Human cytomegalovirus phosphoproteins are hypophosphorylated and intrinsically disordered

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Abstract

Protein phosphorylation has important regulatory functions in cell homeostasis and is tightly regulated by kinases and phosphatases. The tegument of human cytomegalovirus (CMV) contains not only several proteins reported to be extensively phosphorylated but also cellular protein phosphatases (PP1 and PP2A). To investigate this apparent inconsistency, we evaluated the phosphorylation status of the tegument proteins pUL32 and pp65 by enzymatic dephosphorylation and MS. Enzymatic dephosphorylation with bacterial λ phosphatase, but not with PP1, shifted the pUL32-specific signal on reducing SDS-PAGE from ~150 to ~148 kDa, a mass still much larger than the ~118 kDa obtained from our diffusion studies and from the calculated protein mass of ~113 kDa. Remarkably, inhibition of phosphatases through treatment with the phosphatase inhibitors calyculin A and okadaic acid resulted in a shift to ~190 or ~180 kDa, respectively, indicating that a considerable number of potential phosphorylated residues on pUL32 are not phosphorylated under normal conditions. MS revealed a general state of hypophosphorylation of CMV phosphoproteins with only 17 phosphorylated residues detected on pUL32 and 19 on pp65, respectively. Moreover, bioinformatics analysis shows that the C-terminal two-thirds of pUL32 are intrinsically disordered and that most phosphorylations map to this region. In conclusion, we show that important CMV tegument proteins are indeed phosphorylated, though to a lesser extent than previously reported, and the difference in mobility on SDS-PAGE and calculated mass of pUL32 may not be attributed to phosphorylation but more likely due to the partially intrinsically disordered nature of pUL32.

INTRODUCTION

Phosphorylation of cellular proteins plays an important role in the control of cell homeostasis, including cellular signalling and regulating key cellular processes such as metabolism [1–4]. Accordingly, the phosphorylation status of cellular proteins is tightly controlled by a delicate balance of phosphatases and kinases. In viral infections, the tight control of host cell and virus protein phosphorylation is crucial to support microbial replication and survival [5–11]. Cytomegalovirus (CMV), a β-herpesvirus, hijacks two cellular phosphatases, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), from its host cell and incorporates them into the tegument of mature viral particles [11]. After viral entry, these two phosphatases are released into the infected cell and induce a global state of cellular hypophosphorylation within minutes [11]. This cellular hypophosphorylation is further sustained by viral upregulation of PP1 and PP2A expression, which is thought to counteract host cell antiviral responses [7, 11].

In contrast, phosphorylation of critical cellular and viral proteins is crucial for viral replication. The CMV-encoded kinase pUL97 phosphorylates and inactivates the retinoblastoma tumour suppressor to stimulate cell cycle progression in mammalian cells [8]. The viral tegument protein pUL26 is associated with phosphorylation of the tegument phosphoprotein pp28, which is important for stabilization of the tegument in virions and timely expression of immediate-early (IE) genes [12]. The tegument protein pp65 is involved in phosphorylation of the tegument protein pp28 and the IE protein IE72.
RESULTS

Dephosphorylation alters the relative mobility of CMV phosphoprotein UL32 on SDS-PAGE

Dephosphorylation changes the mobility of extensively posttranslational modified proteins such as pUL97 and pUL69 in reducing SDS-PAGE [21, 24]. Accordingly, removal of phosphate residues from the CMV pUL32 should change the relative mobility of pUL32 by ~37 kDa because of the observed relative mobility similar to a 150 kDa protein as compared to its calculated mass of 113 kDa [23, 25]. To test this assumption, a lysate of pUL32-overexpressing HEK293 cells was treated either with increasing concentrations of recombinant mammalian protein phosphatase 1 (PP1) or bacterial lambda phosphatase (λ). Untreated, recombinant pUL32 exhibited a relative mobility as anticipated from previous studies yielding a signal at 150 kDa on immunoblot (Fig. 1a, b). Enzymatic treatment of lysate with PP1 did not appreciably change the relative mobility of pUL32 even at the highest concentration of enzyme (Fig. 1a). Treatment with λ phosphatase yielded a small, although visible, change of ~2 kDa in relative mobility (Fig. 1b).

To confirm the efficacy of the different enzymes used, we also probed the same cell lysates with a mAb specific for a phosphosubstrate on the cellular ribosomal protein S6 (rbS6). RbS6 is intrinsically expressed in all mammalian cells and phosphorylated during translation [26]. We found a clear reduction or elimination in immunoblot signal upon treatment of cell lysates with PP1 or λ, indicative of an efficient dephosphorylation (Fig. 1a, b). Moreover, significant changes in relative mobility of the retinoblastoma tumour suppressor protein (Rb) upon enzymatic treatment with PP1 and λ, respectively, further confirmed the efficacy of the enzymatic dephosphorylation (Fig. 1c).

To validate these observations, CMV-infected human foreskin fibroblast (HFF) cells were treated likewise. Similar to the experiments with recombinant protein, untreated pUL32 yielded a signal at 150 kDa on immunoblot, as expected (Fig. 1d). Treatment of virus-infected cell lysates with PP1 did not result in a change in the relative mobility of pUL32, whereas treatment with λ phosphatase led to only a minimal change in its relative mobility (Fig. 1d).

