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Herpes simplex virus 1 protein pUL21 alters ceramide metabolism by activating the inter-organelle transport protein CERT

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Abstract

Herpes simplex virus (HSV)-1 dramatically alters the architecture and protein composition of cellular membranes during infection, but its effects upon membrane lipid composition remain unclear. HSV-1 pUL21 is a virus-encoded protein phosphatase adaptor that promotes dephosphorylation of multiple cellular and virus proteins, including the cellular ceramide transport protein CERT. CERT mediates non-vesicular ceramide transport from the ER to the trans-Golgi network, whereupon ceramide is converted to sphingomyelin and other sphingolipids that play important roles in cellular proliferation, signaling, and membrane trafficking. Here, we use click chemistry to profile the kinetics of sphingolipid metabolism, showing that pUL21-mediated dephosphorylation activates CERT and accelerates ceramide-to-sphingomyelin conversion. Purified pUL21 and full-length CERT interact with sub-micromolar affinity, and we solve the solution structure of the pUL21 C-terminal domain in complex with the CERT PH and START domains using small-angle X-ray scattering. We identify a single amino acid mutation on the surface of pUL21 that disrupts CERT binding in vitro and in cultured cells. This residue is highly conserved across the genus Simplexvirus. Additionally, we identify a pUL21 residue essential for binding to HSV-1 pUL16. Sphingolipid profiling demonstrates that ceramide-to-sphingomyelin conversion is severely diminished in the context of HSV-1 infection, a defect that is compounded when infecting with a virus encoding the mutated form of pUL21 that lacks the ability to activate CERT. However, virus replication and spread in cultured keratinocytes or epithelial cells is not significantly altered when pUL21-mediated CERT dephosphorylation is abolished. Collectively, we demonstrate that HSV-1 modifies sphingolipid metabolism via specific protein-protein interactions.
Introduction

Herpes simplex virus (HSV)-1 is a human pathogen that is estimated to infect the majority of the human population, causing a life-long latent infection (1). Latent HSV-1 resides in sensory or sympathetic neurons, migrating to the periphery in periodic reactivation events throughout the lifetime of the host. In order to sustain acute (lytic) infection, HSV-1 drastically modifies infected cells (2–5). In particular, the virus extensively remolds the composition and architecture of cellular membranes to facilitate virus assembly and spread. Nascent capsids leave the nucleus via sequential envelopment and de-envelopment at the inner and outer nuclear membranes (6). These cytosolic capsids acquire a proteinaceous layer termed ‘tegument’ and bud into the lumen of post-Golgi membranes that are studded with viral glycoproteins (so called ‘secondary envelopment’) (7). The resultant virus-containing vesicles are transported to cell contact sites where they release the mature virus particles to disseminate the infection (8).

HSV-1 pUL21 is a tegument protein that is conserved in all alphaherpesviruses (9). This multifunctional protein and its homologues are known to interact with multiple cellular and viral partners, including pUL16 (10, 11), pUL11 (12), gE (12), tubulin (13) and Roadblock-1 (14), and it has been implicated in a number of important processes including capsid nuclear egress (15), viral cell-to-cell spread (12, 16), and retrograde transport along axons to the neuronal cell bodies where latency is established (14, 17). Mutant HSV-1 lacking pUL21 expression exhibits a 10- to 100-fold replication defect and severely impaired cell-to-cell spread, both of which can be at least partially ascribed to the phosphomodulatory role of pUL21 as a protein phosphatase 1 (PP1) adaptor (18). PP1 is a highly active and abundant cellular phosphatase (19) and pUL21 recruits PP1 to promote dephosphorylation of multiple substrates, including the viral protein pUL31 that is implicated in viral nuclear egress (18, 20) and the cellular protein CERT that regulates sphingomyelin metabolism (18).

