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ORIGINAL INVESTIGATION

The proteome of human cytomegalovirus virions and dense bodies is conserved across different strains

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Abstract The morphogenesis of human cytomegalovirus (HCMV) particles is incompletely understood. Analysis of the protein composition of HCMV virions and subviral dense bodies (DBs) by mass spectrometry provides valuable information to increase our knowledge about viral morphogenesis. Here we addressed the viral proteome of virions and DBs from two fibroblast-passaged isolates and the widely used endotheliotropic TB4-BAC40 strain of HCMV. The results show a striking concordance of the particle proteomes of different strains. One surprising finding was that only low levels of gpUL128-131A were found in TB40-BAC4 virions. These three proteins, together with gH and gL, form a protein complex that is critical for the endothelial cell tropism of that strain. This indicates that either few molecules of that complex per virion or a small fraction of pentamer-positive virions suffice to retain the tropism. Furthermore, using a pp65-deficient variant of TB40-BAC4, we confirm our previous finding that the major tegument protein serves as a scaffold to support the upload of a fraction of the outer tegument proteins into particles. The results demonstrate that HCMV particle morphogenesis is an orchestrated process that leads to the formation of particles with a largely strain-independent protein composition.

Keywords Cytomegalovirus · Proteome · Viral proteins · Virion · Dense bodies · Mass spectrometry · HCMV strains · UL128-131A

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Introduction

The application of quantitative proteomics in the field of virology has provided a wealth of information regarding virus–host interactions, morphogenesis and composition of viral particles [1, 2]. Initial studies on the particle proteome of the human cytomegalovirus (HCMV) revealed that the virions of that virus display a high level of complexity, containing at least 70 viral and 70 cellular proteins [3, 4]. Subviral particles, termed dense bodies (DBs) which are released in abundance from infected fibroblast cultures, share that complexity, yet to a lower level [3, 4]. We could recently confirm the data from Varnum and colleagues but observed some discrepancies in the abundances of particular viral proteins [5]. The overall particle proteome of different variants of one particular laboratory strains, AD169, revealed a striking conservation [6]. Analogous studies on HCMV mutants that lack expression of the abundant tegument protein pp65 (pUL83) showed that this polypeptide serves as a scaffold protein that supports upload of a fraction of the matrix proteins into virions [5]. The analyses, however, also revealed that the virion of HCMV displays a considerable level of flexibility, tolerating the loss of a substantial portion of the tegument protein mass without abolishing infectivity.

To the best of our knowledge, all proteomic analyses of HCMV virions and DBs published to date focused on derivatives of the AD169 laboratory strain. However, the genomes of such strains that have been propagated extensively in fibroblast cell culture display mutations and rearrangements, leading to a substantial loss in the coding capacity in each case [7–12]. The AD169 strain was the first virus where this deletion phenomenon was discovered. AD169 lacks the genomic region encompassing the UL133 to UL151 open reading frames [13]. In addition, due to a mutation in the UL131A gene, this virus is deficient in its expression of a pentameric protein complex consisting of gH, gL and UL128-131A [14, 15]. This complex is essential for HCMV infection of epithelial, endothelial or dendritic cells [14, 16, 17]. As a consequence of these genetic alterations, the particle proteome of the AD169 strain may not be fully representative for the proteome of other HCMV strains. In this study, we applied a well-established quantitative proteomics approach on virions and DBs from two fibroblast-passaged HCMV isolates as well as on the particles of the widely used, endotheliotropic strain TB40-BAC4. Our data reveal a surprising conservation in the protein composition of virions and DBs from the different HCMV strains. In particular, none of the proteins encoded by UL133 to UL151 seems to substantially contribute to the HCMV particle proteome.

