The C-terminal part of the human cytomegalovirus terminase subunit pUL51 is central for terminase complex assembly

Sebastian Neuber, Karen Wagner, Martin Messerle* and Eva Maria Borst*

Abstract

The cleavage and packaging of the human cytomegalovirus (HCMV) genome is accomplished by the viral terminase, comprising pUL56 and pUL89, and the recently identified pUL51 subunit. Since knowledge about pUL51 is scarce, we aimed at identifying pUL51 domains that are important for terminase assembly. In silico analysis suggested that the N-terminal half of pUL51 is intrinsically disordered, and that α-helices are present in the C-terminal part. Linker-scanning mutagenesis of pUL51 in the context of the viral genome revealed that amino acid insertions into the predicted α-helices are not compatible with viral growth, whereas upon mutagenesis of the putatively disordered parts interaction with pUL56 and pUL89 was retained and viral progeny was produced. Replacement of pUL51 with the closely related M51 protein of mouse cytomegalovirus did not lead to viable virus, indicating that M51 cannot substitute for pUL51, and swapping the M51 and UL51 N- and C-termini demonstrated the critical role of the pUL51 C-terminal part in building the terminase complex. Notably, the pUL51 C-terminus alone turned out to be sufficient to enable terminase assembly, its nuclear localization and plaque formation. Using HCMV mutants expressing differently tagged pUL51 versions, we did not detect oligomerization of pUL51, as has been proposed for the pUL51 orthologues of other herpesviruses. These data provide an insight into the interaction of pUL51 with the other two terminase components, and provide the basis for unravelling the mode of action of novel antiviral drugs targeting the HCMV terminase.

INTRODUCTION

Human cytomegalovirus (HCMV), the prototypic β-herpesvirus, is highly prevalent worldwide. HCMV disease caused by primary infection, reinfection, or reactivation from latency is a serious complication and can lead to life-threatening syndromes in immunocompromised patients, for instance transplant recipients and immunologically immature infants. Following congenital HCMV infection, long-term sequelae, such as hearing loss or neurological disorders, can occur [1]. The approved anticytomegaloviral drugs all target the viral DNA polymerase, yet upon prolonged treatment severe side-effects (e.g. neutropenia, myelosuppression and nephrotoxicity) are observed, as well as selection of resistant virus [2–4]. In addition to replication of the viral genome by the HCMV polymerase, cleavage of viral DNA and its packaging into capsids have emerged as processes vulnerable to drug inhibition [5–10]. One of the drug candidates targeting HCMV genome encapsidation, Letermovir, exhibited a favourable safety profile, was highly efficacious in transplant patients [11–14] and has lately met the primary endpoint in a phase III clinical study [15] (ClinicalTrials.gov ID: NCT02137772). Letermovir targets the pUL56 subunit of the HCMV terminase [16–18], a viral protein complex with no counterpart in mammalian cells, yet its precise mode of action remains to be determined. Until recently, the terminase was considered to be a heterodimeric enzyme consisting of the pUL56 and pUL89 subunits. It was reported that both proteins bind to viral DNA, possess nuclease activity and contain Walker A and B motifs characteristic of ATP-metabolizing proteins [19–23]. So far ATPase activity has only been described for pUL56 [24, 25], which was also found to bind the HCMV packaging signal [26] and associate with capsids [27]. Based on the available data, a model was suggested that implied that pUL56 provides the energy needed to translocate the viral DNA into capsids and for pUL89 to mediate genome cleavage [22].

We have recently shown that a third HCMV protein, pUL51, associates with pUL56 and pUL89, forming a stable complex in infected cells [28]. Following knock-down of pUL51, viral genomes remained uncleaved and only empty B capsids were produced [28]. These data are supportive of pUL51 representing a third component of the HCMV...
terminase complex. Further studies revealed that pUL51 is required to maintain appropriate UL56 and UL89 protein levels, to mediate pUL56/pUL89 interaction and to promote nuclear localization of pUL89 [29]. Remarkably, subsequent analyses then disclosed that this feature is not specific to pUL51, but applies to pUL56 and pUL89 as well, as in the absence of either terminase subunit the others underwent increased proteasomal turnover, did not interact efficiently, and their nuclear targeting was impaired [29]. We therefore proposed that HCMV terminase complex assembly is governed by a folding-upon-binding mechanism, which implies that mutual interactions among the subunits and their loading into the final complex stabilizes the proteins and protects them from degradation. Consistent with this model, in transfected cells the terminase proteins localized most efficiently to the nucleus in the presence of all three components [30], and in vitro studies indicated that the nuclease activity of pUL56 and pUL89 was enhanced upon their interaction [22].

Despite these recent insights into HCMV terminase formation, knowledge about the protein interfaces mediating binding of the individual subunits to each other is scant. Investigation into this topic would, however, help to elucidate the mechanism of action of novel antiviral compounds interfering with terminase complex assembly. So far, only the mapping of a 20-amino-acid sequence in pUL89 as a putative pUL56 interaction domain has been achieved [31], and a short peptide motif within the pUL56 C-terminus putative pUL56 interaction domain has been achieved [31]. Nevertheless, information about the pUL51 domains responsible for association with pUL56 and pUL89 is completely lacking. In this study, we aimed to identify the pUL51 domains responsible for HCMV terminase complex formation. Computer-based structure predictions suggest that the N-terminal half of pUL51 is intrinsically disordered, whereas the C-terminal part mainly consists of α-helices. Construction of HCMV BAC genomes harbouring targeted insertions of a linker sequence at different positions within the UL51 ORF showed that mutations of pUL51 outside of the presumptive α-helical regions do not interfere with pUL56 or pUL89 interaction. Additionally, the pUL51 C-terminus turned out to not only be crucial, but also sufficient to convey terminase complex assembly and promote its nuclear localization. Moreover, experiments using HCMV mutants expressing differently tagged pUL51 versions indicated that pUL51 does not oligomerize when in complex with pUL56 and pUL89. In summary, our data contribute to a better understanding of the role of the pUL51 subunit in assembly of the HCMV terminase complex.

