Improper Tagging of the Non-Essential Small Capsid Protein VP26 Impairs Nuclear Capsid Egress of Herpes Simplex Virus

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Abstract
To analyze the subcellular trafficking of herpesvirus capsids, the small capsid protein has been labeled with different fluorescent proteins. Here, we analyzed the infectivity of several HSV1(17′) strains in which the N-terminal region of the non-essential small capsid protein VP26 had been tagged at different positions. While some variants replicated with similar kinetics as their parental wild type strain, others were not infectious at all. Improper tagging resulted in the aggregation of VP26 in the nucleus, prevented efficient nuclear egress of viral capsids, and thus virion formation. Correlative fluorescence and electron microscopy showed that these aggregates had sequestered several other viral proteins, but often did not contain viral capsids. The propensity for aggregate formation was influenced by the type of the fluorescent protein domain, the position of the inserted tag, the cell type, and the progression of infection. Among the tags that we have tested, mRFPVP26 had the lowest tendency to induce nuclear aggregates, and showed the least reduction in replication when compared to wild type. Our data suggest that bona fide monomeric fluorescent protein tags have less impact on proper assembly of HSV1 capsids and nuclear capsid egress than tags that tend to dimerize. Small chemical compounds capable of inducing aggregate formation of VP26 may lead to new antiviral drugs against HSV infections.

Introduction
Single and dual-color fluorescently tagged strains are valuable tools to elucidate the intracellular trafficking of virions and subviral particles. In an ideal case, the modified strain replicates with the same kinetics and to the same titers as its parental strain, and the tag neither interferes with any step of the viral life cycle, nor changes the biochemical properties of the modified viral structure. For herpesviruses, fluorescent protein (FP) domains attached to the small capsid protein (SCP) have been used extensively to characterize the molecular mechanisms of virus assembly and egress or nuclear targeting of incoming capsids in cells and biochemical assays (c.f. Fig.1; [1–13]). The SCPs are recruited to capsids via the major capsid proteins (MCP). Although similar building principles apply, the amino acid sequences of both, SCPs and MCPs vary considerably among the herpesviruses [14–18]. SCPs are essential for the replication of human and mouse cytomegalovirus, Epstein-Barr virus and Kaposi sarcoma-associated herpesvirus, but not for the alphaherpesviruses herpes simplex virus type 1 (HSV1), pseudorabiesvirus (PrV) or varicella zoster virus (VZV; [7,8,19–23]). However, HSV1 strains lacking the SCP yield lower titers than wild type in the murine eye and trigeminal ganglion after corneal infection as well as in BHK cells [8,20,24]. PrV lacking the SCP is also less neuroinvasive and grows to lower titers in cell culture, while the SCP of VZV is essential for infection of the human skin xenograft murine model and of melanoma cells but not of embryonic lung fibroblasts [7,25].

VP26, the SCP of HSV1, is a basic 12 kDa protein of 112 amino acid residues (aa) with low solubility and encoded by the gene UL35 [26,27]. In solution, it is only 13 to 15% α-helical but is 80% β-sheet, and a secondary-structure algorithm predicts two α-helical regions between aa 13 to 31 and 42 to 72 [26,28,29]. Herpesvirus capsids are assembled in the nucleus and for its nuclear import VP26 requires the interaction with VP5, the MCP of HSV1, and either capsid protein preVP22a or VP19c [30–32]. Hexamers of VP5 form the 150 hexons on the faces and edges, while pentamers of VP5 form the 11 pentons on the vertices of the icosahedral capsid. A virion can harbor up to 900 copies of VP26 as it decorates the top of the hexons in a hexamer [14,16,20]. The C-terminal half of HSV1-VP26, aa 50 to 112 are sufficient for binding to an interface of hydrophobic residues and small charged patches on the upper hexon domain [26,33]. Combined cryoelectron microscopy and ab initio modeling suggest a novel fold of the C-terminal aa 42 to 112 with three short α-helices [29,34]. While the hexons recruit VP26, the pentons serve as attachment sites for...
### HSV1 strains + constructs

| HSV1 strains + constructs | Genomic Organization | FP | Plaque | Nuclear Aggregates | References |
|---------------------------|----------------------|----|--------|-------------------|------------|
| wild type                 | UL34                 |    |        |                   |            |
| KOS-                      | UL34                 |    |        |                   |            |
| LacZ-VP26bsa1-10-102      | UL34                 |    |        |                   |            |
| (17°)blueLox-VP26bsa5-112 | UL34                 |    |        |                   |            |
| KOS-GFPVP26bsa1-7         | UL34                 |    |        |                   |            |
| (F) GFPVP26bsa1-7         | UL34                 |    |        |                   |            |
| (17°)blueLox-mRFPVP26bsa1-7 | UL34                 |    |        |                   |            |
| (17°)blueLox-GFPVP26bsa1-7 -(gDMRFP) | UL34                 |    |        |                   |            |
| (FY)Ebac102-mRFPVP26bsa1-4 | UL34                 |    |        |                   |            |
| (FY)Ebac102-YFPvenusVP26bsa1 | UL34                 |    |        |                   |            |
| (FY)Ebac102-mRFPVP26bsa1-7 | UL34                 |    |        |                   |            |
| (17°)blueLox-GFPVP26bsa1-7 | UL34                 |    |        |                   |            |
| (17°)blueLox-YFPVP26bsa1-7 | UL34                 |    |        |                   |            |
| (17°)blueLox-CFPVP26bsa1-7 | UL34                 |    |        |                   |            |

### References

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### A

### B

| ggc ggc cgc ctc taa aaa agg acg cac cgc cgc cct aat cgc cag tgc gtt cog gac gcc | wt |
| ggc ggc cgc cta taa aaa agg acg cac cgc cgc cct aat cgc cag tgc gtt cog gac gcc | Δaa5-7 |
| ggc ggc cgc cta taa aaa agg acg cac cgc cgc cct aat cgc cag tgc gtt cog gac gcc | Δaa1-7 |

| ttg gcc cca cac agc cct ccc gac cga cac ccc cat att gct tcc cga ctt cog gtc cag | wt |
| ttg gcc cca cac agc cct ccc gac cga cac ccc cat att gct tcc cga ctt cog gtc cag | Δaa5-7 |
| ttg gcc cca cca cca cca cca cca cca cca cca cca cca cca cca cca cca cca cca cca | Δaa1-7 |

| atg gcc gtc cgg cca ttt cac | wt |
| atg gcc gtc cgg cca TGG ACC ATG GTG AGC AAG | Δaa5-7 |
| M A V P H S T M V S K | Δaa1-7 |
the tegument protein pUL36, and it has been suggested that this may be due to similarities between aa 66 to 96 of VP26 and aa 1712 to 1751 of pUL36 [14,29,35,36].