The cytoplasmic ceramide transport protein CERT (a.k.a. Goodpasture's antigen binding protein, GPBP, encoded by the gene COL4A3BP) mediates the non-vesicular trafficking of ceramide from the endoplasmic reticulum (ER) to the trans-Golgi network (TGN) and, in doing so, defines the rate of sphingomyelin synthesis (21–23). CERT contains two well-folded globular domains: an N-terminal pleckstrin homology (PH) domain that mediates its interaction with TGN membranes by binding phosphatidylinositol 4-phosphate, and a C-terminal steroidogenic acute regulatory related lipid transfer (START) domain that directly binds ceramide to mediate its transfer (23). Crystal structures of both domains have been solved (24, 25). The ‘middle region’ (MR) that connects the PH and START domains of CERT does not adopt a globular fold, instead containing a coiled-coil region that is likely to mediate CERT self-association (26), a ‘two phenylalanines in an acidic tract’ (FFAT) motif that recruits CERT to the ER membranes via binding proteins VAPA and VAPB (27), and a serine-rich motif (SRM), hyperphosphorylation of which represses CERT activity (28). In cultured HeLa cells the vast majority of CERT is in an inactive, hyperphosphorylated state (CERTp), available to be mobilised via dephosphorylation to increase the rate of ER-to-TGN ceramide transport in response to stimuli such as sphingomyelin depletion (28).
Ceramide (Cer) and sphingolipids like sphingomyelin (SM) are essential for mammalian cell growth and they play important roles in cell signalling, apoptosis and membrane trafficking (29). Furthermore, sphingolipids and cholesterol participate in the formation of membrane microdomains (including ‘lipid rafts’) that compartmentalise the lipid and protein composition of cellular membranes (30). This compartmentalisation is especially important in highly polarised cells such as neurons (31). Although sphingolipids are crucial to host-cell biology, relatively little is known about the interaction between pathogens and cellular sphingolipid metabolism. The bacteria Chlamydia trachomatis is known to directly recruit CERT to bacteria-containing intracellular inclusions, thereby increasing the abundance of SM in the inclusion membrane (32), and CERT activity is necessary for the biosynthesis of double-membrane vesicles that serve as the sites of hepatitis C virus and polio virus replication (33). However, to the best of our knowledge, the binding of CERT to HSV-1 pUL21 (18) is the only known example of a direct interaction between CERT and a virus protein.

Previous studies have shown that HSV-1 infection increases Cer synthesis (34) and that depletion of sphingomyelinase causes a >30-fold decrease in HSV-1 replication (35). Sphingomyelinase treatment of cultured epithelial cells has only a very modest effect upon HSV-1 entry (36), suggesting that SM is not absolutely required for virus infection. In contrast, recent studies in macrophages demonstrated that acid ceramidase activity is potently antiviral, helping sequester incoming virus particles by promoting their neutralising association with sphingosine-rich intraluminal vesicles within endocytic multi-vesicular bodies (37). Furthermore, siRNA depletion of CERT has been shown to promote secretion of usually cell-associated HSV-1 virions (38). It is therefore increasingly clear that cellular sphingolipid metabolism is important for HSV-1 biology, and that further studies are required to obtain a full picture of how specific sphingolipids support and/or prevent HSV-1 infection.

We sought to define the functional consequences of the pUL21:CERT interaction for HSV-1 infection. Using click chemistry, we demonstrated that pUL21-mediated CERT dephosphorylation increases the rate of Cer to SM conversion in cultured cells. We characterised the solution structure of the pUL21 C-terminal domain in complex with the PH and START domains of CERT, identifying a specific pUL21 amino acid required for the interaction. Functional characterisation of mutant HSV-1 encoding this mutated form of pUL21 confirmed that pUL21-mediated CERT dephosphorylation alters the rate of SM synthesis in infected cells. Furthermore, we observed a dramatic increase in the rate of Cer accumulation in infected cells, which was only partly counteracted by pUL21-stimulated CERT activity, but this increase in Cer abundance does not significantly alter virus replication or spread in cultured keratinocytes or epithelial cells.
Results

Human cells typically maintain lipidome homeostasis via robust feedback and control mechanisms, which respond to perturbations via local activation of specific signalling pathways (39, 40). We hypothesised that pUL21, expressed at late points of infection (3), would likely alter the rate of sphingolipid synthesis and thereby affect lipid-mediated signalling events, rather than altering the overall steady-state lipid composition of infected cells. Synthetic lipids with alkyn-containing acyl chains are efficiently processed by mammalian lipid-modifying enzymes and represent powerful tools to probe lipid metabolism (41). The impact of pUL21-directed CERT dephosphorylation on sphingolipid metabolism was therefore probed using a clickable analogue of sphingosine (Sph), alkyn-Sph, to monitor the rate of sphingomyelin biogenesis in immortalized human keratinocyte (HaCaT) cells. Exogenous Sph is efficiently incorporated into cellular metabolic pathways, being rapidly converted into ceramide (Cer) and then sphingomyelin (SM) or hexosylceramides (HexCer) like glucosylceramide or galactosylceramide (42). It is also converted into phosphatidylcholine (PC) via a so-called ‘salvage’ pathway (Fig 1A) that directs sphingosine to palmitoyl-CoA, which serves as substrate for re-acetylation of lysophosphatidylcholine (43).