RESULTS AND DISCUSSION

Ability of pUL51 linker-scanning mutants to support viral growth

To gain insight into the functional domains of the UL51 protein, linker-scanning mutagenesis was performed by inserting a DNA linker encoding aa GASAG at various positions of the UL51 ORF (Fig. 1a, top). In silico analyses predicted no specific secondary structure elements for the N-terminal half of pUL51, rather this part appeared to be disordered, forming random coils, whereas several α-helices were formed by the C-terminal aa sequence (Fig. 1a). The positions for linker insertion were selected in such a way that the pUL51 sequence was evenly covered, with a preference for stretches comprising hydrophilic aa, which are commonly exposed on the protein surface and are often part of the interface mediating protein–protein interactions. As we described before for the unmodified UL51 protein [28, 29], the ORFs encoding the pUL51 variants plus UL51 promoter sequences were introduced at an ectopic position of the HCMV genome (Fig. 1b, line 2), because due to the close spacing of viral ORFs, mutations within the UL51 ORF at the authentic position may have unwanted effects on the expression of neighbouring essential genes. The HCMV BAC pHG-ΔUL51 [28, 33] that expresses the EGFP marker gene and carries a disrupted UL51 ORF at the original position served as parental construct (Fig. 1b, line 1). BACs pHG-UL51-i1-11 encode the different pUL51 variants, which were all tagged with a Strep/FLAG/HA epitope at the N-terminus to check their expression (Fig. 1a, b, line 2). Notably, the control genome pHG-UL51-SF/Hα encoding the unmodified pUL51 (Fig. 1b, line 3) is known to give rise to a virus that grows with kinetics that are comparable to those of the parental HCMV [28, 29].

In order to assess the consequences of the GASAG insertions, we examined whether the transfection of cells with the BACs pHG-UL51-i1-11 yields infectious progeny virus. RPE-1 cells transfected 4 days earlier by the recently established adenovirus particle-mediated delivery protocol (adenofection, [34]) were added to monolayers of human foreskin fibroblasts (HFF) and 10 days later viral spread was monitored. We chose this approach because the adenofection efficiency was highest with RPE-1 cells, whereas HFF are more productive and allow the viral spread to be assessed more easily than RPE-1 cells. As expected, in cultures of HFF to which RPE-1 cells adenofected with pHG-ΔUL51 were seeded, only single green cells were detected, confirming that pUL51 is essential for the HCMV life cycle [28, 29], while efficient plaque formation was observed in HFF mixed with RPE-1 cells transfected with pHG-UL51-SF/Hα (Fig. 1c, top). As for the pHG-UL51-i1 BAC genomes, the pUL51-i1 and pUL51-i4 versions supported viral growth similarly to the parental UL51 protein, whereas the genomes encoding pUL51-i2, -i3, -i5, -i8 and -i11 resulted in mutant viruses forming smaller plaques, and no virus was reconstituted from BACs carrying ORFs UL51-i6, -i7, -i9 and -i10 (Fig. 1c, top). Measurement of plaque sizes (Fig. 1c, lower part) confirmed that viruses derived from BACs pHG-UL51-i1 and -i4 did not show impaired plaque formation, whereas viral spread was reduced approximately sevenfold for the mutants reconstituted from pHG-UL51-i2, -i3, -i5, -i8 and -i11. Taken together, the linker-scanning mutational analysis of pUL51 revealed that insertions into the predicted α-helices (i6, i7, i9 and i10) are not compatible with viral growth, while viruses
Fig. 1. Linker-scanning mutagenesis of pUL51. (a) Top: amino acid sequence of pUL51 (GenBank accession no. ACL51131) shown with putative α-helical elements depicted as grey cylinders (H1–H3). Secondary structure prediction was performed with the Robetta full-chain protein structure prediction server (http://robetta.bakerlab.org/) [64]. H, α-helix; C, random coil. The arrowheads indicate the positions of the GASAG linker insertions, giving rise to the respective UL51 mutants (i1–i11). Bottom: prediction of intrinsic disorder in pUL51 using the DISOPRED3 tool (http://bioinf.cs.ucl.ac.uk/psipred). Blue lines, intrinsic disorder profile; orange lines, putative protein-binding sites within disordered regions. (b) Schematic drawing of HCMV BACs generation. The abbreviations are as follows: F, FRT site; P, UL51 promoter region; SF/HA, Strep-, FLAG- and HA-tags; Δ, disrupted UL51 ORF; EGFP, enhanced green fluorescent protein; open boxes, repeat regions. The illustration is not drawn to scale. (c) Ability of mutated UL51 protein versions to support plaque formation. Top: HFF were mixed with adenofected RPE-1 cells and the spread of EGFP-expressing virus was monitored 10 days later by UV light microscopy. Scale bars, 100 µm. Bottom: the sizes of 100 plaques were determined on days 6, 8 and 10 post-infection (p.i.) using Jalview software (version 2.8). The means (±SD) are shown.
Interestingly, the presumptive α-helical aa regions are conserved among UL51 orthologues of different herpesvirus families, whereas almost no similarity was seen for the N-terminal sequences [35]. While we performed UL51 mutagenesis in the context of the viral genome, Beilstein et al. analysed the herpes simplex virus type 1 (HSV-1) UL33 protein (the orthologue of HCMV pUL51) by applying transposon mutagenesis to a UL33-encoding plasmid, and tested the functionality of pUL33 variants by trans-complementation of a HSV-1 UL33 null virus [35]. Comparable to our data for UL51, the pUL33 mutations within its C-terminal helices interfered with the growth of the UL33 deletion virus, whereas pUL33 variants harbouring mutations in the N-terminus retained the ability to complement. A recent study reporting on the generation of HSV-1 pUL33 mutants showed that deletions or amino acid substitutions within the conserved C-terminal half of pUL33 likewise interfere with the growth of HSV-1 [36]. Of note, a motif of charged amino acids in the pUL33 C-terminus that was suggested to be important for the release of monomeric genomes from cleaved viral concatamers and is conserved within α-herpesviruses (codons 110–113; [36]) is different in C-MVM pUL51 (KTQL instead of KRER in HSV-1 pUL33). Overall, these results point to an essential role for the predicted α-helices in the function of pUL51 and its orthologues. The N-terminal region appears to be less important, but the growth deficit of the HCMV mutants pHG-UL51-i2, -i3, -i5, -i8 and -i11 suggests that the N-terminus is nevertheless required for full activity of pUL51.