In addition to VP5, HSV1-VP26 can also interact with the capsid proteins VP23 and pUL25 as well as the tegument proteins pUL11, pUL14, pUL16, pUL21, pUL37, VP16, pUL31, and pUS3 in yeast-two-hybrid assays [33,37]. Yet, the incorporation of pUL37 and VP16 into HSV1 virions does not depend on VP26 but on pUL36 [30]. Furthermore HSV1-VP26 can bind to the host proteins tetrasiapin-7 and the dynenic light chain Tctex-1 and RPS5 [33,39,40]. However, incoming capsids of HSV1-AVP26 can still utilize the microtubule motor dynein for transport to the nucleus, and HSV1-AVP26 capsids with inner tegument proteins on their surface can recruit dynen, whereas nuclear capsids devoid of inner tegument, but exposing VP26 cannot [5,13,20,21,41]. Thus, VP26 is not essential for binding dynen to viral capsids, but it may somehow regulate the interaction of dynen light chains with other host factors or the function of dynen.

The N-terminal region of the VP26 of HSV1 or PrV has been successfully used to insert a FP while maintaining infectivity [c.f. Fig. 1A; [2,4,7,8,10,11,42,43]]. The microscope study tomography studies indicate that there are few highly ordered contacts between VP26 and the surrounding tegument, and most reported interaction partners employ its C-terminal half [24,26,33,35,36]. However, the mass of the SCVPs is tripled with additions such as mRFP (monomeric red FP) or GFP (green FP), GFP (cyan FP) or YFP (yellow FP) that have molecular weights of 25 to 27 kDa, and such tags can attenuate the replication of HSV1 and PrV in culture, and its pathogenesis in murine infection models [7,8,42,44].

Since the HSV1[17'] strains that we had constructed previously exhibited prominent nuclear aggregates of GFPVP26 and reduced titers in BHK cells [8,42], we analyzed here several other strains in order to identify a less invasive FP tag. While some insertions were well tolerated, unexpectedly other additions prevented the formation of infectious HSV1 in spite of HSV1-VP26 not being essential. The non-infectious constructs had a very high propensity to form large nuclear aggregates of VP26 and were impaired in nuclear capsid egress, most likely by sequestering other essential proteins into these aggregates. The strain with the least invasive mRFP tag on VP26 induced few nuclear aggregates, and fewer impediments of nuclear egress, secondary envelopment and virion formation. It therefore provides a better backbone to construct new dual-color HSV1[17'] variants with other FPs on tegument or glycoproteins. On the other hand, tags that promote mild VP26 aggregate formation may be used to identify further functional interactions with other structural HSV1 proteins by screening for additional synthetic lethal or rescuing mutations [45,46]. Our data furthermore imply that if compounds could be identified that induce SCP aggregation and thus prevent virion formation; even non-essential viral proteins could provide targets for antiviral therapy.

Materials and Methods

Ethics Statement

For immunofluorescence labeling (see below) we applied human serum of healthy, HSV1-seronegative volunteers. This procedure was approved by the Institution Review Board (Ethikkommission of Hannover Medical School, approval number 993). Written informed consent of the blood donors, according to the Institution Review Boards, was obtained.

Cells, Viruses and Antibodies

BHK-21 (ATCC CCL-10) and Vero (ATCC CCL-81) cells were cultured in Minimum Essential Medium (MEM) with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Gibco, Paisley, UK). All virus stocks were titrated on Vero cells [49]. Rabbit polyclonal antibodies (PAb) directed against empty capsids (a-NC; [50], VP26(370–417) [50], VP23 (a-NC-1; [50], VP22a (a-NC-3,4; [50], VP23 (a-NC-5; [50]), pUL25 (ID1, R8–3; [51,52]), pUL36 aa1408–2112 (#147; [15,41]), pUL36(3005–3057) (C-term; [53]), pUL33 (54), pUL34 (55), or human PML protein aa157–394 (I-283, Santa Cruz Biotechnology Inc., Santa Cruz, CA), as well as the mouse monoclonal antibodies (MAb) against VP5 (3C10, 6F5; [56], LP12; [57], H1:4 [Bioscience International, Saco, ME]), VP23 (ID2; [50]), pUL25 and pUL17 (#166, #203; [59]), or ICp8 (HB8180, ATCC, Rockville, MD, USA; provided by Regine Heilbronn, Charité-Universitätsmedizin, Berlin, Germany) were used.