To monitor Sph metabolism, HaCaT cells, either parental or stably expressing pUL21 (HaCaT21), were incubated with alkyn-Sph for five minutes (pulse) and the rate of alkyn-Sph incorporation into the competing metabolic pathways was monitored for two hours (chase) by high performance thin layer chromatography (HPTLC) separation and detection of lipids conjugated to coumarin-azide via a ‘click’ reaction (Fig 1B and 1C). Alkyn-Sph was very efficiently converted to alkyn-Cer in both cell types, the levels of alkyn-Cer remaining relatively stable throughout the chase, and for both cell types synthesis of alkyn-PC and alkyn-SM was observed, but not synthesis of alkyn-HexCer. HaCaT21 cells exhibited significantly reduced rates of alkyn-Sph conversion and the level of alkyn-Cer is significantly lower (Fig S1). The rate of alkyn-PC synthesis was also reduced, although this reduction was not significant owing to the high inter-experiment variability of the alkyn-PC signal for the parental HaCaT cells. Taken together, these results indicated that lipid metabolism is altered in HaCaT cells constitutively expressing pUL21. However, it remained unclear whether these differences reflected adaptations of cellular lipid metabolism in response to constitutive pUL21 expression rather than a direct effect upon CERT activity. Since CERT-mediated non-vesicular transport of Cer from the ER to the TGN defines the rate of Cer-to-SM conversion (21–23), pUL21-directed CERT dephosphorylation (and thus activation) should increase the rate of SM synthesis. Consistent with this hypothesis, the abundance alkyn-SM as a fraction of total alkyn-Cer plus alkyn-SM was significantly higher in HaCaT21 versus HaCaT cells at early time points (0–30 min) during the chase (Fig 1D). By the 60 min chase time point the rate of alkyn-SM accumulation slows and the difference in relative abundance between cell lines was diminished, consistent with the alkyn-SM levels in both cell types approaching equilibrium. This increase in alkyn-SM accumulation as a fraction of alkyn-Cer plus alkyn-SM abundance was observed despite similar overall signal for alkyn-SM and alkyn-Cer in HaCaT21 cells when compared to parental cells at early time points (0–30 min), the abundance of these lipids being significantly lower in HaCaT21 cells at later time points (Fig 1E). Reduced overall levels of SM plus Cer
in HaCaT21 cells likely results from adaptation to constitutive pUL21 expression (and thus constitutive CERT hyperactivation) via stimulation of feedback pathways that reduce Sph to Cer conversion and/or increased back-conversion of SM and/or Cer to Sph. Treatment of HaCaT cells with the highly-specific CERT inhibitor HPA-12 (44) confirmed that the rate of Cer-to-SM conversion was defined by CERT activity: SM synthesis was significantly decreased in HPA-12–treated cells when compared with untreated cells, and there was concomitant accumulation of alkyne-Cer in treated cells (Fig 1B–D and Fig S1). Immunoblot analysis of protein samples that were re-solubilized following their precipitation during lipid extraction (Fig 1F) confirmed that pUL21 was expressed and that dephosphorylated CERT (CERT\(^{0}\)) predominates in HaCaT21 cells, in contrast to the parental and HPA-12 treated HaCaT cells.