Insertions into the predicted α-helices affect the protein levels of the UL51 variants

We next examined expression of the mutated UL51 proteins in RPE-1 cells transfected with the corresponding HCMV BACs. Immunoblotting of whole-cell lysates prepared 4 days post-transfection revealed that UL51 protein levels were reduced for all mutant genomes, except pHG-UL51-i1 and -i4, when compared to the parental pHG-UL51-SF/HA BAC (Fig. 2a, top). Quantification of the signals showed that the most drastic reduction (90% and more) was seen with pUL51-i6, -i7, -i9 and -i10, which all contain the GASAG insertion within the putative helices, although the levels of pUL51-i2, -i3, -i5, -i8 and -i11, harbouring the mutations at other positions were also diminished (24–46% with respect to the levels of wild-type pUL51). The UL51 protein levels corresponded to the ability of the respective BAC genomes to generate infectious progeny virus (cf. Fig. 1c), i.e. reduction of the pUL51 to approximately one-third of the wild-type quantity resulted in smaller plaques, and no plaques were observed when the pUL51 levels were reduced to 10% and below. This implies that a certain amount of pUL51 is needed to enable viral growth, which is in line with the results of previous studies using a conditional knock-down approach to control pUL51 abundance [28, 33]. We assume that insertion of the GASAG aa sequence, a typical flexible linker, affects the secondary structure elements, such as helices, more strongly, whereas it is better tolerated within disordered regions. Disruption of the α-helical structures would lead to misfolding, triggering cellular protein quality control and the degradation of these pUL51 variants. In silico modelling of the UL51 i6, i7, i9 and i10 protein versions indeed predicted that the corresponding α-helices will be interrupted by the GASAG sequence (not shown). In line with this assumption, treatment of BAC-transfected RPE-1 cells with the proteasome inhibitor MG132 led to higher levels of the respective pUL51 versions (Fig. S1, available in the online version of this article). Taken together, these findings suggest that the UL51 protein is structurally constrained, with the putative α-helices having an important role in the preservation of robust pUL51 levels. Preliminary results obtained through circular dichroism spectroscopy analysis of bacterially produced UL51 protein indeed hinted at the presence of α-helical regions (Neuber, unpublished). Detailed structural analysis of pUL51, ideally in complex with pUL56 and pUL89, will be the topic of future studies. Expression levels and protein stability were not addressed in the mutational analysis of HSV-1 pUL33 [35], but our previous results showed that differences in HCMV terminase protein amounts are uncovered primarily in the context of viral infection [29]. Hence, such differences may be less obvious upon overexpression after plasmid transfection of cells or the usage of heterologous expression systems. Nonetheless, we think that this aspect also deserves attention for pUL51 orthologues of other herpesviruses.

pUL51 variants with mutations outside the putative α-helices support terminase complex formation and nuclear localization of terminase subunits

Next, we tried to pull down the pUL51 variants from the cell lysates depicted in Fig. 2(a) to see whether they interact with the other terminase subunits, pUL56 and pUL89. Due to the partially low abundance of the mutant UL51 proteins, the evaluation of co-purifying pUL56 and pUL89 was inconclusive (not shown). Since reasonable amounts of both pUL56 and pUL89 were present in the lysates, we performed reciprocal immunoprecipitations (IPs). pUL56 and pUL89 were quite efficiently immunoprecipitated from cells adenofected with the pHG-UL51-i6, -i7, -i9 and -i10 were used (Fig. 2b, lanes 9, 10, 12, and 14 to lane 16; Fig. S2) and co-precipitation of the respective other terminase subunits was detected. This was not the case when lysates of cells adenofected with pHG-UL51-i6, -i7, -i9 and -i10 were used (Fig. 2b, lanes 9, 10, 12, 13 and Fig. S2). We recently proposed a folding-upon-binding mechanism for terminase complex assembly, based on the finding that in the absence of one terminase subunit the others do not interact properly [29]. This scenario implies that the terminase proteins only adopt their bona fide conformation upon complex formation, whereas the isolated or pairwise expressed subunits are aberrantly folded. The observation that the antibodies against pUL56 or pUL89 recognize their target proteins less efficiently when pUL51 is
Fig. 2. The expression, interaction and localization of terminase proteins in RPE-1 cells adenofected with the HCMV BACs indicated, and the ability of mutant UL51 proteins to mediate terminase complex assembly in transiently transfected cells. (a) Four days post-transfection, total cell lysates were analysed by immunoblotting using the indicated antibodies. The HCMV late protein pUL52 was

used as a control for both transfection efficiency and viral gene expression, and GAPDH served as a loading control. The asterisks mark specific reactivity with a cellular protein. Quantification of protein levels of mutant pUL51-i variants was performed using ImageJ (version 1.47) as described elsewhere [29], with the signals of the wild-type UL51 protein set to 1.00. (b) pUL56 was immunoprecipitated from the cell lysates, and interacting pUL51-HA or pUL89 was detected by immunoblotting. IgG HC, immunoglobulin heavy chain. (c) RPE-1 cells were transiently transfected with transiently transfected pUL51 variants i6, i7, i9 and i10 or the parental pUL51, either alone or together with pUL56- and pUL89-expressing plasmids (middle). Protein expression was analysed in whole-cell lysates using the indicated antibodies, with GAPDH serving as a loading control. Right: following Streptactin pull-down of pUL51 or the variants, interacting pUL56 and pUL89 was assessed by immunoblotting. The asterisk denotes specific reactivity with a cellular protein. (d) RPE-1 cells were adenofected with the indicated BACs and analysed 4 days later by confocal laser scanning microscopy after labelling with antibodies against the HA-tag of pUL51, against pUL56 or against pUL89. EGFP expression marks BAC-transfected cells, and the contours of the nuclei are denoted by a white dashed line. The numbers below each panel indicate the proportion of cells exhibiting the localization pattern shown in the respective micrograph. Images displaying the same antibody staining were acquired with identical microscope settings. Scale bars, 10 µm.