Materials and methods

Viruses and particle purification

Primary clinical isolates R1 and R5 were obtained from the diagnostic repositories for clinical specimens of the Virology Institute of the University of Erlangen, Germany (kindly provided by Manfred Marschall) [18, 19]. These strains have lost their capacity to infect epithelial cells, likely because of their passaging on fibroblast cultures. The TB40/E strain was originally isolated from a bone marrow transplant recipient [20] and was subsequently cloned into a bacterial artificial chromosome vector, providing strain TB40-BAC4 after reconstitution [21]. The pp65-negative derivative TBUL83stop was generated by en passant mutagenesis as previously described [22, 23]. Briefly, *Escherichia coli* strain GS1783 [24] carrying TB40-BAC4 was transformed with 10 ng of a DpnI-digested and column-purified PCR product generated using plasmid pEPkan-S2 [25] (kindly provided by Jens von Einem, Ulm University) as a template and oligonucleotides 884 (5′CGGCTTTCAGCACGTGCCCCGAAATGGG ACCCAGTACGGATCACTATTCGGGACAACGGCGAC CAACCAATTAACCAATTCTGATTAG-3′) and 885 (5′-C GCAGGCAGCATGGAGTCGCGCGGTCGCCGTTGTCC CGAATAGTGATCCGTACTGGGTCCCATAGGATGACG ACGATAAGTAGGG-3′) as primers [26]. Homologous recombination [24] resulted in a BAC with an I-SceI-Kan cassette inserted in the UL83 coding sequence and stop codons replacing UL83 amino acids 11 and 12. Subsequent "scarless" removal of the I-SceI-Kan sequences by homologous recombination [24] produced pTBUL83stop. The identity and integrity of three pTBUL83stop clones were verified in comparison with TB40-BAC4 by restriction fragment length and DNA sequence analysis, and clone 1 was used for the experiments described in this work. Viruses were reconstituted, and virus stocks were produced upon electroporation of BAC DNA into MRC-5 cells following standard protocols [27]. The bacterial artificial chromosome (BAC)-derived virus BADwt represents the AD169 strain of HCMV and was reconstituted on HFF from pAD-cre (kindly provided by Thomas Shenk, Princeton). All viruses were grown on human foreskin fibroblasts (HFF). Virus purification from the culture supernatants of infected HFF by glycerol–tartrate ultracentrifugation was performed as recently published [5]. Particles were collected after the final centrifugation step in 100 µl PBS. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Bonn, Germany). Twenty micrograms of virions and DBs were subsequently diluted to result in 50–80 µl aliquots and were frozen at −80 °C until mass spectrometry was performed.

Proteomic analyses

The quantitative proteomics analyses of purified viral particles were performed in four technical replicates using ion-mobility-enhanced data-independent acquisition [28] on a Synapt G2-S mass spectrometer as published recently [5].

Results

Different HCMV strains display only subtle variations in the virion proteome

Virions from fibroblast-passaged isolates R1 and R5, from the TB40-BAC4 strain and, for reference, from BADwt were purified from the cell culture supernatants of 6- to 7-day-infected HFF and were subsequently subjected to proteomic analyses by mass spectrometry. Average parts per million (ppm) values of quadruplicate runs were calculated for each detected viral protein. These average values were correlated with the average ppm values of the major capsid protein (MCP), set as 1. The 40 most abundant viral proteins found in the virions of the reference strain BADwt were pairwise compared to the virions of strains R1 and R5 and to the virions of TB40-BAC4, resulting in data representing molar ratios (Fig. 1). A threshold level for the differences between two values was set at twofold.