Construction of HSV1 Strains Tagged with VP26

In our previously described strains HSV1[17']blueLox-mRFPVP26 and -GFPVP26, the fluorescent protein sequences had been inserted into the VP26 sequence by deleting a 65 bp sequence including the first seven VP26 codons for Met-Ala-Val-Pro-Glu-Phc-His (c.f. Fig. 1B; [8]; P. Desai, personal communication). Therefore, in the present study they are called HSV1[17']blueLox-mRFPVP26(Asa1-17) and -GFPVP26(Asa1-17). In HSV1[17']blueLox-GFPVP26(Asa1-17)DmRFP, glycoprotein D has also been labeled with monomeric RFP at its C-terminus;
this virus has been named HSV1(17\textsuperscript{+})blueLox-GFPVP26-gDmRFP previously [8]. To construct additional HSV1 strains in which aa 5 to 7 (Glu-Phe-His) of VP26 were replaced by the fluorescent protein sequence as originally proposed by Desai & Person (1998), first an XhoI site was inserted into the ORF UL35. To this end, 500 bp upstream of the insertion site were amplified with XhoI/XmaI (GGT GGA CGA GGA TGG GAT GAT GAG and XhoI/XmaI (GGT GGA CGA GGA TGG GAT GAT GAG and digested with BsrGI and cloned into pUC18 (non-annueling nucleotides in italics, restriction sites underlined; all oligonucleotides were obtained by MWG, Ebersberg, Germany). The 500 bp downstream of the insertion site were amplified with XhoI/NcoI (ACG CGC GCC GAT GAT GG and XhoI/NcoI (ACG CGC GCC GAT GAT GG and ligated into the XhoI site of pUC18-UL35, resulting in pUC18-UL35NB. The fluorescent protein sequences from pEGFP-N1, pEYP-N1 or pECFP-N1 (Clontech; Mountain View, CA) were excised with NotI and BsrGI and inserted into pUC18-UL35NB to generate pUC18-GFPUL35. -YFPUL35 or -CFPUL35. The complete constructs were amplified from these plasmids with ATG CCC AGG CCC TCG AG CTG AGT GAG TGG, and ligated into the pUC18-UL35 cassette of the bacterial artificial chromosome (BAC) pHHSV1(17\textsuperscript{+})blueLox-ΔVP26 (Fig. 1A; [9]). The resulting BACs were named pHHSV1(17\textsuperscript{+})blueLox-GFPVP26\textsubscript{AAA57}+ , -YFPVP26\textsubscript{AAA57}+, or -CFPVP26\textsubscript{AAA57}, respectively (Fig. 1). From these BACs the modified UL35 ORFs were amplified, and the PCR products were sequenced (Seqlab; Goettingen, Germany). For transfection, BAC-DNA was prepared from 500 ml overnight E. coli cultures using the NucleoBond\textsuperscript{TM} BAC 100 kit (Macherey & Nagel; Düren, Germany). 5×10\textsuperscript{5} Vero cells in 3.5 cm dishes were grown overnight, transfected with 2 μg BAC-DNA (MBS Mammalian Transfection Kit; Stratagene, La Jolla, CA), and cultured for several days until cytopathic effects developed.

Immunoblot

Vero cells grown to a density of 5.7×10\textsuperscript{5} per 3.5 cm dish were synchronously infected with 10 plaque forming units (PFU)/cell in 500 μL for 2 h on ice, and then further cultured in regular growth medium at 37°C. After 1 h, the cells were washed for 3 min with citrate buffer (40 mM citric acid, pH 3, 135 mM NaCl, 10 mM KCl) to inactivate any extracellular virions that had not been internalized [60], and returned to fresh medium to initiate a synchronous infection. At different time points, the culture supernatants were collected and tittered. Infected cells and their complete supernatants were also harvested at 48 h post infection (p.i.). Viral particles released from infected cells into the culture medium were pelleted in a Beckman TL-A100.3 rotor at 50,000 rpm for 30 min and resuspended in 100 μL hot SDS-PAGE sample buffer [61] with protease inhibitors. The cells were scraped into 200 μL of hot sample buffer with protease inhibitors and sheared by 50 passages through a 24 gauge needle. After SDS-PAGE on linear 5 to 15% polyacrylamide gradient gels and transfer onto a nitrocellulose membrane, viral proteins were probed with specific primary antibodies and alkaline-phosphatase-coupled secondary antibodies (Dianova, Hamburg, Germany).

Fluorescence Microscopy

Vero cells grown on cover slips to a density of 5 to 10×10\textsuperscript{4} per 2 cm\textsuperscript{2} were synchronously infected as described above at a multiplicity of infection (MOI) of 10 PFU/cell. At 9 h p.i., cells were fixed with 3% (v/v) para-formaldehyde (PFA) in PBS and permeabilized with 0.1% Triton X-100 (TX-100) for 5 min, fixed with absolute methanol, or fixed and permeabilized with PHEMO-fix (3.7% [v/v] PFA, 0.05% [w/v] glutaraldehyde, 0.5% [v/v] TX-100 in PHEMO buffer [68 mM PIPES, 25 mM HEPES, pH 6.9, 15 mM EGTA, 3 mM MgCl\textsubscript{2}, 10% DMSO]) for 10 min. The HSV1 Fc receptor was blocked with 10% (v/v) human serum of healthy, HSV1-seronegative volunteers, and the samples were immunolabeled as described before [5,8,47,49,62,63]. All secondary antibodies were highly pre-absorbed against cross-reactivities against other species than the intended one, and were coupled to lissamine-rhodamine-sulfonyl chloride, RedX, fluorescein isothiocyanate, or Cy5 (Dianova, Hamburg, Germany). The cells were analyzed on an Axiovert 200M microscope equipped with a LSM 510 META confocal laser scanning unit (ZEISS, Jena, Germany) using a plan-apochromat 63× oil-immersion objective with an 1.4 numeric aperture. Image acquisition and processing was performed using the ZEISS LSM imaging software, ImageJ 1.35 (Wayne Rasband; National Institute of Health, USA; http://rsb.info.nih.gov/ij/), and Adobe\textsuperscript{®} Photoshop CS (Adobe Systems, San Jose, CA, USA). For classification and quantification of the intranuclear VP26 phenotype, we used a primary data set obtained from an analysis of at least 85 randomly chosen cells fixed at several time points from 4 to 12 h p.i. [8]. The degree of nuclear aggregate formation was classified based on the density and appearance of the anti-VP26 or GFPVP26 signals into the categories “none”, “single”, “grainy”, and “aggregated”.