missing or substantially diminished is in line with this model, as the respective epitopes may be less accessible when the proteins are not folded correctly. However, due to the low amounts of pUL51-i6, -i7, -i9 and -i10 present in the cell lysates and the inefficient IP of pUL56 and pUL89, a conclusion about their interaction cannot be drawn. To overcome this issue, pUL51-i6, -i7, -i9 and -i10 were transiently expressed in RPE-1 cells, either alone or in combination with pUL56 and pUL89 (Fig. 2c, left and middle). Indeed, the terminase subunits were detected at high levels under these conditions. This is in agreement with our previous observation that proteasomal turnover of the terminase components is most obvious in the viral genome as compared to transient transfection [29], which we attributed to the fact that the number of active proteasomes is increased in HCMV-infected cells. Streptactin pull-down of the mutant UL51 proteins and investigation of co-purifying pUL56 and pUL89 showed that terminase complex formation was strongly impaired (Fig. 2c, right). No pUL56 was associated with pUL51-i6, -i7 and -i9, and only very little pUL56 was detected with pUL51-i10. Likewise, the amounts of co-purifying pUL89 were strongly diminished, and were near the detection limit in case of pUL51-i6 and -i7. A similar outcome was observed when performing IP with the pUL56-specific antibody (Fig. S2; please note that due to limited efficiency of anti-pUL89 IP from transiently transfected cells [29] we performed the reciprocal IP with the pUL56 antibody only). These results indicate that the C-terminal part of pUL51 provides interaction domains for the other two terminase proteins, which become disrupted by the linker sequences. The impaired interactions, along with the assumed misfolding of the non-functional pUL51 versions (see above), then evidently result in proteasomal degradation in BAC-adenofected cells.

In HCMV-infected cells, the terminase proteins pUL51, pUL56 and pUL89 localize to the cell nucleus [28]. To assess the consequences of the pUL51 mutations for the subcellular distribution of the terminase components, adenofected RPE-1 cells were analysed by confocal laser scanning microscopy (Figs 2d and S3). As is exemplarily shown for pUL51-i2, UL51-i protein forms that were able to interact with pUL56 and pUL89 (see Fig. 2b) led to distinct nuclear localization of all terminase subunits. Conversely, following the transfection of pHG-UL51-i6 (as well as pHG-UL51-i7, -i9 and -i10), pUL89 was mainly seen in the cytoplasm, and pUL51 was barely detectable (Figs 2d and S3), which was consistent with the strongly decreased pUL51 levels observed with these BAC genomes (compare Fig. 2a). pUL56 was found in the nucleus throughout, demonstrating that none of the pUL51 mutations interfered with its correct subcellular targeting, and confirming that it can localize to the nucleus in the absence of the other terminase subunits [29, 37]. Taken together, only the pUL51 variants in which the integrity of the predicted structural elements within the C-terminus was preserved were able to promote the assembly of terminase subunits and their nuclear targeting.

Neither the C-terminal part of M51 nor the full-length protein is able to take over pUL51 function

Since our data pointed to an important role for the C-terminal half of pUL51, and this aa sequence is well conserved between HCMV pUL51 and its herpesvirus orthologues, we tested whether it can be substituted by the corresponding part of the mouse CMV (MCMV) M51 protein. M51 is a 233 aa protein with a predicted molecular mass of 25 kDa (Fig. S4). The conserved region in M51 starts at position 155 and exhibits 55 % aa identity with pUL51 (Fig. 3a). Remarkably, three helices, as predicted for pUL51, appear to be present within the M51 C-terminus as well. We constructed the BAC pHG-UL51-M51 that expresses a chimeric protein consisting of the pUL51 N-terminus (aa 1–70) fused to the M51 C-terminus (aa 150–233) (Fig. 3b, first line). Additionally, to examine whether the pUL51 C-terminus aa 1–70 can be replaced by the N-terminal part of M51 (aa 1–149), BAC pHG-M51-UL51 was generated (Fig. 3b, second line), and in pHG-M51 the complete M51 ORF replaced UL51 (Fig. 3b, third line). Of these mutant BACs, only pHG-M51-UL51 produced plaques upon transfection, whereas pHG-UL51-M51 and pHG-M51 did not (Fig. 3c, left). Thus, in the context of the HCMV genome the closely related M51 protein is not able to take over the task of pUL51. Likewise, a swap of the pUL51/M51 C-termini is not compatible with HCMV growth. As already suggested by the results for the pUL51 insertion mutants, the pUL51 N-terminal half appears to be less important for pUL51 function and could be replaced by the respective M51 sequence, albeit the resulting mutant displayed slower viral
Fig. 3. Properties of BAC genomes expressing chimeras between HCMV pUL51 and MCMV M51, or expressing the full-length M51 protein. (a) Amino acid sequence alignment of pUL51 and M51 (GenBank accession no. ADD10433) using the BLAST program revealing conservation of the C-terminal regions (aa 76–153 of pUL51 and aa 155–232 of M51, respectively). Predicted α-helices are indicated. (b) Left: schematic illustration of BAC constructs generated, with UL51-NT representing pUL51 aa 1–70 and UL51-CT aa 71–157, M51-NT signifying M51 aa 1–149 and M51-CT standing for aa 150–233. Black lines, HpaI restriction fragments characteristic of each BAC genome. The illustration is not drawn to scale. Right: restriction analysis of the HCMV BAC genomes displayed to the left. BAC DNA was cut with HpaI and separated by agarose gel electrophoresis, followed by ethidium bromide staining. Relevant fragments as shown in the schemes to the left are labelled with white dots. Numbers above the lanes correspond to the HCMV genomes depicted in the schematic drawing. (c) Assessment of plaque formation following adenofection of the indicated BACs was performed as described in Fig. 1(c). Scale bars, 100 µm.
spread (Fig. 3c, right). The aa sequence preceding the α-helical structures in M51 is considerably longer than that of pUL51, and in contrast to its presumably disordered pUL51 counterpart, is predicted to comprise several secondary structure elements (Fig. S4). These differences could account for the diminished growth capacity of the pHG-M51-UL51 mutant. In summary, our data imply that it is not merely the α-helical structures, but also the genuine C-terminal aa sequence that is necessary for pUL51 to fulfil its function.