To exclude that the pUL32-specific mAb was binding only the phosphorylated protein and fails to recognize fully dephosphorylated ones, we also probed λ-treated and untreated recombinant protein with polyclonal immune sera and immunoglobulin preparations (IVIG). Immune sera react with multiple epitopes distributed over the pUL32 protein, and overall binding may be therefore less prone to false-negative results [27]. Probing with polyclonal immune sera yielded the same change in relative mobility as observed for the mAb with a change of ~2 kDa (Fig. 1e). In addition, the CMV tegument protein pp65 has a calculated mass of 62.9 kDa, but its relative mobility suggests a 65 kDa protein. We therefore also overexpressed pp65 in HEK293 cells and treated this protein identically to pUL32 with the λ phosphatase. Similar to the observation with pUL32, untreated recombinant pp65 exhibited a relative mobility as anticipated from previous studies yielding a signal at 65 kDa on immunoblot. Dephosphorylation of recombinant pp65 did not yield a detectable difference in relative mobility on immunoblot despite a calculated difference of ~2 kDa (Fig. 1f).

Hyperphosphorylation of recombinant pUL32 decreases relative mobility and reduces immunoblot signals

The minor change in relative mobility of dephosphorylated pUL32 despite 217 potential phosphorylated residues (Table 1) raised the question whether these residues may be
Fig. 1. Enzymatic digestion of CMV tegument proteins pUL32 and pp65 modifies the relative mobility of pUL32 but not of pp65. Stable pUL32-transfected HEK293 cell lysate was treated with (a) 3.1, 1.6, 0.8 and 0.4 U of protein phosphatase 1 (PP1) or (b) with 500, 250, 125 and 62.5 U of lambda phosphatase (λ). (c) Validation of the relative mobility of the control phosphoprotein, retinoblastoma (Rb) protein. Mock-infected G1/S phase-synchronized HFF cell lysate was treated with 500 U λ, 3.1 U PP1 or with phosphatase inhibitor (PPI) only. Anti-retinoblastoma (Rb) protein mAb were used to identify Rb. (d) AD169-infected HFF cell lysate was treated with 500 or 125 U of λ or 3.125 or 0.8 U PP1. (e) Stable UL32-transfected and (f) transient pp65-transfected cells were lysed and treated with 500 U of λ. Anti-pUL32 mAb was used to detect pUL32 (a–c), anti-ribosomal protein S6 (rbS6) mAb was used to verify successful phosphatase digest by decreasing signal strength (a, b) and anti-β-tubulin served as loading control (a, b, d). Two human immune sera and two intravenous immunoglobulin (IVIG) preparations were used as primary antibodies for evaluation (e, f). Cell and viral lysates were generated by two freeze–thaw cycles followed by sonication of samples; prior to lysis, a protease and phosphatase inhibitor cocktail was added to all samples not subjected to dephosphorylation, whereas protease inhibitor was added to samples subjected to dephosphorylation. Final protein concentrations in the digest were 1 µg µl⁻¹ (a, b, d, e) or 0.2 µg µl⁻¹ (c).
Table 1. Human cytomegalovirus phosphoproteins identified by LC-MS/MS

Data were processed using MaxQuant. peptide spectrum match (PSMs) and proteins were filtered at 1% false discovery rate. Phosphopeptides were additionally filtered at a score threshold of >40 and a delta score of >7. Class I sites are defined by a localization probability >0.75. The lower sequence coverage of proteins identified from whole-cell lysates results also in a lower number of identified phosphosites for these proteins. Thus, the listed numbers of observed and expected phosphosites for these proteins should not be considered absolute but rather provide a general overview of the data. Detailed information available in Table S1 (available in the online Supplementary Material).

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phosphorylated in vitro. To address this question, pUL32-expressing HEK293 cells were treated with the PP1/PP2A inhibitors calyculin A (CA), okadaic acid (OKA) or the PP2B inhibitor tacrolimus (FK506) and compared to vehicle control (1% ethanol) or control (no vehicle). Treatment of cells with CA and OKA yielded a pUL32-specific signal at ~190 and ~180 kDa on the immunoblot, respectively (Fig. 2a). In contrast, treatment with FK506 did not yield a visible change in the relative mobility of the pUL32-specific signal (Fig. 2a). As a control, cell lysates were also probed with a phospho-Akt-specific mAb because Akt is a target of PP1 and inhibition of PP1 should increase the signal of phosphorylated Akt protein [28]. Treatment of cells with the PP1 inhibitors CA and OKA but not with the PP2B inhibitor FK506 yielded a more intense phospho-Akt-specific signal than untreated cells (Fig. 2b). Cell viability assays confirmed that the different phosphatase inhibitors did not reduce cell viability significantly during these 4 h treatments (Fig. 2c).

To confirm that the weaker signal intensities measured for hyperphosphorylated pUL32 on immunoblots were not due to lower relative quantities of pUL32 in OKA-treated cells, OKA-treated cell lysate was dephosphorylated and analysed by immunoblotting after normalizing to equal total protein concentration (Fig. 3a). The signal intensity and relative mobility of pUL32 on immunoblot was similar between dephosphorylated OKA-treated and dephosphorylated

![Image of immunoblots and cell viability assay](https://via.placeholder.com/150)

**Fig. 2.** Inhibition of protein phosphatase 1 (PP1) and 2 (PP2A) changes the relative mobility of pUL32 significantly. (a-c) Stable pUL32-expressing cells were treated for 4 h with the phosphatase inhibitors CA, OKA and FK506 with final concentrations of 1, 10 and 1 µM, respectively, or 1% ethanol (vehicle control) or remained untreated (control). Equal protein amounts (20 µg) of each cell lysate were loaded onto SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked and incubated with (a) anti-pUL32 mAb to analyse differentially modified forms of pUL32 after phosphatase inhibitor treatment and (b) anti-phospho-Akt mAb to analyse inhibition of PP1 and PP2A. (c) Evaluation of the effect of phosphatase inhibitors on cell viability. A total of 35 000 stable pUL32-expressing cells per well were plated into 96-well plates, incubated overnight and subsequently treated for 4 h with increasing concentrations of the phosphatase inhibitors OKA, CA or FK506. Error bars indicate SEM and represent triplicates.
untreated cell lysate, indicative of equal relative amounts of pUL32 in the different cell lysates.