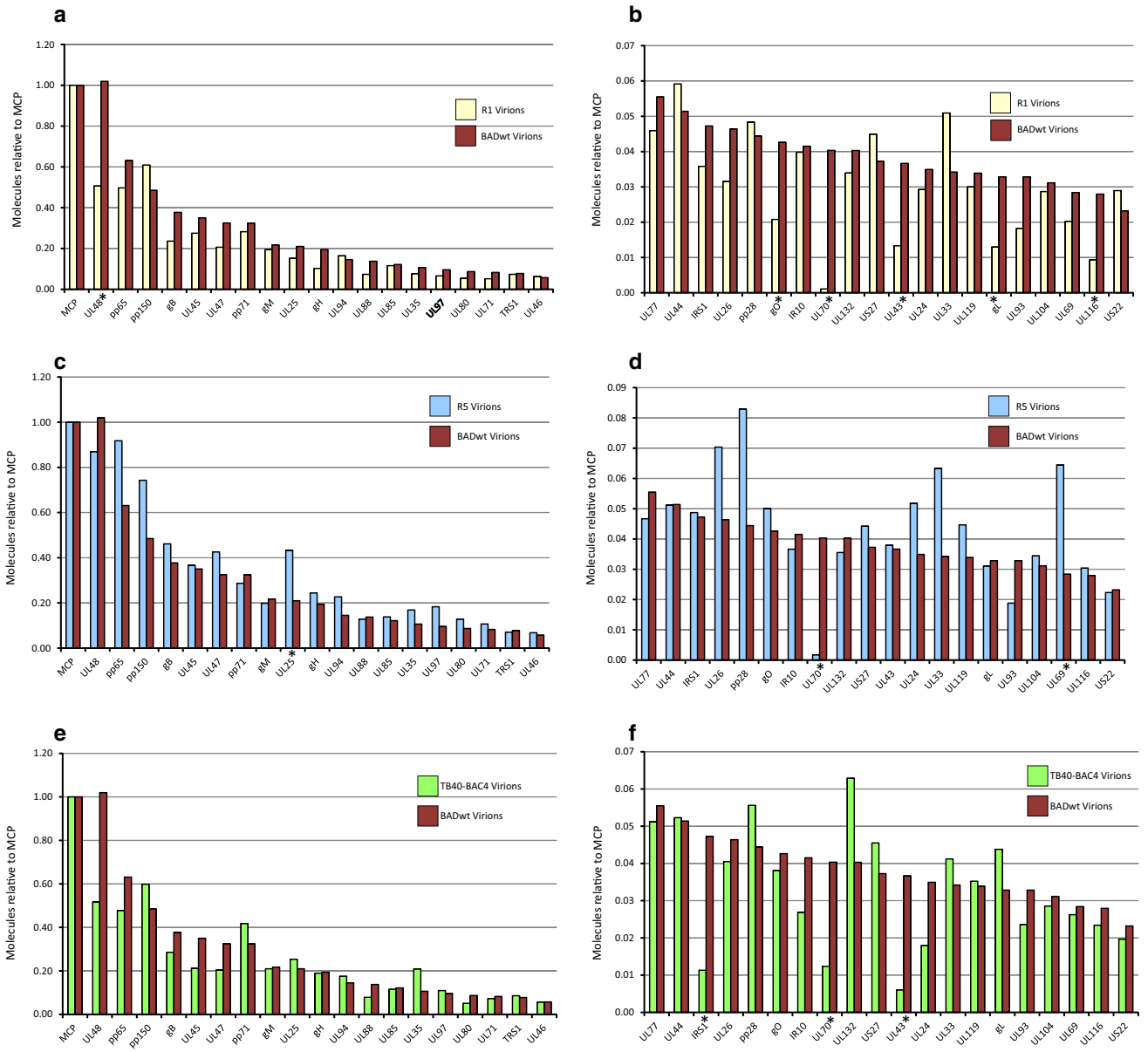

Fig. 1 Mass spectrometry of viral proteins contained in purified virions of strains R1 (**a**, **b**) and R5 (**c**, **d**), and in virions of the TB40-BAC4 (**e**, **f**). Analyses were performed in quadruplicate runs for each sample on a Synapt G2-S mass spectrometer. Results were compared to the virion proteome of BADwt, a derivative of the AD169 laboratory strain of HCMV. Proteins were calculated in molar ratios relative to the major capsid protein (MCP; set as 1). Comparisons were performed against the forty most abundant virion proteins detected in the reference strain BADwt. Proteins showing ratios that differed twofold or more are marked by an *asterisk*. Note the different scales in the charts in (**a**), (**c**) and (**e**) versus those in (**b**), (**d**) and (**f**)

Remarkably, this threshold level was not reached for most of the viral proteins. This indicated that the proteome of the viral proteins was widely conserved in virions from different HCMV strains. Of the proteins that were found in the molar range of 10–100 % with regard to the MCP, only pUL48 showed markedly different levels in R1 and TB40-BAC4 virions, relative to virions of the reference strain BADwt (Fig. 1a, e). This difference was not seen for strain R5 (Fig. 1c). The pUL48 is the largest tegument protein and is tightly associated with the capsid structure [29]. The results indicate that virions of HCMV tolerate different levels of that abundant protein without abrogating infectivity.

