antiviral therapies, especially regarding the cross-resistances of the current available compounds, the use of vector-mediated shRNA as an antiviral strategy for HCMV has a great potential.

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