To examine the effect of hyperphosphorylation of pUL32 on antibody recognition, we probed in parallel native and hyperphosphorylated pUL32 on immunoblot with immune sera of healthy CMV-seropositive blood donors (n=28, Fig. 3b). The signal intensity was clearly reduced when hyperphosphorylated pUL32 was probed with this immune sera as compared to native pUL32 (Fig. 3b).

Identification of phosphorylation sites on CMV proteins

In order to determine phosphorylation sites of pUL32 and pp65, we subjected lysates of purified viral particles (CMV strain AD169) to MS. In addition, pUL32 and pp65 were analysed in more depth by gel-based purification from purified viral particles. We reached sequence coverage for pUL32 of 92.56 % (970 of total 1048 amino acids (aa)) and for pp65 of 99.29 % (557 of total 561 aa), respectively (Tables 1, 2 and S1, Fig. S1). The phosphorylated residues identified on pUL32 with a high confidence for correct site localization (i.e. class I sites defined by a localization probability ≥0.75, see also [29]) included 16 serine residues (aa426, 497, 504, 533, 535, 708, 744, 765, 857, 865, 869, 888, 923, 966, 991, 1008) and one threonine residue (aa556). Evaluation of pp65 revealed 12 serine residues (aa207, 248, 364, 404, 406, 430, 439, 441, 442, 448, 472, 554) and seven threonine residues (aa32, 368, 421, 452, 468, 502, 555), respectively (Tables 1, 2 and S1, Fig. S1). Additionally, seven phosphosites for pUL32 and four phosphosites for pp65 were identified with a localization probability <0.75 (=class II or III, see Table S1). For these sites, the localization of the phosphoresidue was not possible with high confidence; however, the probability that the respective peptides are indeed phosphopeptides is still very high (i.e. at 1 % false discovery rate). Moreover, in Table S1, we have added the ratio of modified (phosphorylated) to non-modified (unphosphorylated) peptide for each site (as calculated by MaxQuant) as a rough approximation of site occupancies. Although this ratio can be influenced to some extent by the specific physicochemical properties of a given peptide pair [30] and thus has to be interpreted carefully, it is evident that only five sites for pUL32 and three for pp65 display a ratio of 0.05 or higher, whereas the majority of sites is below 0.01. This suggests that most of the identified phosphosites are likely to occur at substoichiometric levels on pUL32 and pp65. The NetPhos 2.0 program predicted 98 phosphorylated residues for pUL32 and 41 for pp65, which exceeds the measured sites by approximately six- and twofold, respectively (Tables 1 and 2, Fig. S1). Moreover, several identified class I sites were not included in the NetPhos prediction. Four phosphorylated residues on pUL32 (S923, S966, S1008; T556) and four phosphorylated residues on pp65 (S554; T368, T452, T502) were not predicted (Tables 1 and 2, Fig. S1).
MS analyses identified 17 class I sites on pUL32 and 19 class I sites on pp65.

### Table 2. Class I sites on pUL32 and pp65

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MS analysis of whole virions for other phosphorylated proteins revealed further phosphosites on 21 further CMV proteins. Phosphoproteins were found on envelope proteins UL55/gB, UL100/gM, UL132, transmembrane proteins IRL10, US18, UL41A, tegument proteins UL24, UL25/pp85, UL35, UL47, UL48, UL69, UL97, UL99/pp28, as well as on US27, UL45, UL80, UL98, UL112/113 and the ORF shared by UL122/IE2/IE86 and UL123/IE1/IE72 (Tables 1 and S1). However, due to the different sample preparation (pUL32 and pp65 were additionally gel purified), the coverage of these proteins was lower than for pUL32 and pp65 and ranged between 41.1 % (IE1) and 89.5 % (UL99) (Tables 1 and S1). In addition to phosphorylation, we also searched the data for other post-translational modifications including acetylation, methylation, ubiquitination and dimethylation. However, we did not find evidence for lysine acetylation, ubiquitination or dimethylation of CMV proteins. Methylation was almost exclusively found on CMV proteins that were gel purified and very likely to phosphorylated residues, we analysed these sequences by using a WebLogo tool (http://weblogo.berkeley.edu/) (Fig. S2). However, we found no specific region that was conserved.

### pUL32 and pp65 are predicted to be partially intrinsically disordered

The limited number of phosphorylated residues on pUL32 does not explain the difference between observed relative mobility of a 150 kDa protein on SDS-PAGE and the calculated mass of 113 kDa based on predicted primary amino acid sequence. Intrinsically disordered proteins (IDPs), however, may lead to overestimation of molecular masses on SDS-PAGE because of erratic mobility characteristics [32, 33]. Bioinformatics analysis of the protein sequence of pUL32 (CMV strain AD169) employing the protein disorder prediction servers IUPred (http://iupred.ensembl.hu/; prediction type ‘long disorder’) and PrDOS (http://prdos.hgc.jp/cgi-bin/top.cgi) [34] showed that the C-terminal two-thirds of pUL32 have a disorder tendency of >0.5 and thus are highly likely to be intrinsically disordered (Fig. 4a, b). Remarkably, all 17 (IUPred) or 15 of 17 (PrDOS) class I phosphorylation sites are located within this potential disordered part of pUL32 (Fig. 4a, b). In the case of pp65, 14 of 19 (IUPred) or 13 of 19 (PrDOS) class I sites mapped to the disordered region located at the C-terminal part of the protein (Fig. 4c,d).