The levels of pUL25 were elevated in the virions of R5 (Fig. 1c). This was concordant with an elevated level of pp65, which, however, did not reach the twofold threshold. Recent work has shown that packaging of pUL25 depends on pp65 expression [5, 26]. The increase in pUL25 in virions of R5 was thus likely caused by the variable packaging

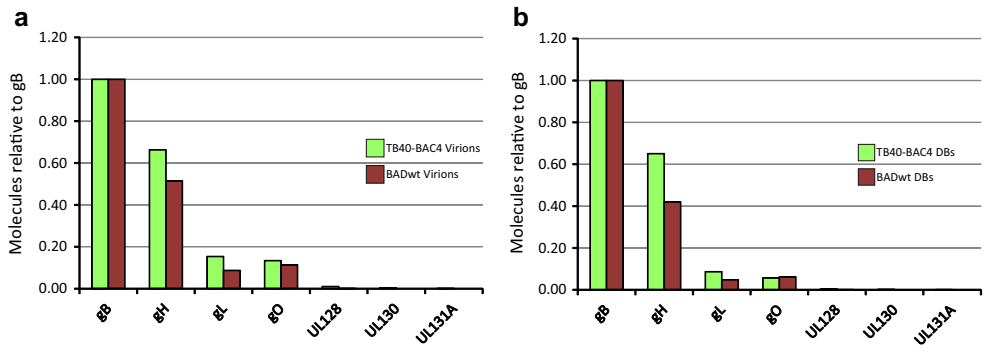

Fig. 2 Mass spectrometry of virions (**a**) and DBs (**b**) from TB40-BAC4 and BADwt with regard to the levels of the envelope proteins gH, gL, gO, gpUL128, gpUL130 and gpUL131A. Analyses were performed in quadruplicate runs for each sample. Proteins were calculated in molar ratios relative to gB (set as 1)

of pp65 into virions of different viruses, a feature of HCMV strains established a long time ago [30].

Some differences between the strains were found in the lower range of the relative molar ratios (Fig. 1b, d, f). Notably, pIRS1, pUL70, pUL43, pUL69 and pUL116 were found in different levels in some of the strains. Only for pUL70, the levels in virions from R1, R5 or TB40-BAC4 were consistently lower, compared with BADwt. The pUL70 is part of a protein complex together with pUL102 and pUL105 that shares homologies with DNA helicase–primase complexes of other herpesviruses [31]. As pUL102 was found in very low amounts and pUL105 remained undetectable in virions of the different strains, the relatively high level of pUL70 in BADwt likely reflects fortuitous packaging rather than representing the result of true differences between the strains. The pIRS1 was found in virions of TB40-BAC4, yet at low levels. The detection of this protein in TB40-BAC4 was unexpected, as its gene should be deleted as a consequence of BAC insertion [21]. However, pIRS1 shares a great deal of sequence identity with its counterpart pTRS1. As the latter protein is expressed in all strains, peptides common to both proteins may have led to the annotation of pIRS1 in virions of TB40-BAC4. Further investigations are required to elucidate this and, in addition, to address the different ratios of pUL43, pUL69 and pUL116 in the virions.

Interestingly, the glycoproteins gH, gL and gO were all found in reduced amounts in R1 virions. The gH/gL complex is considered to regulate the fusion activity of gB [32]. The concordant reduction in gH/gL/gO in only R1 indicates that there are variations in the density of expression of the trimeric complex in the envelope of the HCMV strains investigated here. This is in agreement with the result of others, using immunoblots for detection [33]. Of note, none of the proteins encoded by the open reading frames UL133 to UL151 were found in relevant amounts in virions or DBs of R1, R5 or TB40-BAC4. These genes are deleted in BADwt (AD169). The level of conservation of the virion protein in the strains indicated that the loss of UL133 to UL155 had no impact on the particle composition of AD169 strain, thereby reinforcing the relevance of previously performed proteomic analyses on the latter virus [3, 5].

Low levels of gpUL128-131A in virions and DBs of the endotheliotropic TB40-BAC4

The ability of HCMV strains to infect epithelial or endothelial cells is related to the expression of an envelope protein complex consisting of gH, gL and gpUL128, 130, 131A (pentamer) [14, 17]. Laboratory strains are pentamer deficient [3, 16, 32]. The widely used endotheliotropic strain TB40/E, however, is pentamer positive [20, 21]. The TB40-BAC4, a derivative of TB40/E, cloned in a BAC vector, was used in our analyses. It had retained its tropism to epithelial cells (data not shown), indicating that pentamer expression was preserved. Looking at the data sets, surprisingly small amounts of gpUL128-131A were, however, detectable in both virions and DBs of TB40-BAC4 (Fig. 2; Table 1). Some gpUL128 was also found in the virions of the laboratory strain BADwt, indicating that this protein can be packaged independent of gpUL130/131A. It remains, however, unclear whether gpUL128 was membrane bound in this instance or was included as a component of the tegument.