Correlative Light and Electron Microscopy

RPE cells grown to 4×10\textsuperscript{5} cells per 8.5 cm\textsuperscript{2} on glass bottom dishes (MatTek Corporation, Ashland, MA, USA) with self-made marks to aid the localization of individual cells were synchronously infected with HSV1/KOS(-)GFPVP26\textsubscript{AAA57}+ at an MOI of 10 PFU/cell. After 19.5 h of infection, the fluorescence signals were documented with a Zeiss Axiovert 200 microscope prior to fixation for 1 h at room temperature with 2% (w/v) glutaraldehyde in 130 mM cacodylate buffer at pH 7.4 containing 2 mM CaCl\textsubscript{2} and 10 mM MgCl\textsubscript{2}. The cells were washed and further fixed and contrasted with 1% (w/v) OsO\textsubscript{4} in 165 mM cacodylate buffer at pH 7.4 containing 1.5% (w/v) K\textsubscript{2}Fe\textsubscript{3+}(CN)\textsubscript{6} for 1 h followed by incubation in 0.5% (w/v) uranyl acetate in 50% (v/v) ethanol overnight. The cells were flat-embedded in Epon, and semi-thin sections cut parallel to the substrate were used to identify cells characterized by a high amount of nuclear aggregates and an indicative nuclear morphology that had been documented by epifluorescence microscopy prior to fixation. Such cells were then relocated in ultrathin sections of 50 nm and analyzed with a FEI Tecnai G2 T20 electron microscope. We analyzed ultrathin sections of more than 50 cells, and furthermore in detail by correlative light and electron microscope 8 cells each containing more than 5 nuclear aggregates.

Results

Construction of HSV1 Strains with Tagged VP26

To identify strains whose capsid formation and intracellular trafficking mimic that of their untagged parent, we compared several constructs with different FP tags on VP26 that we had introduced into the HSV1(17\textsuperscript{+})blueLox BAC. The
HSV1(17)blueLox-GFPVP26Δaa1-7 strain, as the previously reported HSV1(KOS)-GFPVP26Δaa1-7, lacks 65 bp upstream of the start codon of GFPVP26 including the seven N-terminal aa of VP26 (Fig. 1; P. Desai, personal communication and data not shown; [8,42]). Both strains propagate to similar titers in BHK cells [8].

In this study, we have generated the new strains HSV1(17)blueLox-CFPVP26Δaa5-7, -GFPVP26Δaa5-7, and -YFPVP26Δaa5-7 (c.f. Fig. 1A, B) according to a strategy originally described by Desai & Person (1998). The codons for aa 5 to 7 of VP26 were replaced by different FP with the linkers His-Ser-Thr and Asn-Ser-Ser at their respective N- and C-termini (Fig. 1B). The viral genomes of these strains contained the engineered changes in UL35 (arrowheads in Fig. 2A) but not any major unwanted alterations as shown by restriction enzyme digestion with NotI, EcoRI, EcoRV or SfiI (Fig. 2A and data not shown). There was some heterogeneity among strains in the NotI joint fragments around 3.3 kb (asterisks in Fig. 2A) that do not seem to have any influence on infectivity as reported previously [8,63,64]. Sequencing the UL35 region of the BACs confirmed the correct insertion of the FP sequences (not shown). But after transfection of BHK cells, sequencing the UL35 region of the BACs confirmed the correct fragments around 3.3 kb (asterisks in Fig. 2A) that do not seem to have any influence on infectivity as reported previously [8,63,64]. Sequencing the UL35 region of the BACs confirmed the correct insertion of the FP sequences (not shown).

To further characterize potential differences between these strains, we compared their growth kinetics and protein expression. While HSV1(17)blueLox-AVP26 and -mRFPVP26Δaa1-7 were attenuated in BHK cells [8], there was no growth difference between HSV1(17)blueLox, -AVP26 and -mRFPVP26Δaa1-7 in Vero cells (Fig. 2B). But compared to those, -CFPVP26Δaa5-7 and -GFPVP26Δaa1-7 were slightly attenuated, and -mRFPVP26Δaa1-7 replicated with delayed kinetics (Fig. 2B). Likewise, HSV1(17)blueLox-GFPVP26Δaa1-7 is delayed in BHK cells when compared to -mRFPVP26Δaa1-7 [8]. Thus, the growth properties of these HSV1 strains varied depending on the type of the tag, the location of the tag, and the cell line. After synchronous infection of Vero cells with an MOI of 10 PFU/cell (Fig. 2C), the parental HSV1(17) synthesized more proteins and secreted more virions into the medium than HSV1(17)blueLox and its derivatives. An antibody raised against VP26 detected a band around 12 kDa (Fig. 3A) or HSV1(17)blueLox-GFPVP26Δaa1-7 and -mRFPVP26Δaa1-7 did not yield virions. But an analysis of transfected cells revealed small nuclear GFPVP26Δaa1-7 and -YFPVP26Δaa5-7 flocs of the typical capsid size as well as large nuclear GFPVP26Δaa5-7 aggregates, which were both also labeled by antibodies against VP5 or VP26 (Fig. 3D).

After transfection with the BACs pHSV1(17)blueLox-GFPVP26Δaa5-7 or -YFPVP26Δaa5-7 did not yield virions. But an analysis of transfected cells revealed small nuclear GFPVP26Δaa1-7 and -YFPVP26Δaa5-7 flocs of the typical capsid size as well as large nuclear GFPVP26Δaa5-7 aggregates, which were both also labeled by antibodies against VP5 or VP26 (Fig. 3D). However, there were much fewer cytoplasmic GFPVP26Δaa1-7 capsids than after infection with HSV1(17)blueLox-GFPVP26Δaa1-7 (c.f. Fig. 3C with Fig. 3D). The nuclear aggregates after transfection with -YFPVP26Δaa5-7 were even larger and of irregular shape, and there were no individual nuclear or cytoplasmic capsids (data not shown). In contrast, the strain HSV1(17)blueLox-CFPVP26Δaa5-7 induced fewer and smaller nuclear aggregates, and there were single nuclear and cytoplasmic capsids, both after transfection and infection (not shown). The difference between HSV1(17)blueLox-GFPVP26Δaa1-7 and -GFPVP26Δaa5-7 might have been due to different expression levels as the protein GFPVP26Δaa1-7 had been expressed from viral genomes, whereas the mRNAs for GFPVP26Δaa5-7 were transcribed from the transected BAC plasmids. However, in the case of HSV1(17)blueLox-GFPVP26Δaa1-7, capsids were export ed to the cytoplasm after both, infection for 9 h (Fig. 3C), or transfection for 24 h (Fig. 3E). While GFPVP26Δaa5-7 was largely confined to the nucleus (Fig. 3D), GFPVP26Δaa1-7 particles even spread from the transfected to neighboring cells and were transported to the neighboring nucleus (Fig. 3Ei, white arrows). After PHEMO fixation, DNA could be detected in the nuclear aggregates by TO-PRO-3 staining, whereas after PFA fixation this was not the case (Fig. 3Eii) as described previously [4].