**Diffusion and rotational properties of pUL32 in solution correspond to a 118 kDa protein corroborating the predicted partially intrinsic disordered nature of pUL32**

To determine the molecular mass of pUL32, we subjected lysates of pUL32-EGFP-HCMV particles to fluorescence correlation spectroscopy (FCS; Figs 5 and S3). Our protocol allowed us to detect translational diffusions of nanomolarities of EGFP and pUL32 tagged with EGFP, giving diffusion times of 132.5±3 µs and 245.7±7 µs, corresponding to estimated molecular masses of 22±2 kDa and 140±10 kDa, respectively, assuming spherically shaped molecules (Tables 3 and S2). A molecular mass of ~118±10 kDa for pUL32 was obtained from the translational diffusion coefficients of EGFP and EGFP-tagged pUL32, using the Stokes–Einstein equation (see equations 3 and 4 in Methods) and assuming spherical shape. The experimentally determined molecular mass is in very good agreement with the theoretically calculated one of 113 kDa, but significantly lower than the observed 150 kDa on immunoblotted SDS-PAGE gels.

In addition, fluorescence anisotropy was performed to monitor rotational motions and calculate the molecular weight or apparent volume of the protein. Anisotropy values of 0.345±0.006 for pUL32-EGFP, 0.301±0.007 for EGFP-bound IgG and 0.254±0.008 for single EGFP were obtained, corresponding to molecular masses of ~21 kDa (hydration degree of 0.1 ml g⁻¹ protein) for EGFP alone, ~178 kDa (assuming hydration degree of 0.1 ml g⁻¹ protein) for EGFP-bound IgG and ~141 kDa (hydration degree of 0.3 ml g⁻¹ protein) for pUL32-EGFP using the Perrin equation [35]. The significantly higher anisotropic values for EGFP-tagged pUL32 compared to those for a structured EGFP-IgG complex (with theoretical molecular weight 173 kDa) are caused by a larger solvation shell for pUL32-EGFP, which is due to its high content of solvent-exposed polar residues, which are the landmark of IDP regions [33]. Taken together, our calculated molecular...
mass of pUL32 alone (~118 kDa) is in good agreement with the translational diffusion analysis.

DISCUSSION

The assumption of an extensive phosphorylation of CMV tegument proteins was based on phosphate-specific labelling of proteins and a significant discrepancy between calculated UL32 molecular weight of ~113 kDa and its relative mobility on reducing SDS-PAGE of 150 kDa [16, 23]. However, the molecular mass of a phosphate moiety is only 80 Da [36], and even with all 217 potential phosphorylated residues modified on pUL32, the additional mass would only make up for 17.4 kDa. By using enzymatic dephosphorylation, we found that the change in relative mobility was even smaller and yielded a shift of the signal on reducing SDS-PAGE of ~2 kDa (Fig. 1b, d and e) in contrast to pp65 (Fig. 1f) despite the identification of 19 phosphorylated residues equivalent to 1.5 kDa (Tables 1, 2 and S1, Fig. S1) [36]. Accordingly, unexpected mobility patterns of CMV proteins on SDS-PAGE may not always be attributed to extensive posttranslational modifications.

Phosphorylations have been previously identified on several CMV proteins using qualitative methods with the exception of one study that used MS to identify phosphosites on pp65 [13, 16–18]. The signal strength of different proteins was variable between studies and very likely attributable to differences in methodologies [13, 16, 18]. Using MS, we could confirm that the previously described CMV phosphoproteins are indeed phosphorylated (Tables 1, 2 and S1, Fig. S1). Nevertheless, we also found that phosphorylation was clearly less extensive on pUL32 or pp65 than expected from previous studies [13, 16]. Although shotgun MS can hardly yield complete information on the stoichiometry of posttranslational modifications of a given protein, the high coverage reached for pUL32 and pp65 allows for a thorough assessment of the phosphorylation status of these proteins and, indeed, we could confirm previously found phosphorylation sites on pp65 [17]. Accordingly, multiple CMV proteins that were considered hyperphosphorylated are actually hypophosphorylated.

The significant difference of 37 kDa between the calculated protein mass of ~113 kDa and the relative mobility of pUL32 on reducing SDS-PAGE that migrates with an apparent mass of 150 kDa was subject of discussion and still requires explanation [37]. In silico analysis of the protein-coding sequence of pUL32 strongly indicates that the C-terminal two-thirds of the protein are intrinsically disordered (Fig. 4a, b). In contrast to folded proteins, IDPs do not possess a unique three-dimensional structure and are best described with an ensemble of rapidly interconverting conformations [38]. Their disordered nature is due to a large content of hydrophilic amino acid residues compared with hydrophobic ones, preventing the hydrophobic collapse that leads to a folded globular protein [39]. IDPs have outstanding biochemical and biophysical properties – they lack a three-dimensional structure and cover a large spectrum of degrees of disordered states ranging from random coils and globules to large multi-domain proteins with domains connected by flexible linkers [33, 40]. Because of their unusual amino acid composition, IDPs bind less SDS than usual proteins and their apparent molecular weight is often 1.2–1.8

Fig. 4. Majority of class I sites of pUL32 and pp65 are located on predicted disordered amino acids. Disorder tendency of full-length pUL32 calculated with IUPred (a) and PrDOS (b) and disorder tendency of full-length pp65 calculated with IUPred (c) and PrDOS (d). Amino acid residues with a disorder tendency higher than 0.5 (dashed horizontal line) have a high probability to be intrinsically disordered or unstructured. Identified class I sites are indicated by solid vertical lines (serine) or dashed vertical lines (threonine).
times higher than the real one calculated from amino acid sequence data or measured by MS, resulting in unusual relative mobility patterns on SDS-PAGE [32, 33]. Hence, the relative mobility of pUL32 on reducing SDS-PAGE that mimics a mass of 150 kDa is consistent with its intrinsically disordered character (Fig. 4).