Opposed to the pentamer, the constituents of the trimeric complex consisting of gH, gL and gO were found in abundance in both virions and DBs of TB40-BAC4 and BADwt (Fig. 2; Table 1). The gH–gL complex is an essential component of the fusion machinery of HCMV. The gO is thought to promote the trafficking of gH and gL, resulting in the insertion of the proteins into virion membranes [34]. The detection of gO in all samples confirms the finding of others that this protein forms a stable trimeric complex together with gH and gL in the virions of different

Table 1 HCMV envelope glycoproteins detected by nano-UPLC mass spectrometry in virions and DBs from TB40-BAC4 and BADwt (classification according to Ref. [46])

ORF	Synonym	Molecular weight	Max score[a]	Reported peptides[b]	TB40-BAC4 virions[c]	BADwt virions[c]	TB40-BAC4 DBs[c]	BADwt DBs[c]
UL55	gB	102,797	35,347	80	1.000	1.000	1.000	1.000
UL75	gH	85,119	8301	36	0.663	0.514	0.650	0.420
UL115	gL	31,384	21,807	15	0.153	0.087	0.086	0.048
UL74	gO	55,043	4839	17	0.134	0.113	0.057	0.061
UL128	gpUL128	20,260	8106	14	0.010	0.002	0.004	0.001
UL130	gpUL130	25,107	789	8	0.003	0.000	0.003	0.000
UL131A	gpUL131A	15,274	1680	2	0.001	0.000	0.001	0.000

[a] Maximum protein lynx global server (PLGS) identification score; tandem MS search algorithm (http://waters.com)

[b] Number peptides detected for each protein

[c] Calculated copy numbers of molecules per gB molecule. Proteins are listed according to decreasing frequencies in virions of BADwt

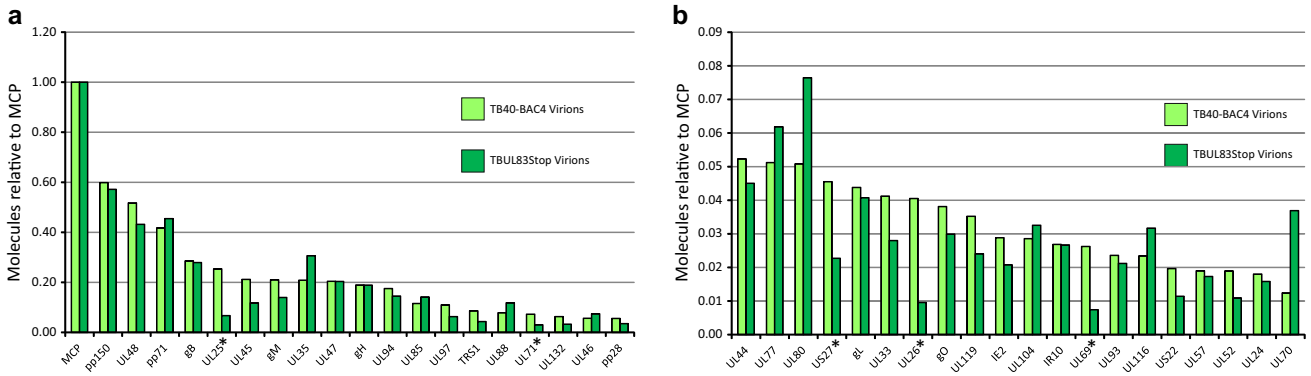

Fig. 3 Mass spectrometry of viral proteins contained in purified virions of the pp65-negative strain TBUL83stop. Analyses were performed in quadruplicate runs for each sample. Results were compared to the virion proteome of the parental strain TB40-BAC4. Proteins were calculated in molar ratios relative to the major capsid protein (MCP; set as 1). Comparisons were performed against the forty most abundant virion proteins found in the reference strain TB40-BAC4. Proteins showing ratios that differed twofold or more are marked by an *asterisk*. Note the different scales in the charts in (**a**) versus those in (**b**)

HCMV strains [33]. In addition, the gO was also found in DBs of TB40-BAC4 and BADwt, indicating that a trimeric complex is also formed in the membrane of these particles (Fig. 2b). The levels of individual glycoproteins were comparable between virions of DBs of individual strains, indicating that their abundance was conserved when normalized to gB. These data show that virions of TB40-BAC4, released from fibroblasts, contained very little of the pentamer compared with the levels of the trimer, as shown before by others [35]. Further to that, the levels of individual glycoproteins showed remarkable conservation between strains and between virions and DBs.