pUL21 has multiple functions during infection, promoting the PP1-mediated dephosphorylation of not just CERT but of multiple different cellular and viral proteins (18). It was therefore essential to identify a point mutant of pUL21 with impaired binding to CERT, but not to other cellular or viral partners, to dissect the functional significance of increased CERT activity during infection. Defining the interaction surfaces of transient protein complexes presents a considerable challenge (45), made more difficult in the case of pUL21 and CERT by their containing multiple domains and regions of intrinsic disorder (18). The minimal region of CERT required for pUL21 binding was thus probed using pull-down experiments where pUL21-GST purified following bacterial expression (bait) was used to capture CERT or truncations thereof (preys) expressed by in vitro coupled transcription/translation in wheat germ extract (Fig 2A). The longer cytoplasmic isoform of CERT (CERT\(_{L}\)), where expression of exon 11 extends the amino terminal region of the START domain by 26 amino acids (START\(_{L}\)), was used since both CERT and CERT\(_{L}\) have been shown to possess ceramide transfer activity (23) and a contribution of these additional 26 amino acids to pUL21 binding could not be excluded. Full-length CERT\(_{L}\) was efficiently captured by pUL21-GST. The PH and START\(_{L}\) domains were also captured by pUL21-GST when expressed individually, albeit less efficiently, suggesting that the CERT\(_{L}\) possesses multiple pUL21-binding motifs. The CERT middle region, MR, was not captured by pUL21-GST and is thus likely to be dispensable for binding. A truncated CERT\(_{L}\) construct that retained the pUL21-binding PH and START\(_{L}\) domains, but lacked the majority of the highly flexible MR, was thus designed for use in subsequent biochemical and structural studies (miniCERT\(_{L}\)) (Fig 2B). The extent of the MR retained to link the two domains was informed by the crystal structure of the CERT PH:START domain complex (46), defining a minimal distance of at least 55 Å between the PH domain C terminus and the START domain N terminus, and the desire to exclude the predicted coiled-coil region that may cause oligomerisation (26). MR residues 132–350 were thus excluded from miniCERT\(_{L}\). The first 19 amino acids of the PH domain were also omitted from miniCERT\(_{L}\) as this region is predicted to be highly disordered and was excluded in previous structural studies (24, 47).

Previous studies identified that the MR mediates CERT trimerisation (26, 48). Size-exclusion chromatography with inline multi-angle light scattering (SEC-MALS) (Fig 2C) confirmed that hyperphosphorylated StreptII-tagged CERT\(_{L}\)^\(^{p}\), purified following expression in mammalian (Freestyle 293F) cells, has an observed mass (184.3 kDa) approaching that expected for a trimer (215.7 kDa). Similarly, a constitutively hypophosphorylated form of CERT\(_{L}\) where S132 is mutated to alanine, and
thus cannot become phosphorylated to initiate the SRM phosphorylation cascade (28), had an observed mass (199.2 kDa) approaching that expected for a trimer. In contrast, H6-miniCERTL purified following bacterial expression was predominantly monomeric and monodisperse (observed mass 43.9 kDa, expected monomeric mass 44.9 kDa), consistent with trimerization of CERTL being driven by the MR and not being dependent upon CERTL phosphorylation.

Isothermal titration calorimetry (ITC) demonstrated that pUL21-H6 (18) binds StreptI-CERTL with approximately micromolar affinity (Fig 2D,E, Table 1 and Table S1), the observed affinity not differing significantly between the hyperphosphorylated (CERTL(P)) and hypophosphorylated (CERTL(S132A)) forms of the protein. While StreptI-CERTL(S132A) forms a 1:1 complex with pUL21, the observed binding stoichiometry (N) was consistently lower for StreptI-CERTL(P) (0.74), consistent with a proportion of the pUL21 binding sites on CERTL being sterically occluded when the protein is hyperphosphorylated. H6-miniCERTL and pUL21-H6 form an equimolar complex, binding with micromolar affinity similar to the full-length protein (Fig 2F; Table 1). However, the thermodynamics of binding differ significantly; whereas pUL21 binding to CERTL is endothermic and entropically driven, binding to miniCERTL is exothermic and enthalpically driven with minimal change in overall entropy (Table 1). This is consistent with CERTL undergoing significant conformational rearrangement upon binding to pUL21, whereas the conformational changes to miniCERTL (if any) upon pUL21 binding are likely to be much more modest.