The role of disordered viral proteins in viral replication is relatively unexplored. The conformational adaptability of the adenoviral E1A, for example, facilitates the simultaneous binding of the p300-CBP coactivator family and Rb. As a result, the CBP histone acetyl transferase may acetylate Rb, which again enhances binding of MDM2, a cellular ubiquitin ligase, causing Rb degradation and thus uncontrolled onset of S-phase genes [41–45]. UL32 has been shown to bind tightly to the capsid, interact with the microtubule system and play an important role in maturation of viral particles [23, 46, 47]. Hypothetically, the IDP character of pUL32 is also important for binding multiple proteins and connecting capsid with proteins of the assembly compartment is similar to that described for the adenoviral E1A.

Phosphorylation of pUL32 is highly conserved among different clinical CMV strains as class I sites are located only outside regions of CMV interstrain variability [48]. Moreover, the majority of phosphorylations identified on pUL32 mapped to the predicted disordered C-terminal two-thirds (Fig. 4). Similarly, the majority of phosphorylation sites on pp65 was located in the predicted intrinsically disordered region, previously described as the linker region [17]. These observations are in concordance with previous studies that described phosphorylation of disordered domains as being common and more frequent than in ordered protein regions [49].

We observed that the digest of pUL32 derived from mature virions with PP1 did not result in a detectable difference in relative mobility (Fig. 1). In contrast, Bogdanow et al. suggested recently that cellular phosphatases such as PP1, which is incorporated in the mature virion, dephosphorylate pUL32 [50]. We also found that three of the phosphoacceptor sites described by Bogdanow et al. (S504, S991 and S1008) are actually phosphorylated in the mature virion (Fig. S1, Table S1). We may only hypothesize on the potential reasons for this discrepancy. Potentially, the use of mature virions in our study versus the use of a prokaryotic expression system that included cellular but not viral gene products (particularly the viral kinase UL97, which acts similarly to cellular cyclin-associated kinases [8]) by Bogdanow et al. may explain the discrepant findings.

In order to assess the intrinsically disordered nature of pUL32, we determined its translational diffusion coefficient, which is directly related to proteins’ hydrodynamic radius, which in turn depends on the size, shape and compactness of the molecule. Following the Stoke–Einstein relation, translational diffusion is mainly dependent on the particle size, viscosity and temperature and independent of any net charge of the molecule or matrix effects, which appear in gels or chromatography columns, and translational diffusion time is proportional to the molecular hydrodynamic radius (Fig. 5, Table 3). The molecular mass of pUL32-EGFP calculated from the observed hydrodynamic radius is larger than predicted because its hydrodynamic radius is larger compared to a globular protein of equivalent mass due to its intrinsically disordered nature.

To further corroborate its intrinsically disordered nature, we used a complementary approach, i.e. anisotropic analysis, on rotational motion of pUL32-EGFP, which provides information about the effective size of the molecule in aqueous solution (Table 2). Anisotropy is related to the molecular size and its hydrodynamic shell and showed a high hydration (0.3 ml g⁻¹ of water per pUL32-EGFP) compared to 0.1 ml g⁻¹ for a globular EGFP or complex of structured proteins EGFP-bound to IgG (Table 3). This high hydration of pUL32-EGFP can be attributed to the high content of solvent accessible polar residues of pUL32 and hence its partial intrinsically disordered nature.

Additionally, large distribution of translational diffusion times of pUL32-EGFP was observed by focusing on single molecules diffusing through the confocal volume (Table S2). This heterogeneity of the diffusion behaviour may result from different conformations of a single molecule in the confocal volume, suggesting the presence of a conformational ensemble, which is in line with its partially intrinsically disordered nature [51]. This heterogeneity can be reduced to two populations of pUL32-EGFP with different diffusion times and hydrodynamic radii of the molecule (Table S2). The conformation of these two populations can be interpreted with a compaction factor [52], taking hydrodynamic radii for the native (C value of 1) and fully denatured states (C value of 0), respectively, into account. A compaction factor between 0.972±0.004 and 0.984±0.004 was obtained from hydrodynamic radii, assuming partially disordered parts of the protein, suggesting two populations with significantly different compaction states.

Posttranslational modification of immune targets on infectious pathogens may mask epitopes from immune recognition [6]. Potentially, phosphate modification of pUL32 could impair antibody recognition and mask epitopes. We found that native and dephosphorylated pUL32 yielded equally strong signals when probing with immune sera, suggesting that the 17 identified phosphate modifications do not mask detectably pUL32 epitopes (Fig. 1e). Nevertheless, hyperphosphorylation of pUL32 clearly decreased signal intensity on the immunoblot indicating that extensive phosphorylation of pUL32 interferes with antibody binding (Fig. 3a). Hence, extensive phosphorylation of the CMV antigen pUL32 could mask epitopes from immune recognition, but the pUL32 protein is actually hypophosphorylated in vivo.

In the present study, we found clear differences in the efficacy of phosphatases. Despite use of a more than sixfold higher concentration of PP1 than of λ, PP1 did not remove
phosphate residues as efficiently as λ (Fig. 1). One difference between the phosphatases is their molecular mass – PP1 has a molecular weight of 37.5 kDa, whereas λ has a molecular weight of 25 kDa. Perhaps, due to its smaller size, λ phosphatase could have access to phosphoresidues not accessible by PP1 and thus eliminate additional phosphates.