Depletion of pp65 results in reduced packaging of a fraction of outer tegument proteins

Deletion of the gene encoding the major tegument protein pp65 in the genome of the laboratory strain AD169 leads to reduced packaging of a fraction of the outer tegument proteins into virions [5]. To test whether this was strain independent, the virion proteomes of TB40-BAC4 and a pp65-negative variant of that strain (TBUL83stop) were compared. Concordant with the previous study, pUL25, pUL71, pUS27, pUL26 and pUL69 were reduced in virions of TBUL83stop to levels exceeding the threshold twofold (Fig. 3). The pUL45 was also found in lower amounts in the pp65-mutated virus, yet to levels not reaching the threshold. The pUL70 was found in higher amounts in TBUL83stop. This protein had not been reported before to be a constituent of AD169 virions [3, 5]. All other proteins out of the set of the 40 most abundant virion constituents appeared to be present in similar amounts in both TB40-BAC4 and TBUL83stop. Taken together, the data confirm the previously postulated scaffold function of pp65 that supports the packaging of a defined subset of outer tegument proteins [5].

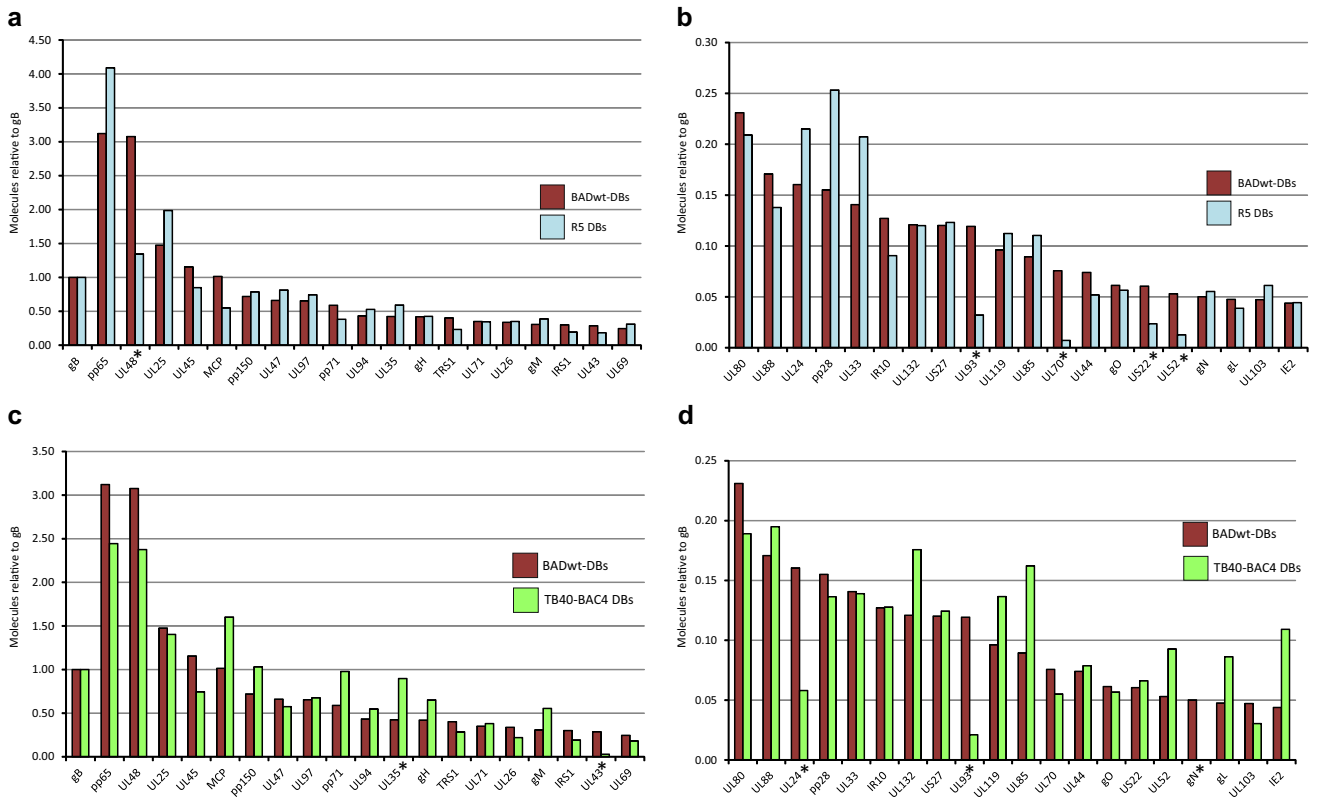

Fig. 4 Mass spectrometry of viral proteins contained in purified DBs of strain R5 (**a**, **b**) and of strain TB40-BAC4 (**c**, **d**). Analyses were performed in quadruplicate runs for each sample. Results were compared to the DB proteome of BADwt. Proteins were calculated in molar ratios relative to the glycoprotein B (gB; set as 1). Comparisons were performed against the forty most abundant virion proteins found in the reference strain BADwt. Proteins showing ratios that differed twofold or more are marked by an *asterisk*. Note the different scales in the charts in (**a**) and (**c**) versus those in (**b**) and (**d**)

Dense body morphogenesis appears to be a conserved process between HCMV strains

DBs are subviral structures that are released in abundance from HCMV laboratory strain-infected fibroblasts. They lack capsids and are less complex than virions in their composition of viral proteins [3]. It was, however, unclear at this point whether DB morphogenesis and protein composition was strain-dependent. To address this, DBs were purified from the culture supernatants of HFF that were infected with TB40-BAC4 or with R5. The particles were analysed by mass spectrometry. The two data sets that were obtained were compared to that of the DBs from BADwt. In this instance, the