In a similar way to that described for the UL51 insertion mutants, we checked expression of the chimeric proteins and M51, and also examined their ability to interact and promote nuclear localization of the other terminase subunits (Fig. 4). Each of these proteins was detected in adenofected RPE-1 cells, though with slightly reduced abundance compared to the native UL51 protein (Fig. 4a, left). Interestingly, while migration of the tagged M51 and the M51-UL51 protein version in the gel was almost in concordance with their predicted molecular masses (31.5 and 31.7 kDa), the UL51-M51 chimeric protein migrated much more slowly than expected (predicted molecular mass, 23.3 kDa). In fact, its apparent size (~38 kDa) was similar to that of pUL51, which we have previously described as exhibiting an aberrant mobility [28]. We hypothesized that this feature is due to the low pl value of pUL51 and the high content of proline residues [28], yet the current results suggest that its potentially intrinsically disordered N-terminal part also contributes to this aspect, as only the proteins comprising the pUL51 N-terminus performed quite differently in SDS-PAGE. The impact of intrinsically disordered protein regions (IDPRs) on electrophoretic mobility is known for many other proteins [38], including the HCMV tegument protein UL32/pp150 [39], and is due to the high content of hydrophilic aa within IDPRs, which results in less SDS binding and consequently slower migration through the gel.

Pull-down experiments utilizing the Strep-Tag of the UL51 variants (Fig. 4a, right), as well as immunoprecipitations with UL89 and UL56 antibodies (Fig. S5), revealed that only the M51-UL51 chimeric protein associated with pUL56 and pUL89, yet upon pull-down of either the UL51-M51 protein variant or M51, no binding to pUL56 was seen, and only minute amounts of interacting pUL89 were found (Fig. 4a, right). These data strongly suggest that the pUL51 C-terminus mediates the interaction with the other HCMV terminase subunits and that the corresponding part of M51 cannot provide this function. The fact that pUL51-M51 and M51 are non-functional in terms of terminase assembly and promotion of virus growth was also corroborated by immunofluorescence analysis of adenofected cells (Fig. 4b). Localization of pUL89 to the nucleus was only observed when M51-pUL51 was present. For pUL51-M51 as well as for M51, pUL89 was mainly cytoplasmic, and the signals obtained for all three terminase subunits were weak. As discussed elsewhere [28], during fixation and antibody labeling single components of a multi-protein entity may be lost from cells when their interaction is abolished. Overall, building of the HCMV terminase appears to rely on the presence of the authentic C-terminal half of the UL51 protein, which cannot be replaced by the analogous region of the closely related M51, which probably possesses a comparable secondary structure.

The pUL51 C-terminus is sufficient for interaction with pUL56 and pUL89

Next, we asked whether the C-Terminal part of pUL51 might be sufficient for ensuring viral growth. To address this point, we constructed BACs pHG-UL51-NT and pHG-UL51-CT, which encode either the first 70 aa or aa 71–157 of pUL51 (Fig. 5a). Following adenofection of RPE-1 cells, only pHG-UL51-CT turned out to be infectious (Fig. 5b), although the plaques were considerably smaller than those of the parental virus (Fig. 5b, lower left). Analysis of the growth kinetics revealed that the mutant expressing the pUL51 C-terminal half only produced about one order of magnitude fewer infectious progeny than the parental virus (Fig. 5b, lower right). The deficiency of pHG-UL51-NT with respect to plaque formation was not due to lack of expression of pUL51-NT, as it was detected in the adenofected cells (Fig. 5c, lane 1). The N-terminally truncated pUL51-CT seemed to be even less abundant (Fig. 5c, lane 2), but the amount was sufficient to enable plaque formation. Due to the apparently low amounts of the truncated pUL51 versions in BAC-transfected cells further analysis was performed upon the transfection of cells with expression plasmids encoding the respective proteins. As can be seen in Fig. 5(d), left, the truncated proteins were detected in higher abundance in relation to the authentic pUL51 than upon adenofection. In cells transfected with the pUL51-expressing plasmid, in addition to the expected 38 kDa band, another one of ~23 kDa (Fig. 5d, lane 4) was seen, which was not detected in infected or adenofected cells and might be a proteolytic cleavage product of pUL51 produced in transiently transfected cells. Interestingly, as has been described for the full-length pUL51 [29], pUL51-CT is also obviously able to stabilize and augment the level of the co-expressed UL56 protein in cells transfected with plasmids encoding the terminase subunits, whereas this was not the case in cells expressing the UL51-NT protein (Fig. 5d, left, lanes 2–4). Pull-down experiments revealed that, besides pUL51, pHG-UL51-CT is also able to mediate interaction with pUL56 and pUL89 and thus terminase complex assembly (Fig. 5d, right, lanes 3 and 4). In contrast, no pUL56 or pUL89 was associated with pUL51-NT (Fig. 5d, right, lane 2). The ability of pUL51-CT to convey terminase formation also resulted in the recruitment of all terminase subunits to the nucleus in transiently transfected cells (Fig. 5e, left). Conversely, pHG-UL51-NT was distributed throughout the cell, and residual pUL89 was detected outside of the nucleus (Fig. 5e, right). As described previously [28], the low amount of pUL89 often seen in immunofluorescence (IF), despite the high levels found in immunoblotting, is probably due to its diffuse cytoplasmic distribution in the absence of terminase assembly, as well as its being inability to form an ordered structure.
more prone to extraction during the IF procedure when it is not being incorporated into its genuine stable complex. In conclusion, terminase complex assembly can be accomplished by the C-terminal half of pUL51, and the N-terminal sequences appear to be dispensable for this task. Even so, the growth deficit of the virus derived from the BAC genome encoding the pUL51 C-terminal sequences only indicates that the pUL51 N-terminal part also has a role in the viral replication cycle. Although low levels of pUL51-CT could be a possible cause for the growth deficit, it stands to reason that the pUL51 N-terminal sequences are also of relevance. IDPRs, as predicted within the N-terminal part of pUL51, are known to take over various tasks, e.g. they can increase the stability of a protein or regulate its function through intramolecular interaction [40–42]. Moreover, IDPRs are typical for hub proteins that display multiple interaction partners within protein–protein interaction networks [43]. Indeed, yeast two-hybrid screens and other interaction analyses suggested that the pUL51 orthologues of other herpesviruses bind to various viral proteins, thereby constituting hubs within herpesviral protein networks that might link genome cleavage-packaging to nuclear egress [44–47]. In general, disordered protein regions enable the association with many different proteins of various conformations [48, 49]. Thus, intrinsic disorder within pUL51 would be supportive of the supposed hub-protein model, yet most of the suggested interaction partners of the pUL51 orthologues await their confirmation in infected cells. Of note, interactions between IDPRs and their target proteins are often highly dynamic and short-lived, of low affinity, and yet highly specific [50, 51], all characteristics to be expected from hub proteins that sequentially associate with a number of different proteins. However, such interactions are easily missed by standard IP procedures and their detection may require more elaborate techniques, such as cross-linking of proteins. In this context, it should be mentioned that to date no additional proteins other than pUL56 and pUL89 have been identified as being stably associated with pUL51 in HCMV-infected cells, and a recent study reporting on purification of the HSV-1 terminase complex from infected cells did not reveal further interaction partners other than the major capsid protein [52]. Altogether, the presumably intrinsically disordered N-terminus of pUL51 appears not to be directly involved in terminase complex