Fig. 5. FCS analysis of EGFP and pUL32-EGFP in lysis buffer. A drop of EGFP in solution and of viral pUL32 tagged with EGFP in lysis buffer were positioned on a glass surface and excited with a 488 nm Ar laser line. (a) Fluorescence intensity fluctuations of EGFP and pUL32 tagged with EGFP through a confocal volume of 0.65 fl were recorded for 30 s. (b) The normalized autocorrelated dataset was fitted with a one-component model with high precision (c) and the diffusion time was obtained from the point of inflection of the fitted curve. The respective molecular mass was calculated from the hydrodynamic radius obtained from the characteristic diffusion time and coefficient using the Stoke–Einstein equation.
Table 3. Biophysical parameters of EGFP, EGFP-bound IgG and pUL32-EGFP protein obtained by FCS and fluorescence anisotropy (T=20°C)

The standard deviation mentioned at each parameter is obtained from 20 experiments (ten experiments for two different preparations). For FCS, the diameter of the pinhole was set to 35 µm. \(D_{\text{trans}}\) and \(R_h\) are calculated from equations 3 to 5 using \(\tau_0\) obtained by FCS. Steady-state fluorescence anisotropy is directly calculated from the measured fluorescence polarization values using equations 5 and 6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(r)</th>
<th>Hydration (ml g(^{-1}))</th>
<th>(\tau_0) (ns)</th>
<th>(D_{\text{trans}}) (µm(^2) s(^{-1}))</th>
<th>(R_h) (Å)</th>
<th>MW/MW(_{\text{theor}}) (kg mol(^{-1})/kg mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP</td>
<td>0.25±0.008</td>
<td>0.14±0.04</td>
<td>132.5±3</td>
<td>104.3±2.3</td>
<td>20.3±0.4</td>
<td>22±2/21</td>
</tr>
<tr>
<td>pUL32-EGFP</td>
<td>0.345±0.006</td>
<td>0.31±0.02</td>
<td>245.7±7</td>
<td>56.3±1.5</td>
<td>38.1±1.1</td>
<td>140±10/131</td>
</tr>
<tr>
<td>EGFP-anti-EGFP-IgG</td>
<td>0.301±0.007</td>
<td>0.12±0.02</td>
<td>266.4±2</td>
<td>51.9±0.4</td>
<td>51.3±0.3</td>
<td>178±4/173</td>
</tr>
<tr>
<td>pUL32=pUL32-EGFP – EGFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>=118±10/113</td>
</tr>
</tbody>
</table>

(Fig. 1c). Notably, the 17 identified phosphoresidues on pUL32 account for 1360 Da [36], suggesting that λ digest, which increased the relative mobility of pUL32 to ~2kDa, successfully dephosphorylated pUL32 (Fig. 1b, d and e). Inhibition of phosphatases with the use of CA or OKA yielded proteins with very different relative mobilities on SDS-PAGE (Fig. 2a). Apparently, CA was more efficient in inhibiting cellular protein phosphatases than OKA, as indicated by a slower relative mobility of pUL32 on SDS-PAGE (Fig. 2a). These observations clearly demonstrate that pUL32 and perhaps other tegument proteins with such as pp65. Significant changes in pUL32 relative mobility in vitro, suggesting that a number of potential phosphorylated residues are accessible to kinases. Nevertheless, a limiting factor to this posttranslational modification was the cell toxicity of the phosphatase inhibitors or the presence of hyperphosphorylated cellular proteins (Fig. 2b, c).

In conclusion, our data suggest that pUL32 is indeed phosphorylated but to a clearly smaller extent than previously thought [23]. The significant discrepancy between calculated protein mass of the protein and its relative mobility on reducing SDS-PAGE may be more likely explained by a proposed IDP character than by posttranslational modifications. Moreover, we provided evidence about the extent and location of phosphorylated residues on CMV proteins such as pp65. Significant changes in pUL32 relative mobility on SDS-PAGE following CA or OKA treatment indicate that pUL32 and perhaps other tegument proteins with unphosphorylated serine, threonine or tyrosine residues can be hyperphosphorylated in vitro. The present observations solve a long-standing set of contradictory evidence about the contribution of phosphorylations and intrinsically disordered regions to the obtained molecular mass of pUL32.

**METHODS**

**Cell culture, transfection and establishment of single cell expression clones**

Human embryonic kidney cells (HEK293; ATCC) and HFF (kindly supplied by Dr Thomas Mertens, University Ulm, Germany) were cultured in Dulbecco’s modified Eagle’s medium (GlutaMAX; Thermo Fisher Scientific) with 10% FCS plus antibiotic–antimycotic mix (Thermo Fisher Scientific). For recombinant protein expression, HEK293 cells were transfected with Lipofectamine 2000 and plasmids according to the manufacturer’s instructions (Thermo Fisher Scientific). The expression vector, which encodes full-length His-tagged UL32, was used as described previously [53]. The pp65 plasmid was a kind gift from Dr Deborah H. Spector, San Diego. The stable cell clone expressing pUL32 was generated by antibiotic selection of transiently transfected (UL32His vector) HEK293 cells, dilution to receive single cell clones and subsequent evaluation by immunoblotting to verify pUL32 expression. For the evaluation of changes in relative mobility of Rb, G1-synchronized HFF cells were used because phosphorylation of this protein occurs during cell cycle progression towards S phase [2].