data were calculated using gB as reference, set as 1. Remarkably little variations were seen for the 20 most abundant proteins (Fig. 4a, c). Differences of more than twofold were only detectable for pUL35 and pUL43 between DBs from TB40-BAC4 and BADwt. The pUL48 was found in greater abundance in DBs from BADwt as opposed to DBs from R5. None of the other viral proteins in this group was found in amounts that differed by more than twofold between TB40-BAC4 and BADwt or R5 and BADwt, respectively.

There were some proteins in the lower molar range that differed in abundance of more than twofold between the strains (Fig. 4b, d). The only difference consistent for both pairs of DBs, however, was the reduced amount of pUL93 in both TB40-BAC4 and R5, as opposed to BADwt. It is unclear whether that was caused by enhanced packaging into BADwt virions or overexpression of the protein in BADwt infected cells. The latter appears more likely though, as pUL93 is associated with the capsid structure which is absent in DBs. Along these lines, all other alterations for the proteins that were found in only low amounts have to be considered with care, as subtle differences in expression levels in the cell may lead to such differences. Functional confirmation for a role of these proteins in DBs morphogenesis and structure is warranted.

Taken together, the data show that the morphogenesis of DBs is a strain-independent, regulated process that leads to the assembly of tegument components comparable to the tegumentation of virions.

Discussion

Mass spectrometry is a powerful technique to elucidate the protein composition of complex viral particles such as those from herpesviruses. Using that strategy, numerous viral and cellular proteins were found to be associated with virions and DBs of HCMV laboratory strains [3–6]. Using an optimized data-independent acquisition approach here [28], we addressed the proteome of viral particles packaged into virions and DBs of different strains including the endotheliotropic strain TB40-BAC4. The high level of conservation that was observed in the proteomes of these particles argues in favour of an orchestrated process of morphogenesis for both virions and DBs. It appears that a network of strain-independent protein–protein interactions in the cell drives the tegument assembly process, leading to a widely predefined composition of the particles. However, there are some issues that may warrant a more differentiated appreciation of the data.