Fig. 4. Interaction of full-length M51 or chimeric UL51-M51 proteins with pUL56 and pUL89, and influence on nuclear localization of the terminase subunits. (a) Left: expression of the protein chimeras and the indicated proteins in RPE-1 cells adenofected with the given BAC genomes was examined on day 4 after transfection by immunoblotting. Asterisks mark unspecific bands. Right: SF/HA-tagged proteins (UL51-M51 chimeras, M51, or pUL51) were pulled down from the lysates using Streptactin sepharose, and co-purifying pUL56 or pUL89 was identified by immunoblotting. (b) Impact of M51 and UL51-M51 chimeric proteins on nuclear targeting of terminase subunits. Analysis of BAC-adenofected RPE-1 by confocal laser scanning microscopy was performed as denoted in Fig. 2(d). Scale bars, 10 µm.
Fig. 5. Role of the pUL51 C-terminus in terminase complex assembly. (a) Schematic illustration of the HCMV BAC genomes expressing either aa 1–70 of pUL51 (pHG-UL51-NT) or aa 71–157 (pHG-UL51-CT). (b) The plaque-forming ability of the genomes depicted in (a) (top; scale bars, 100 µm), with measurement of plaques sizes (lower left) and analysis of growth kinetics (lower right). Evaluation of plaques was performed as described in Fig. 1(c). For growth curves, HFF were infected at an m.o.i. of 0.05, and virus in the supernatants collected at the time points indicated was assessed by standard plaque assay. (c) Expression of the C- or N-terminally truncated pUL51 forms (UL51-NT or -CT) in RPE-1 cells 4 days post-adenofection was investigated by immunoblotting. The late protein pUL52 served as a control for adenofection efficiency and viral gene expression (asterisk, unspecific signal). (d) Interaction of truncated pUL51 versions with pUL56 and pUL89 in transiently transfected cells. Expression of the indicated proteins was examined in lysates of cells 2 days after transfection (left), and following Streptactin pull-down of pUL51 and its variants, co-purification of pUL56 and pUL89 was analysed (right). (e) Influence of truncated UL51 proteins on subcellular distribution of the terminase subunits. Transiently transfected cells were examined on day 2 post-transfection by confocal laser scanning microscopy. An EGFP-expressing plasmid was included in each setting to mark the transfected cells. Scale bars, 10 µm.
Assessment of pUL51 self-interaction

By using different assays the pUL51 orthologues were found to interact with themselves [44–47], although this aspect has not yet been addressed in viral infection. pUL51 shares structural similarities with several viral proteins, e.g. the small terminase subunit of phage 44RR [53], the KSHV ORF 57 protein [54], or HSV-1 ICP27 [55], inasmuch as they all contain an unstructured N-terminal region followed by an α-helical C-terminus, and these proteins were shown to form homodimers or oligomers through their C-termini. To examine potential self-interaction of pUL51, we generated HCMV mutants in which pUL51 was fused to either the V5- or the myc-epitope, and which expressed the red fluorescent protein mRFP instead of EGFP (Fig. 6a), and then performed co-infections with PHG-UL51-SF/HA. The micrographs in Fig. 6b show that the majority of cells took up both virus mutants and 4 days post-infection expressed both the EGFP and the mRFP markers (although due to the different expression levels of the marker proteins in individual cells some cells do not appear as bright yellow; examples are marked with arrows). The pUL51-SF/HA and pUL51-V5 variants were readily detected in the corresponding cell lysates (Fig. 6b), whereas due to the lower sensitivity of the myc-antibodies, expression of pUL51-myc could only be verified following enrichment with myc-trap beads (cf. Fig. 6d, bottom). Upon pull-down of pUL51-SF/HA from lysates of cells co-expressing either pUL51-V5 or pUL51-myc, it was possible to isolate the SF/HA-tagged pUL51 very efficiently (Fig. 6c, first panels), yet neither pUL51-V5 nor pUL51-myc co-purified with pUL51-SF/HA (Fig. 6c, top and bottom, lane 2). The weak signal obtained for pUL51-myc is similar to the background of the negative control (Fig. 6c, bottom, compare lanes 1 and 2), and probably represents pUL51-myc adhering non-specifically to the Streptactin matrix). Consequently, this experiment did not hint at self-interaction of pUL51. As a positive control, we checked for pUL56 interacting with pUL51-SF/HA, which did indeed co-purify as expected (Fig. 6c, top, lanes 1 and 2, and bottom, lane 2). Reciprocal co-IPs gave the same results, as following IP of pUL51-V5 no interacting pUL51-SF/HA was found (Fig. 6d, top, lane 2), and, likewise, after IP of pUL51-myc, pUL51-SF/HA did not co-purify (Fig. 6d, bottom, lane 2). Again, ample quantities of the control protein pUL56 were bound to the pUL51 variants in both settings, as anticipated (Fig. 6d, top, lanes 2 and 3, and bottom, lanes 1 and 2). In summary, these data argue against self-interaction of pUL51 in HCMV-infected cells, at least when pUL51 is part of the terminase complex. With regard to this, it was recently proposed that the terminase of varicella zoster virus exists as a 1:1:1 heterotrimer in which each of the three subunits is represented once, but nonetheless higher-order assemblies of these trimeric complexes may eventually engage in genome cleavage-packaging [56]. Still, when performing its supposed function as a hub protein, pUL51 may form short-lived protein complexes in which it is present as a dimer or an oligomer, but investigation into this aspect requires alternative experimental approaches, as outlined above.