We have reported previously that the capsids of HSV1(17)blueLox accumulate faster in the cytoplasm than those of HSV1(17)blueLox-GFPVP26Δaa1-7-DmRFP (Fig. 8C in [8]). Here, we have analyzed also the time course of nuclear aggregate formation using the same data set of randomly collected confocal fluorescence microscopy images. The cells were classified by their phenotypes as having (i) no nuclear VP26 as in uninfected cells (none), (ii) individual nuclear capsids (single), (iii) many capsids filling the entire nucleus (grainy), or (iv) nuclear aggregates containing VP26 (Fig. 4A). Already 4 h after infection with HSV1(17)blueLox (Fig. 4B), more than 30% of the cells contained nuclear capsids, and by 8 h almost 90% of the nuclei...
contained many nuclear capsids. By 12 h, the first nuclear aggregates appeared in the nuclei of some few cells. In contrast, after infection with HSV1(17)blueLox-GFPVP26Daa1–7-gDmRFP (Fig. 4C), there were already prominent nuclear aggregates in 25% of the nuclei after 6 h, and by 10 h this had risen to 70% [8]. In summary, our data indicate an inverse correlation between the amount of nuclear aggregate formation and the efficiency of nuclear capsid egress and thus infectivity. The extent of inhibition depended on the type of the tag with mRFP or CFP being almost non-invasive, on the location of the tag with XFPVP26Daa1–7 being better tolerated than XFPVP26Daa5–7, and on the cell line with Vero cells being less prone to aggregate formation than HeLa, RPE or neuronal cells.

Nuclear Aggregates Contain Capsid Proteins but not Capsids
To further characterize the protein composition of the aggregates, we determined the subcellular localization of other HSV1 proteins in infected or transfected Vero cells (summarized

Figure 2. Characterization of HSV1(17)blueLox-VP26 strains. (A) Restriction digestion analysis of different BAC clones with NotI and EcoRI. Band shifts resulting from modifications in the UL35 ORF (arrowheads) and the NotI joint fragments containing the viral α-sequences (asterisks) are indicated. DNA sizes in kb. (B) For single-step growth kinetics, Vero cells were infected in duplicates with 10 PFU/cell, and the amount of secreted infectious virus at a given time point was determined by duplicate plaque assays. (C) Vero cells were infected at an MOI of 10 PFU/cell with HSV1(17) or HSV1(17)blueLox and its derivatives as indicated. After 48 h, the cells and virions secreted into the culture medium were harvested, and analyzed by SDS-PAGE and immunoblotting for expression of VP5 (α-NC-1) and VP26 (α-VP26). The signals in the molecular weight range below 25 kDa were further contrast enhanced.

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Figure 3. Nuclear aggregates induced by HSV1-XFPVP26 impair nuclear capsid egress. Vero cells were infected (inf.) with 10 PFU/cell of HSV1(17)+blueLox (A, wild type), HSV1(17)+blueLox-mRFPVP26_Daa1–7 (B), or HSV1(17)+blueLox-GFPVP26_Daa1–7 (C), and fixed at 9 h with PFA. Alternatively, cells were transfected (transf.) with pHHSV1(17)+blueLox-GFPVP26_Daa5–7 (D) or pHHSV1(17)+blueLox-GFPVP26_Daa1–7 (E), and fixed at 24 h. In addition to the intrinsic fluorescence of the XFPVP26 constructs (mRFPVP26 or GFPVP26), the subcellular localization of VP26 (α-VP26) and VP5 (MAb 5C10) were analyzed after permeabilization with TX-100 and immunolabeling by confocal fluorescence microscopy. The nuclei were stained with TO-PRO-3 (A, E). Arrows highlight cytoplasmic capsids (A–D) or incoming capsids at the nuclear rim of a neighboring cell (Ei). Scale bar: 10 μm. doi:10.1371/journal.pone.0044177.g003
in Table 1). Antibodies against two minor capsid associated proteins, pUL25 (Fig. 5Ai), or pUL17 (Fig. 5Bi), as well as VP26 (Fig. 5ii, 5iii, 5iv), co-localized with the XFPVP26 throughout the nuclear aggregates, both after infection with HSV1(17)blueLox-GFPVP26\textsubscript{AA5–7} (Fig. 5A-D), or after transfection with pHSV1(17)blueLox-GFPVP26\textsubscript{AA5–7} (Fig. 5E). The MAbs 5C10 directed against mature hexon epitopes also highlighted the entire volume of the aggregates (Fig. 5Aii, 3Aii, 3Dii, 3Eii), while MAb 8F5, also recognizing mature hexon epitopes, was enriched on the periphery of the aggregates (Fig. 5Cii, 5Dii, 5Fii). Since de Oliveira et al. (2008) detected a high amount of XFPVP26 on the periphery of the aggregates [4], we also imaged them with less sensitive detection settings at higher magnification (Fig. 5D). Occasionally, large aggregates appeared to consist of smaller entities but the GFPVP26 or anti-VP26 signals did not seem to be particularly enriched on the rims of the aggregates (Fig. 5Dii and 5Diii). Nevertheless, MAbs LP12 and MAbs H1.4, two other antibodies directed against VP5, also preferentially labeled the periphery but not the center of most aggregates (not shown; summarized in Table 1). Surprisingly, in cells transfected with pHSV1(17)blueLox-GFPVP26\textsubscript{AA5–7}, the mature hexon epitope recognized by MAb 8F5 was not detected at all (Fig. 5Eii), whereas the other antibodies directed against VP5 resulted in similar patterns as after infection with HSV1(17)blueLox-GFPVP26\textsubscript{AA5–7} (summarized in Table 1). This was not due to different expression levels in infected or transfected cells, as the MAb 8F5 epitope was formed after both, infection or transfection with pHSV1(17)blueLox-GFPVP26\textsubscript{AA5–7} (Fig. 5Fii).