Small-angle X-ray scattering (SAXS) in batch mode was used to probe the solution structure of H6-miniCERTL (Fig 2G and Table S2). The frequency distribution of real-space distances (p(r) profile) is largely symmetric (Fig 2H) and the dimensionless Kratky plot (Fig 2I) has a bell-shaped peak at sRg = √3, consistent with miniCERTL having a compact globular conformation in solution. Ab initio modelling of the H6-miniCERTL scattering profile using GASBOR (49) yields a dummy-residue model (Fig 2J,K) that closely resembles the crystal structure of CERT START and PH domain complex (46). A pseudo-atomic model of the H6-miniCERTL solution structure, generated using the crystal structures of the CERT PH and START domains determined in isolation (24, 25) as rigid bodies, confirms the similarity of the H6-miniCERTL solution model to the crystal structure of the CERT PH:START domain complex (Fig 2L,M). Collectively, the MALS, ITC and SAXS analysis demonstrates that miniCERTL forms a compact monomeric protein with high affinity for pUL21, representing an excellent tool for structural studies.

Structural characterisation via X-ray crystallography and SAXS has revealed pUL21 to comprise two domains joined by a highly flexible linker (Fig 3A) (18, 50, 51), and immunoprecipitation experiments mapped CERT binding to the pUL21 C-terminal domain (18). The C-terminal domain of pUL21 (pUL21C), spanning amino acids 275–535, was expressed with an N-terminal hexahistidine tag and purified following bacterial expression. In contrast to a previous report (50), we did not observe copurification of nucleic acid with H6-pUL21C, the purified protein having an A260/280 ratio of ~0.6. SEC-MALS and SAXS analysis (Fig S2) confirmed that H6-pUL21C is monomeric and monodisperse, adopting a compact structure in solution that matches the previously determined crystal structure (χ² = 0.99, CorMap p = 0.133) (50). SEC analysis of a pre-formed complex between purified H6-miniCERTL
and a 1.34-fold molar excess of Hc-pUL21C confirmed that the two proteins form a stable complex (Fig 3B), suitable for structural studies. ITC demonstrated that Hc-pUL21C forms an equimolar complex with Hc-miniCERTL with a dissociation constant (Kd) of 4.3 ± 1.2 μM (Fig 3C, Table 1 and Table S1). The approximately 4-fold reduction in miniCERTL binding by pUL21C versus full-length pUL21 is consistent with previous immunoprecipitation results, where transfected pUL21C-GFP captured endogenous CERT slightly less efficiently than did pUL21-GFP in HEK293T cells (18). These ITC experiments confirm that the C-terminal domain of pUL21 is the major determinant of CERT binding. Attempts to predict the structure of pUL21C in complex with miniCERTL using AlphaFold-Multimer (52) did not yield high confidence models, presumably because of a paucity of co-evolutionary signal between these viral and human proteins, and structural characterisation of this complex thus required an experimental approach.

To probe the structural basis of the CERT recruitment by pUL21, a pre-formed complex of Hc-miniCERTL and Hc-pUL21C was subjected to SEC with inline SAXS measurement (SEC-SAXS, Fig 3D). SAXS data were processed by averaging frames with a consistent calculated radius of gyration (Rg) and then subtracting averaged buffer frames to yield the Hc-miniCERTL:Hc-pUL21C complex scattering profile (Fig 3E). The probable frequency of real-space distances (ρ(r) profile) of the complex is moderately asymmetric (Fig 3F), in contrast to the highly symmetric ρ(r) profiles of Hc-pUL21C (Fig S2D) or Hc-miniCERT (Fig 2H) alone, suggesting a less spherical particle, and the dimensions of the complex (Rg 3.5 nm, Dmax 13.6 nm) are substantially larger than for Hc-pUL21C (Rg 2.2 nm, Dmax 8.5 nm) or Hc-miniCERT (Rg 2.7 nm, Dmax 9.1 nm). The peak of the dimensionless Kratky plot is slightly higher, with its peak away from sRg = √3 (Fig 3G), suggesting some flexibility in the system granted either by modest dissociation of the complex or some mobility of the Hc-miniCERTL domains with respect to each other and Hc-pUL21C. Ab initio modelling using GASBOR indicated an elongated molecule (Fig 3H). While initial pseudo-atomic models of the complex generated using a fixed conformation of Hc-miniCERTL did not fit the SAXS profile acceptably, allowing the PH and STARTL domains freedom to move with respect to each other and to Hc-pUL21C yielded three pseudo-atomic models with high-quality fits to the SAXS profile (Fig 3I,J and Fig S3). In all three top models miniCERTL binds ‘end-on’ to the pUL21 molecule, forming an ellipsoidal ‘rugby ball’ like particle. In two of the top three models miniCERTL binds the ‘left wing’ of the dragonfly-shaped pUL21C domain (50), whereas in the other it binds the ‘right wing’ (Fig 3I,J and Fig S3). These models all have a similar overall shape, and thus all explain the SAXS scattering data well, but the relative orientations of Hc-miniCERTL and Hc-pUL21C differ. All three top models were thus used to design specific pUL21 mutations that might disrupt (mini)CERT binding.