One is that this study and all other published studies on the proteomes of HCMV particles focused on material obtained from human fibroblast cultures. The cellular environment is likely to impact on HCMV particle composition. Thus, virus released e.g. from epithelial or endothelial cells may be different to virus released from fibroblasts [35]. It will be necessary to analyse HCMV particles from other cell types to evaluate the role of the cell in the process of morphogenesis. Further to that, the discrimination level of mass spectrometry is dependent on a number of variables. This includes the length of the protein and, related to that, the number of peptides that can be detected. The components UL128, UL130 and UL131A of the pentameric complex of surface glycoproteins, for instance, should be present in equimolar amounts. Still, UL130 and UL131A were detectable in lower ratios, relative to UL128. Looking at those data reveals that UL128 provided 14 peptides for analysis, whereas UL131A provided only two (Table 1). The components of the trimeric complex gH, gL and gO should also be present in equal molar ratios. Still, gH was consistently detectable in higher amounts compared with gL and gO in this and other studies (Fig. 2, [3, 5]). The reason for this discrepancy is unclear, but the level of glycosylation or other factors influencing mass spectrometry have been discussed for explanation [3]. Again, only half as many peptides were detectable for gL and gO, compared with gH, indicating that indeed technical limitations rather than biological effects were causing the differences. Still, the latter cannot be totally excluded given that, at least for gH versus gL, concordant differences were obtained by different laboratories, using different proteomic methodologies [3, 5 and this work].

Some of the low-abundant proteins were unequally packaged into virions. According to the arguments discussed above, it remains unclear whether that was reflecting the limitation of the technology or was representing true strain-dependent differences. For these proteins, corroboration of the findings by functional assays will be necessary.

Recent reports have shown that most of the HCMV strains tested package vastly more of the trimeric gH/gL/gO complex into virions, compared with the pentameric gH/gL/gpUL128-131A complex [33, 36]. Our proteomic analyses confirm these findings. Although, as alluded to above, ratios between individual proteins in one sample cannot be considered as being an absolute measure, large differences can certainly be appreciated as being relevant. The molarity of gH and gL in virions of TB40-BAC4 was 66-fold and 15-fold increased, respectively, relative to that of gpUL128 (Table 1). Given the fact that, for example, roughly the same number of peptides was detectable for both gL and gpUL128, it appears that molar ratios in this case truly reflect a relevant dominance of the trimeric complex over the pentameric complex in TB40-BAC4 virions and DBs.

The low levels of the pentameric complex in TB40-BAC4 virions were surprising, as this strain readily infects various cell types like those of epithelial or endothelial origin. The infection of these cells requires the pentamer. The data presented here thus suggest that low levels of that complex suffice to mediate the extended cell tropism of that strain. It has to be considered, however, that there is evidence for the release of a heterogenous population of HCMV particles from fibroblasts [35]. We thus cannot exclude that a fraction of the virions in the material used for proteomic analysis contained larger amounts of the pentameric complex and was thus capable of infecting epithelial cells. Still, the very limited total amount of gpUL128-131A suggests that also a limited number of molecules of the pentameric complex on one particle may suffice to mediate attachment and entry into epithelial cells. Thus, the detection of even small amounts of any given protein in HCMV virions can be of a relevance that has to be tested by functional assays.

The capacity of HCMV strains to infect a broad array of cell types is mediated by the pentameric complex and is considered to be a determinant of pathogenicity. Consequently, the neutralizing antibody response towards this complex has attracted considerable attention [37]. Several studies have demonstrated the extraordinary potential of pentamer-specific antibodies for virus neutralization and even for pathogenesis [38–45]. The relative low level of the pentamer in TB40-BAC4, which is considered to be a clinically relevant strain, may provide one explanation for the efficiency of these antibodies. Since only few complexes on the surface of cell-free TB40-BAC4 virions may be necessary and sufficient to mediate cell entry, low concentrations of neutralizing antibodies targeting the pentamer could already suffice

to mediate a marked neutralizing effect. The availability of strains that express different levels of the pentameric complex [36] enables studies to address this hypothesis.

The proteome of DBs from the different strains was remarkably conserved. This argues in favour of a regulated process, comparable to virion morphogenesis that leads to the formation of these particles. Opposed to virion formation, however, there is no capsid in DBs that serves as a primary structure for the attachment of different layers of tegument proteins. The high level of conservation thus supports the idea of a self-assembly of the tegument structure of DBs that is orchestrated by a network of protein–protein interactions. The formation of the DB core is then followed by envelopment. One striking finding of this work is that the levels of membrane glycoproteins, including gpUL128-131, were comparable between virions and DBs of the same strain. This indicates that the same membranes, containing viral glycoproteins, serve as substrate for both virion and DB envelopment. Although postulated before, the results of this work now substantiate this assumption.

In conclusion, the quantitative proteomic analysis performed here demonstrates a high level of strain-independent conservation in the viral protein composition of virions and DBs from different HCMV strains. This provides the basis for further analyses to understand the molecular mechanisms of tegumentation and envelopment of HCMV particles.

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