Using correlative light and electron microscopy, we further analyzed RPE cells after infection with HSV1(KOS)-GFPVP26\textsubscript{AA5–7} (Fig. 6), a virus-cell combination that was particularly prone to nuclear aggregate formation. Prior to fixation, the subcellular localization of the nuclear aggregates was documented by fluorescence microscopy (Fig. 6A). Corresponding electron micrographs of ultrathin sections through such cells showed (Fig. 6B), particularly at higher magnifications (Fig. 6C-E) that these aggregates contained amorphous electron dense material (arrows) but no capsids, and that capsids (arrowheads) were found rarely at the periphery of the aggregates. The aggregates reacted specifically with the uranyl acetate stain that is applied after sectioning for contrast, indicating that this material was rich in proteins and/or nucleic acids (data not shown). The different types of A, B, and C capsids had all been generated, and sometimes also clustered into larger assemblies (Fig. 6C and E, arrowheads). In summary, immune labeling, the TO-PRO-3 staining after PHEMO fixation (not shown), and the electron microscopy analysis demonstrated that the nuclear aggregates comprised most, if not all capsid proteins and DNA, but many of them only abut HSV1 capsids, and the MAb 8F5 epitope was only formed on the aggregate periphery, or not at all after transfection with pHSV1-GFPVP26\textsubscript{AA5–7}.

Nuclear Aggregates do not Sequester pUL36, pUS3 or pUL34 and are Neither Replication Compartments nor Promyelocytic Leukemia Protein Nuclear Bodies

Like pUL17 and pUL25, the large inner tegument protein pUL36 has also been implicated in nuclear capsid egress [63–67]. After infection with HSV1(17)blueLox (Fig. 7A) or HSV1(17)blueLox-GFPVP26\textsubscript{AA5–7} (Fig. 7B), pUL36 as detected by antibodies raised against a middle region (pUL36\textsubscript{AA1408–2112}) or its C-terminus (pUL36\textsubscript{AA3048–3057}; not shown) was enriched in the perinuclear cytoplasm and localized in the cell periphery, where it co-localized with VP5 (arrows in Fig. 7Aii and 7Aiii), as reported previously [63]. The host chromatin, stained with TO-PRO-3, was marginalized to the nuclear periphery (Fig. 7A), as reported previously [68]. pUL36 did not colocalize with nuclear aggregates after infection with HSV1(17)blueLox-GFPVP26\textsubscript{AA5–7} (7Bii), after transfection with pHSV1(17)blueLox-GFPVP26\textsubscript{AA5–7} that does not release capsids from the nucleus into the cytoplasm (Fig. 7Cii), pUL36 was similarly distributed in the cytoplasm (Fig. 7Ciii). In few cells transfected with pHSV1(17)blueLox-GFPVP26\textsubscript{AA5–7}, pUL36 was enriched on the periphery of the nuclear aggregates (Fig. 7Ciii). In contrast, the HSV1 protein kinase pUS3 and the integral membrane protein pUL34 that function in nuclear capsid egress (reviewed in [66,67]) were localized at the nuclear envelope as after infection with HSV1 wild type, and not redirected to the nuclear aggregates (not shown).

The morphology of the nuclear aggregates seemed to have some resemblance to nuclear DNA replication compartments [69–71]. After infection with HSV1(17)blueLox, the single-strand DNA binding protein ICP8, an essential component of the HSV1
Figure 5. Nuclear aggregates contain capsid proteins. Vero cells were infected (inf.) with 10 PFU/cell of HSV1(17)\textsuperscript{blueLox-GFPVP26\textsubscript{last1-7} for 9 h (A–D) or transfected (transf.) with pHSV1(17)\textsuperscript{blueLox-GFPVP26\textsubscript{last1-7} (E) or pHSV1(17)\textsuperscript{blueLox-GFPVP26\textsubscript{last1-7} (F) for 24 h. The cells were fixed with PFA and permeabilized with TX-100. GFPVP26 was detected by its intrinsic fluorescence (left column). Furthermore, the cells were labeled with different VP5 antibodies (Mab 8F5 or 5C10) and with α-VP26, α-pUL25 or α-pUL17, and analyzed by confocal fluorescence microscopy. For row D, the specimen was scanned at higher magnification and lower photomultiplier settings. The arrows point to cytoplasmic capsids. Bars: 5 μm (D), 10 μm (F).

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Table 1. Characterization of HSV1-GFPVP26 nuclear aggregates.

| Antigen   | Antibody   | HSV1[17\textsuperscript{+}]blueLox-GFPVP26 | Subcellular localization | Detection in nuclear aggregates |
|-----------|------------|------------------------------------------|--------------------------|--------------------------------|
| VP26      | pAb aa 95–112 | Δaa1–7 infection | mainly nuclear, cytoplasmic dots | +++ entire |
| VP5       | mAb5C10    | Δaa1–7 infection | mainly nuclear, cytoplasmic dots | +++ entire |
|           | mAb 8F5    | Δaa1–7 infection | mainly nuclear, cytoplasmic dots | ++ periphery |
| mAb H1.4  | Δaa1–7 infection | mainly nuclear, cytoplasmic dots | ++ periphery |
| mAb LP12  | Δaa1–7 infection | mainly nuclear, cytoplasmic dots | ++ periphery |
| VP22a     | pAb NC-3,4  | Δaa1–7 infection | cytoplasmic dots | +++ (MeOH) |
|           |            | Δaa5–7 transfection | ++ (PHEMO) | periphery |
| VP23      | pAb NC-5   | Δaa1–7 infection | nuclear, nuclear rim, cytoplasmic dots | – – |
| mAb 1D2   | Δaa1–7 infection | perinuclear region | – – |
| pUL25     | mAb ID1, aa 154–703 | Δaa1–7 infection | nuclear, nuclear rim, cytoplasmic dots | – – |
| pUL17     | pAb #203 | Δaa1–7 infection | mainly nuclear, cytoplasmic dots | +++ entire |
| light capsid | pAb anti-LC  | Δaa1–7 infection | mainly nuclear, cytoplasmic dots | +++ periphery |
| pUL36     | pAb aa 1480–2112 | Δaa5–7 transfection | cytoplasm | + periphery |
|           | pAb C-term | Δaa1–7 infection | mainly perinuclear, cytoplasm | – – |
| pU53      | pAb aa 98–364 | Δaa1–7 infection | nuclear, nuclear rim | – – |
| pUL34     | pAb aa 1–252 | Δaa5–7 transfection | perinuclear region | – – |
| ICP8      | mAb HB6180 | Δaa1–7 infection | nuclear | – – |
| PML       | pAb H-238  | Δaa1–7 infection | degraded | – – |