In conclusion, our data broaden our knowledge of the formation of the tripartite HCMV terminase complex, especially with respect to the pUL51 domains involved. Further dissection of the protein interfaces mediating assembly of the terminase subunits will add to a deeper understanding of the mode of action of antiviral drugs targeting HCMV genome encapsidation.

METHODS

Cell culture and transient transfection

Human foreskin fibroblasts (HFF), hTERT-RPE-1 cells (Clontech Laboratories, Palo Alto, CA, USA) and HeLa cells were propagated as reported previously [29, 34, 57]. RPE-1 or HeLa cells were transfected by use of the jetPEI reagent (PolyPlus company, distributed by VWR, Darmstadt, Germany) as outlined previously [29].

Plasmid construction

The oligonucleotides used in this study are listed in the Table S1. For ectopic insertion of GASAG-modified UL51 sequences into HCMV BAC pHG-ΔUL51 by Flp-mediated recombination [58], the respective sequences were transferred into pORi6K-derived shuttle plasmids containing a kanamycin-resistance cassette, a flippase (Flp) recognition target (FRT) site and the R6Kα bacterial origin of replication [59]. The UL51-i2, -i3, -i5, -i6, -i7, -i8, -i10 and -i11 insertion mutants were generated by gene synthesis (Eurofins MWG Operon, Martinsried, Germany) and cloned into the SpeI and SacI sites of pORi6K-UL51-P-SF, a derivative of pORi6K-UL51-SF [28] that provides 255 nt of UL51 upstream promoter sequences, as well as Strep-FLAG-HA (SF/HA) epitope-encoding sequences for tagging of the UL51 variants. Shuttle plasmids harbouring UL51-i1, -i4 and -i9 were constructed by PCR-based mutagenesis using 5′-phosphorylated oligonucleotides containing the GASAG linker sequence. Following the amplification of plasmid pORi6K-UL51-SF using UL51-i1.for and UL51-i1.rev, UL51-i4.for and UL51-i4.rev, or UL51-i9.for and UL51-i9.rev, ligation of the PCR products was performed, and all plasmids comprising the R6Kα origin of replication were propagated in the Escherichia coli strain PIR1 (Invitrogen/Thermo Fisher Scientific, Dreieich, Germany).

Shuttle vectors expressing chimeric HCMV UL51-MCMV M51 proteins fused to the SF/HA-tag were constructed by Gibson assembly. M51 sequences corresponding to aa 1–149 or aa 150–233, respectively, were PCR-amplified with the primer pairs M51-1-149.for and M51-1-149.rev, or M51-150-233.for and M51-150-233.rev using pSM3fr [60] as a template. Vector sequences plus either the 5′-region of ORF UL51 representing aa 1–70 or the UL51 3′-region encoding aa 71–157 were obtained from pORi6K-UL51-SF using the primer pairs UL51-1-70.for and UL51-1-70.rev, or UL51-71-157.for and UL51-71-157.rev. Ligation of PCR
Fig. 6. Assessment of pUL51 self-interaction in infected cells. (a) Schematic drawing of the two HCMV genomes encoding mRFP (RFP) and either myc- or V5-tagged pUL51, and also of the pHG-UL51-SF/HA BAC. (b) Top: HFF were infected with the indicated viruses and fluorescence microscopy was performed 4 days later. Arrows denote examples of cells that did not appear as bright yellow due to variable expression of either the mRFP or the EGFP marker. Scale bars, 50 µm. Bottom: expression of the indicated viral proteins in infected cells at day 4 was tested with the HA-, V5- and pUL56-specific antibodies. (c) Streptactin pull-down of pUL51-SF/HA from the cell lysates shown in (b) and analysis of interacting pUL51-V5, pUL51-myc, or pUL56 by immunoblotting. (d) pUL51 tagged with either the V5 or the myc epitope was immunoprecipitated from the cell lysates and co-purification of pUL51-SF/HA or pUL56 was examined.
products was performed using the Gibson Assembly Master Mix according to the manufacturer’s instructions (New England Biolabs, Frankfurt, Germany), giving rise to pOri6K-UL51-M51 and pOri6K-M51-UL51, respectively. pOri6K-M51 encoding full-length Strep-FLAG-HA-tagged UL51 was similarly generated by Gibson assembly. The M51 ORF was PCR-amplified from pSM3fr using M51.for and M51.rev, followed by ligation through applying Gibson assembly to a PCR product representing the vector backbone, which was obtained from pOri6K-M51-SF with M51-backbone.for and M51-backbone.rev. pOri6K-UL51-M51 encoding full-length Strep-FLAG-HA-tagged UL51 and pOri6K-M51-UL51, respectively.