**CMV propagation and purification**

Virus propagation and concentration measurements were performed as described elsewhere [54]. For purification of virions, virus culture supernatant was clarified by low-speed centrifugation and subsequently ultracentrifuged at 70,000 g for 40 min (min) at 16°C. Thereafter, virus pellets were resuspended in PBS and applied to 1000 kDa cut-off centrifugation columns (Vivaspin VS2061; Sartorius) to further concentrate virions and eliminate low-cut-off contaminants such as bovine serum albumin (BSA). For MS, virus samples (strain AD169) were freeze-thawed twice at −20 °C for virus inactivation, loaded on SDS-PAGE, stained with Coomassie to excise specific protein bands or lysed with urea buffer as described below. For FCS analysis, UL32-EGFP-HCMV, a TB40E strain of CMV expressing pUL32 with C-terminal tagged EGFP (pUL32-EGFP) [55], kindly provided by Professor Sinzger (University of Tübingen, Germany), was used.

**Sample preparation for MS**

Excised gel bands were prepared as described by Shevchenko et al. [56] in a slightly modified version and digested with either trypsin (Trypsin Gold; Promega) or chymotrypsin (Roche). Peptides were cleaned up on C18 StageTips [57] and then submitted to LC-MS/MS. Inactivated whole virus samples were lysed in PBS by adding solid urea to a final concentration of 8 M. After reduction with 10 mM dithiothreitol (DTT) for 30 min at room temperature and alkylation with 20 mM iodoacetamide for another 30 min at room temperature in the dark, the remaining iodoacetamide was quenched with 5 mM DTT. The sample was 8× diluted.
with 50 mM ammonium bicarbonate and split in two equal halves. Both halves were digested with either trypsin or chymotrypsin overnight at 37 °C and at a 1:30 enzyme to total protein ratio. The digest was stopped with 1% trifluoroacetic acid and cleaned up using C18 StageTips. Ten percent of the sample was kept for direct injection, and the remaining 90% was subjected to enrichment of phosphopeptides using TiO$_2$, as described by Mazanek et al. [58].

**MS and data analysis**

Peptides were separated using an UltiMate 3000 RSLCnano liquid chromatography system (Thermo Fisher Scientific) with an Acclaim PepMap pre-column (100 Å pore size, 5 µm particle size, 3005 mm) coupled to an Acclaim PepMap analytical column (100 Å pore size, 2 µm particle size, 75 × 500 mm; Thermo Fisher Scientific). The column temperature was maintained at 50 °C and the column was coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) via a nano-electrospray source. The peptides were loaded on the pre-column and separated over a 120 min segmented linear gradient from 3 to 80% buffer B (80% acetonitrile, 0.1% formic acid). The mass spectrometer was operated in data-dependent mode; survey scans were obtained in a mass range of 380–1650 m/z at a resolution of 70,000 at 200 m/z and an automatic gain control target value of 3E6. The 12 most intense ions were selected for fragmentation, the peptide match and exclude isotope ratio of all HCMV class I phosphopeptides were additionally manually validated with respect to correct identification and site localization (Fig. S4, Table S1). To identify other possible candidates of posttranslational modifications, a second search was run with mono- and dimethylation (arginine and lysine), lysine acetylation and lysine ubiquitination as variable modifications in addition to phosphorylation. The sequence logo for class I sites of HCMV was created using the online tool from Crooks et al. [60] (http://weblogo.berkeley.edu, version 2.8.2) using the frequency plot option (Fig. S2).

**Enzymatic treatment of proteins**

UL32-expressing HEK293 cells and virus-infected HFF were washed twice with TBS pH 7.4, resuspended in tris-buffered saline (TBS) buffer and lysed by two freeze–thaw cycles followed by sonication. Protein concentrations were measured by using a BCA kit (Thermo Fisher Scientific). Lambda phosphatase (λ) and protein phosphatase 1 (New England Biolabs; #P0753S and #P0754S, respectively) were used to dephosphorylate the lysates. Each reaction was performed according to the manufacturer’s recommendations and maximum protein concentration did not exceed 1 µg µl$^{-1}$. Maximum concentrations used for experiments with λ and PP1 were 500 or 3.125 U, respectively, for 50 µg protein. According to the manufacturer, 100 U λ should have the same effect as 0.1 U PP1. Enzymatic reactions were stopped by the addition of reducing sample buffer and incubation at 99 °C for 5 min. Protease inhibitor (Calbiochem, #539134) was used in all experiments and phosphatase inhibitor without EDTA (Thermo Fisher Scientific) additionally in all experiments except the samples using phosphatases.

**Inhibition of cellular phosphatases in vitro**

Stable pUL32-expressing HEK293 cells were seeded onto a 96-well plate, 35,000 cells per well, incubated overnight and treated for 4 h with different phosphatase inhibitors as indicated for the respective experiment [CA ab141784, Abcam; OKA, Calbiochem; tacrolimus (FK506), Astellas Pharma]. Cell viability under the influence of the different phosphatase inhibitors was evaluated by using CellTiter-Blue (Promega). CellTiter-Blue was added 2 h after the start of treatment and incubated for two additional hours to maintain a total of 4 h of inhibitor treatment. Subsequently, plates were measured and viability was calculated based on comparison with the vehicle control (1% EtOH). For cell experiments, 1.12 × 10$^6$ cells per well were seeded in six-well plates, incubated with the different phosphatase inhibitors and harvested after 4 h of treatment. Error bars indicate SEM calculated over triplicates.