Vero cells were infected with HSV1[17\textsuperscript{+}]blueLox-GFPVP26\textsubscript{mouse} -7 at an MOI of 10 PFU/cell and fixed at 9 h, or transfected with pHSV1[17\textsuperscript{+}]blueLox-GFPVP26\textsubscript{mouse} -7 or -GFPVP26\textsubscript{mouse} -7 and fixed at 24 h. The cells had been fixed with PFA unless indicated otherwise, and subsequently labeled with different antibodies. MeOH, methanol fixation; PHEMO, PHEMO fixation; –, not detected.

We therefore tested whether the nuclear HSV1 aggregates or capsids might have been trapped by this intrinsic antiviral defense mechanism. However, while uninfected cells contain several ND10 domains in each nucleus, the promyelocytic leukemia protein bodies were degraded after infection with HSV1[17\textsuperscript{+}]blueLox-GFPVP26\textsubscript{mouse} -7 as well as with HSV1[17\textsuperscript{+}]blueLox (not shown), as reported before for HSV1 wild type [75]. Therefore, the nuclear aggregates do not represent anti-viral promyelocytic leukemia protein bodies.
Discussion

Tagging of HSV1-VP26

Our attempts to tag the N-terminus of the non-essential small capsid protein VP26 of HSV1(17+)blueLox revealed that subtle differences in the FP domain defined whether a transfected BAC replicated with wild type kinetics or was non-infectious (c.f. Fig. 1). The degree of nuclear aggregate formation correlated with an impediment of nuclear capsid egress and depended on the presence of upstream non-coding sequences, the tag with mRFP or CFP being less invasive than GFP or YFP, the location of the tag with XFPVP26,AAaa1,7 being better tolerated than XFPVP26,AAaa5,7, and the cell type with Vero cells generating fewer aggregates than HeLa, RPE or primary neurons.

In cells infected with HSV1(17+), immunolabeling for VP5 or VP26 does not reveal such nuclear aggregates up to 15 h p.i., but rather results in a grainy pattern that most likely represents individual capsids (Fig. 3A; 4B; 7A; [62,63]). Notably, release of

Figure 6. Nuclear aggregates do not contain capsids. RPE cells were infected with HSV1(KOS)-GFPVP26,AAaa1,7 at an MOI of 10 PFU/cell. At 19.5 h, the cells were analyzed by fluorescence microscopy to identify nuclei with bright fluorescence spots (A). After fixation, embedding and sectioning these cells were further analyzed by electron microscopy (B–E). The nuclei contain nuclear capsids (arrowheads) as well as the amorphous electron dense material representing the aggregates (arrows) identified by fluorescence microscopy. The aggregates do not contain capsids (arrowheads). Bars: 10 μm (A), 5 μm (B), and 500 nm (C–E).
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Figure 7. Nuclear GFPVP26 aggregates do not sequester pUL36 and are not DNA replication compartments. Vero cells were infected (inf.) for 9 h with 10 PFU/cell of HSV1(17\textsuperscript{+})blueLox (A) or HSV1(17\textsuperscript{+})blueLox-GFPVP26\textsubscript{Daa1–7} (B, D), or transfected (transf.) with the pHSV1(17\textsuperscript{+})blueLox-GFPVP26\textsubscript{Daa5–7} for 24 h(C, E). The cells were fixed with PFA and permeabilized with TX-100. GFPVP26 was detected by its intrinsic fluorescence. Furthermore, the cells were labeled with anti-pUL36\textsubscript{aa1408–2112} and with MAb 5C10 against VP5 (A–C), against the single-strand DNA binding protein ICP8 and VP26 (D, E), or with TO-PRO-3 to stain the DNA (A). The arrows point to cytoplasmic capsids. Scale bar: 10 \textmu m.
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infectious particles from the infected cells commences as early as 12 h p.i. [Fig. 2B; [9]]. Therefore, we consider the nuclear VP26 aggregates that formed with certain FP tags as early as 6 h p.i. to be non-physiological. Since the subcellular distribution of mRFPVP26aa1–7 resembles that of the authentic VP26 more closely than the GFP variants, HSV1-mRFPVP26aa1–7 strain seems best suited to investigate the assembly and intracellular trafficking of HSV1.

The distinct phenotypes of the FPVP26 constructs may be related to differences in their quaternary structure. While GFP and YFP tend to dimerize with dissociation constants around 0.1 mM, the N146I mutation in CFP does not only increase its brightness but also its dissociation constant to 3 mM [76–79]. mRFP is not related to GFP and has been designed to remain monomeric [80]. Thus, dimerization and aggregate induction of GFPVP26aa5–7 and YFPVP26aa5–7 may be favored when compared to CFPVP26aa5–7 and mRFPVP26aa1–7, given the high concentration of capsid proteins in the nucleoplasm, and given an intrinsic dissociation constant of 0.02 mM for recombinant VP26 [28]. Furthermore, deleting non-coding sequences upstream of the UL35 gene reduced expression, and thus possibly the toxicity of GFPVP26aa1–7 when compared to GFPVP26aa5–7. Indeed, these 50 bp encompass a sequence with perfect consensus to the Inr element YYANWWY required for efficient transcription from HSV1 late promoters [81]. Another similar sequence within the deletion and two other sequences upstream of the deletion diverge from the Inr consensus in the last base pair (Fig. 1B).

In a triple-color HSV1(F) strain, VP26 has been tagged at codon 2 with YFPVYPA206K, with this YFP having a mutation that also increases its dissociation constant from 0.11 mM to 74 mM [11,77,78,82]. This strain is indeed infectious when the extent of infectivity and efficient nuclear capsid egress when the extent of nuclear aggregate formation is kept low.