Bacterial artificial chromosome (BAC) mutagenesis and virus reconstitution

The recombinant viral genomes constructed in this study are based on the BAC-cloned HCMV strain AD169 [57]. Mutant HCMV BACs relying on the integration of pOri6K-derived shuttle vectors were generated by inserting the respective plasmid at the ectopic UL1-10 locus of pHG-ΔUL51 through Flp recombinase as reported earlier [28, 58, 59].

pHR-ΔUL51 designated for the insertion of either the myc- or the V5-tagged pUL51 version was constructed by en passant mutagenesis [61]. The mRFP ORF, together with a kanamycin-resistance cassette and an I-SceI restriction site, was amplified with the primer pair mRFP1.for and mRFP1.rev from pMCMV3-mRFP1 (Messerle, unpublished). The resulting PCR product was recombined with pHG-ΔUL51 by red-α-, β- and γ-mediated homologous recombination in E. coli strain GS1783 (kindly provided by Gregory Smith, Northwestern University, Chicago, IL, USA), resulting in pHR-ΔUL51, in which the EGFP expression cassette in the US region was replaced by the mRFP sequences. Successful mutagenesis was confirmed by restriction analysis and sequencing of relevant parts within the respective BACs. pHG-UL51-SF/HA expressing the original pUL51 fused to the SF/HA-tag has been reported previously [29]. Virus mutants were reconstituted from recombinant BAC genomes upon the adenofection of RPE-1 cells [29, 34, 62], which were mixed 4 days post-transfection with HFF followed by further incubation until complete cytopathic effect was observed.

Monitoring of plaque formation

BAC-adenofected RPE-1 were harvested on day 4 post-transfection, and 8×10^6 cells were added to a confluent monolayer of HFF cells in six-well plates. Ten days later, EGFP expression was visualized using an Axio Observer Z1 fluorescence microscope (Zeiss, Oberkochen, Germany), and images were further processed using AxioVision (version 4.9.1.0), Photoscape (version 3.7) and Adobe Photoshop CS4 (version 11.0). To evaluate plaque sizes, cells were cultivated in semi-solid medium containing 0.75 % (w/v) carboxymethylcellulose (Sigma-Aldrich, Taufkirchen, Germany). Images of virus plaques were taken with a Nikon Eclipse TS100 fluorescence microscope, and plaque areas were measured using ImageJ software (version 1.47 v).

Immunoblotting and immunofluorescence microscopy

For immunoblot analysis, cells were lysed in Roti-Load 1 (Carl Roth, Karlsruhe, Germany), and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The primary antibody dilutions were 1 : 1000 for the rabbit HA monoclonal antibody (mAb) (C29F4, Cell Signalling Technologies/NEB, Frankfurt, Germany), the V5 rabbit mAb (D3HQ8, Cell Signalling Technologies) and the myc-tag rabbit mAb (71D10, Cell Signalling Technologies); 1 : 100 for the anti-pUL56 and the anti-pUL89 mouse
hybridoma supernatants [28, 29]; 1: 200 for the anti-pUL52 mouse hybridoma supernatant [28]; and 1:2000 for the rabbit GAPDH mAb (14C10, Cell Signalling Technologies). The secondary horseradish peroxidase-conjugated antibodies employed were goat anti-mouse IgG (1:3500; Pierce, Dreieich, Germany, catalogue no. 32230) and goat anti-rabbit IgG (1:2000; Pierce, catalogue no. 32260), and signals were detected by chemiluminescence using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and an LAS-3000 imager (Fujifilm, Düsseldorf, Germany). Images were further processed with Adobe Photoshop CS4.

Immunofluorescence microscopy of transfected cells was performed on day 4 (BAC-adenofected RPE-1) or day 2 post-transfection (transiently transfected HeLa cells) as delineated elsewhere [63]. The primary antibody dilutions were 1:500 for the rabbit HA mAb, and 1:10 for the anti-pUL56 and anti-pUL89 mouse hybridoma supernatants. The secondary antibodies employed were Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, catalogue no. A11036) and Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, catalogue no. A11031), both at a dilution of 1:500. Images were taken with a confocal laser scanning microscope (LSM 510 Meta, Zeiss) and processed using AxioVision and Adobe Photoshop CS4.

Affinity purification and immunoprecipitation
RPE-1 cells were harvested on day 4 after adenofection or day 2 post-transfection with expression plasmids. Cells were lysed in 50 mM Tris-HCl/pH 7.4 –0.5 % NP-40 in the presence of protease inhibitors (Cocktail Set III, Calbiochem, Darmstadt, Germany). Insoluble material was removed by centrifugation (16 000 g, 15 min, 4 °C). Pull-down of SF/HA-epitope-tagged UL51 or M51 protein variants with Strept-Tactin sepharose (IBA Lifesciences, Göttingen, Germany) and co-immunoprecipitations using either the pUL56- or the pUL89-specific antibody plus Protein G Sepharose 4 Fast Flow (GE Healthcare, Darmstadt, Germany) were performed as reported previously [28]. To analyse potential self-interaction of pUL51, HFF were infected with the respective virus mutants at an m.o.i. of 1 followed by centrifugal enhacement (30 min, 950 g), and cell lysates were prepared as described above. For the pull-down of myc-tagged pUL51 or the immunoprecipitation of pUL51-V5, Myc-Trap_A beads (ChromoTek, Martinsried, Germany) or 3 μg of the V5 rabbit mAb per sample were used.

Funding information
This work was funded by the German Research Foundation (DFG; grant BO4176/1-2 to E. M. B.), and in part by the German Center for Infection Research (DZIF; grant ICH07.0802 to M. M.).

Acknowledgements
We thank Thomas Goldner and Peter Lischka (AICuris, Wuppertal, Germany) for providing expression plasmids for pUL56 and pUL89, and Jennifer Kleine-Albers for contributing to BAC mutagenesis. We are grateful to Christiane Ritter (Helmholtz Centre for Infection Research, Braunschweig, Germany) for performing the circular dichroism spectra measurement, and to the Research Core Unit for Laser Microscopy (ReCoLa) at Hannover Medical School for continuous support.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


---

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.