**SDS-PAGE and immunoblotting**

Samples were diluted in 5× reducing sample buffer (300 mM Tris, pH 6.8, 50% glycerol, 0.05% bromophenol blue, 10% SDS and 10% 2-mercaptoethanol) and boiled at 99 °C for 5 min. Subsequently, SDS-PAGE was performed with the use of gradient gels (Bio-Rad) or fixed percentage gels as appropriate for an optimum resolution of target proteins. After transfer to a polyvinylidene difluoride (PVDF) membrane (#IPFL00010; Merck Millipore), membranes
were blocked with StartingBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific), incubated with the different primary antibodies, sera or intravenous immunoglobulin preparations, washed three times with PBS-T (0.05% Tween-20), incubated with secondary antibodies and finally visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific). In selected experiments, membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) and re-probed. Signal intensity on immunoblots were measured by using a ChemiDoc Imaging System (Bio-Rad) and analysed with the software Image Lab 5.0 (Bio-Rad). All comparisons of signal intensity between different protein preparations were done in parallel and on the same immunoblot. The approximate mass (kDa) of proteins on immunoblots was calculated by the relative mobility distance of reference proteins and marker lanes and subsequent extrapolation as recommended by Bio-Rad (www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5576C.pdf).

Antibodies, sera and intravenous immunoglobulin preparations

The anti-pUL32/pp150 mAb was generated by immunization of mice with the XP1 antigen expressed in E. coli as previously described [61]. The β-tubulin HRP antibody (ab21058) was obtained from Abcam, and the Phospho-S6 Ribosomal Protein (Ser235/236) (#2211), the Phospho-Akt (Ser473) (#4060) and the Retinoblastoma (Rb) (4 H1, #9309) antibody were obtained from Cell Signaling Technology. Secondary antibodies were from Jackson ImmunoResearch. Two human immune sera of healthy donors and two commercially available intravenous immunoglobulin preparations (IVIG) (Baxter and CSL Behring) were applied for analysis of immunological impact after enzymatic dephosphorylation. For analysis of reactivity of immune sera with hyperphosphorylated and native pUL32, 28 different immune sera of healthy donors were used.

FCS

The virus pellet of pUL32-EGFP-HMCV was lysed in a lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 2% NP-40, 0.02% SDS, 10 mM DTT, 1 mM PMSF, 1 mM protease inhibitor cocktail) modified from Yu et al. [47] for 10 min at 4°C followed by ultracentrifugation at 80,000 g for 45 min at 4°C to remove cell debris. The diffusion characteristics of pUL32-EGFP-HMCV in clear lysate were studied by FCS. The calibration of the confocal volume of the ConfoCor2 fluorescence correlation microscope (Carl Zeiss-Evotec) was performed with the same optical setup as previously published [62].

Fluorescence fluctuations of pUL32-EGFP-HMCV were monitored over a time period of 30 s (Fig. 5a) and autocorrelated by the function \( G(\tau) \);

\[
G(\tau) = \frac{\langle \delta F(t) \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}
\]

where the angular brackets denote the ensemble average, \( \delta F(t) \) the corresponding variance and \( \tau \) the delay or correlation time interval over which the fluctuations are compared. Characteristic translational diffusion times of \( \tau=245.7\pm5\mu s \) (mean±SEM) for pUL32-EGFP-HMCV and of \( \tau=132.5\pm5\mu s \) (mean±SEM) for EGFP through the confocal volume were obtained from the FCS ACCESS Fit (Carl Zeiss-Evotec) software package using a one-component fit model [62].

\[
G(\tau) = 1 + \frac{1}{N(1 + \frac{\tau}{\tau_D})^2}
\]

where \( N \) is the particle number and \( \tau_D \) the molecular translational diffusion time of the excited fluorophores moving in a three-dimensional confocal volume through an axial \( (z) \) to radial \( (\omega) \) dimension.

The molecular diffusion coefficient \( D \) (cm² s⁻¹) of the protein was calculated from the translational diffusion time \( \tau_D(s) \) by

\[
D_{\text{trans}} = \frac{\omega_D^2}{4 \tau_D}
\]

and its respective average hydrodynamic radius \( R_h \) from the Stokes–Einstein relation.

\[
R_h = \frac{k_B T}{6 \pi \eta D_{\text{trans}}}
\]

where \( D_{\text{trans}} \) denotes the translational diffusion coefficient, \( k_B \) is the Boltzmann constant \((1.38\times10^{-23} \text{ J K}^{-1})\), \( T \) is the temperature \((293 \text{ K})\), \( \eta \) is the viscosity of the solvent \((0.001 \text{ kg m}^{-1} \text{ s}^{-1})\) and \( R_h \) is the hydrodynamic radius of the molecule. Finally, the molecular weight of the protein can be estimated by

\[
\text{MW} = \frac{4}{3} R_h^3 \pi \rho N_A
\]

where \( N_A \) is Avogadro’s number \((6.023\times10^{23} \text{ mol}^{-1})\) and \( \rho \) is the mean density of the molecule.

Steady-state anisotropy measurements

The rotational movement of pUL32-EGFP-HMCV, EGFP-bound IgG and EGFP, respectively, was studied by means of fluorescence anisotropy, which provides information on the size as well as the shape of a protein. Since fluorescence occurs in the nanosecond timescale, steady state is reached almost immediately. All steady-state fluorescence anisotropy measurements were carried out on a Perkin Elmer LS50B Fluorimeter using a quartz cuvette of 10 mm pathlength (Hellma Analytics). The EGFP proteins were excited at 481 nm and the emission intensity of polarized light detected at 509 nm. The steady-state anisotropy \( r \) is experimentally measured following:
where \( G \) denotes the grating factor of the instrument, \( I_v \) is the emission intensity of vertically (v) or horizontally (h) polarized light parallel to the plane of excitation and \( I_{\perp} \) is the intensity of vertically (v) or horizontally (h) polarized light perpendicular to the plane of excitation.

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

Ethical statement
Written informed consent was obtained from all patients at the time of enrolment. The study protocol was approved by the local institutional review board of the Medical University of Vienna in accordance with the Declaration of Helsinki (EK26/2012).

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