Nuclear Aggregates do not Contain Capsids

In contrast to de Oliveira et al. [2008], we could detect the tagged VP26, VP5 (MAb 5G10), pUL17 and pUL25 not only on the rim but also within the nuclear aggregates. The aggregates resemble so-called assemblons that can form with HSV1(F) as early as 6 h but become prominent at 16 h of infection, and that also contain the capsid proteins VP5 and VP19c [84,85]. Ward et al. [1996]) therefore suggested that assemblons represent the nuclear site of capsid assembly [84]. Consistent with this hypothesis the nuclear aggregates also contained the scaffolding protein VP22a and the minor capsid proteins pUL17 and pUL25 (c.f. Table 1).

In our analysis, we used four different MAbs raised against VP5: LP12 and H1.4 that detect both, soluble VP5 and capsids, as well as 5C10 and 8F5 that recognize mature hexon epitopes on capsids whose formation requires ATP, proteolytic cleavage of the capsid scaffold and VP26 [5,56,62,86–90]. However, only 5C10 labeled the entire aggregates whereas 8F5, LP12, and H1.4, and also the PAb anti-LC were restricted to their periphery, and after transfection with pHSV1[17’]blueLox-GFPVP26aa5–7, the 8F5 epitope could not be detected at all (c.f. Table 1). Chi & Wilson (2000) suggested that the tag on GFPVP26aa1–7 may interfere with 8F5 but not 5C10 binding, and we have also reported that 8F5 and 5C10 prefer untagged over HSV1(KOS)-GFPVP26aa1–7 capsids [3]. However, PAb anti-LC recognizes capsids irrespective of the tag, and LP12 even prefers HSV1(KOS)-GFPVP26aa1–7 over wild type capsids [5], whereas the nuclear aggregates described here exposed LC and LP12 epitopes only in their periphery. These immunological data are inconsistent with the notion that the nuclear aggregates are conglomerations of authentic progeny capsids. Furthermore, our correlative light and electron microscopy studies showed that many of the nuclear aggregates lack capsids. We therefore conclude that the aggregates are not some sort of capsid assemblons, but may represent an accumulation of dead-end products as also suggested by Lamberti & Weller (1990) and de Bruyn Kops et al. (1990) [69,72]; nevertheless, they may abut areas enriched for capsids.

Therefore, in contrast to what we [8] and others have suggested, a recruitment of viral or host proteins to these nuclear aggregates containing VP5, VP22a, VP26, pUL17 and pUL25 does not indicate that they interact with authentic capsids. Our data rather suggest that these aggregates confiscate so much capsid proteins and possibly other proteins interacting with VP26 that proper capsid assembly and nuclear egress are no longer possible. In our experiments, even pUL36 had been sequestered by the nuclear aggregates in some few cells, although pUL36 usually does not associate with nuclear but only with cytoplasmic capsids [63]. Furthermore, Luxton et al. (2006) [91] reported that deleting UL36 in a PrV strain whose capsids have been tagged with GFPVP26 results in impaired nuclear egress whereas capsids of untagged PrV or HSV1 strains are still exported to the cytoplasm in the absence of pUL36 [63,92–94]. Thus, two mutations with moderate phenotypes, namely a tag on VP26 and deletion of UL36, if combined might potentiate and result in a synthetic phenotype [45,46], in this case impediment of nuclear egress. Such a crosstalk between different mutated proteins might be used to reveal subtle phenotypes which cannot be elucidated otherwise.

Moreover, small chemical compounds fostering aggregation of UL35 gene reduced expression, and thus possibly the toxicity of GFPVP26aa1–7, given the high concentration of capsid proteins in the nucleoplasm, and given an intrinsic dissociation constant of 0.02 mM for recombinant VP26 [28]. Furthermore, deleting non-coding sequences upstream of the UL35 gene reduced expression, and thus possibly the toxicity of GFPVP26aa1–7 when compared to GFPVP26aa5–7. Indeed, these 50 bp encompass a sequence with perfect consensus to the Inr element YYANWWY required for efficient transcription from HSV1 late promoters [81]. Another similar sequence within the deletion and two other sequences upstream of the deletion diverge from the Inr consensus in the last base pair (Fig. 1B).

In a triple-color HSV1(F) strain, VP26 has been tagged at codon 2 with YFPVYPA206K, with this YFP having a mutation that also increases its dissociation constant from 0.11 mM to 74 mM [11,77,78,82]. This strain is indeed infectious in contrast to our HSV1[17’]blueLox-YFPVP26aa5–7, but HSV1(F)-YFPVYPA206K-VP26aa1–7 also replicates less well than its parental strain, and induces nuclear aggregates even in Vero cells [11]. An HSV1(F) strain with N-terminal mRFP on VP26 lacking only 4 initial codons but not the authentic non-coding upstream sequences is also infectious; however, in contrast to our HSV1[17’]blueLox-mRFPVP26aa1–7 reported here, this strain also induces nuclear aggregates late in infection, and it is a bit attenuated in Vero cells, too [4]. Furthermore, a PrV-mRFPVP26 strain with authentic upstream sequences and mRFP located between codon 2 and 3 also induces nuclear aggregates and forms smaller plaques [44,83].

Thus, it seems more likely that the reduced protein expression due to 50 upstream bp missing, and not the removal of the 4 N-terminal codons contributes to the almost wild type properties of our HSV1[17’]blueLox-mRFPVP26aa1–7. We considered that further compensatory mutations might have allowed efficient capsid assembly and nuclear egress of some XFPVP26 mutants. However, when we monitored several independent lots of cells transfected with BAC DNA, there were no signs of any adaption periods during which GFPVP26aa5–7, mRFPVP26aa1–7 or GFPVP26aa1–7 changed from initial slower growth to faster replication cycles. Furthermore, all experiments were conducted with viron of passage 2 or 3 post transfection, and GFPVP26aa1–7 and YFPVP26aa5–7 did not replicate even after prolonged culture. In summary these studies suggest that VP26 can be successfully tagged with FP domains while maintaining infectivity and efficient nuclear capsid egress when the extent of nuclear aggregate formation is kept low.
of an infection resembles more that of untagged capsids when compared to the other tags on VP26.

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Author Contributions

Conceived and designed the experiments: CHN KD RB BS. Wrote the paper: CHN KD BS.

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