Mutations in pUL21C were designed to identify whether miniCERTL binds the left or right wing of this domain. Amino acids in helix α4 or the subsequent loop of pUL21C were mutated to test binding to the left wing (Fig 3I), whereas amino acids in helices α5 and α9 were used to test binding to the right wing (Fig 3J). Immunoprecipitation experiments in transfected HEK293T cells that had been infected with HSV-1 lacking pUL21 expression (HSV-1 ΔpUL21) demonstrated that 4 of 5 substitution in the left wing of pUL21C (P365D, V368E, R373E and V382E) disrupted the ability of CERT to co-precipitate with
pUL21-GFP (Fig 4A), whereas none in the pUL21C right wing disrupted CERT co-precipitation (Fig 4B). These results are consistent with CERT binding the left wing of pUL21C. They are also consistent with observations that CERT binding is lost when pUL21C has a bulky N-terminal GFP tag but retained when the GFP tag is C terminal (18), as the left-wing models place the pUL21C amino terminus but not the carboxy terminus in close proximity to miniCERTL (Fig 3I and Fig S3).

Of the pUL21 substitutions that disrupted CERT binding (Fig 4A), V382E appeared to cause the largest decrease in CERT binding while maintaining the ability of pUL21 to co-precipitate its other known binding partners, PP1 (18) and pUL16 (10). The presence of a valine residue at this position is absolutely conserved in HSV-1 and HSV-2, and is highly conserved across members of the simplexvirus genus (Fig S4). Hc-pUL21V382E was purified following bacterial expression (Fig 4C). Differential scanning fluorimetry (a.k.a. Thermofluor) showed pUL21V382E to be well folded as its thermal stability is similar to wild-type Hc-pUL21 (Fig 4D). ITC analysis demonstrated that Hc-pUL21V382E has approximately 8-fold reduced binding affinity for Hc-miniCERTL when compared to wild-type pUL21 (Fig 4E, Table 1 and Table S1). The effect of the V382E substitution upon the ability of pUL21 to promote CERT dephosphorylation, converting CERTP to CERTO, was probed using an in vitro dephosphorylation assay with all-purified reagents (Fig 4F,G). The dose-dependent acceleration of GST-PP1 mediated CERT dephosphorylation is significantly greater for wild-type pUL21 than pUL21V382E (p = 0.040; two-way ANOVA with Sidak’s multiple comparison test), consistent with the GST-PP1:pUL21V382E complex having lower affinity for the substrate CERTP (EC50[pUL21] = 0.491 ± 0.123 µM, EC50[pUL21V382E] = 3.329 ± 0.7874 µM; three parameter dose-response curve, n = 2 independent experiments).

While mutation of the pUL21C right wing did not prevent CERT binding, one substitution (L490E) abolished binding to pUL16 (Fig 4B). pUL21L490E retains the ability to bind CERT and PP1, suggesting that the mutant is stable and maintains a correct overall fold. A model of pUL16 in complex with pUL21C was generated using AlphaFold-Multimer (52), revealing that pUL16 is predicted to bind the right wing of pUL21C, with helix α9 spanning residues 486–497 being central to this interaction (Fig 4H). The structures of pUL16 and pUL21C, and their relative orientations in the complex, were predicted with high overall confidence (Fig 4I). pUL16 is predicted to comprise two domains, an N-terminal domain (residues 35–172) that is joined by a short linker to a C-terminal domain (residues 179–371) (Fig S5A). A structural homology search using DALI (53) revealed that the pUL16 C-terminal domain shares greatest structural similarity to the N-terminal domain of pUL21 (Fig S5B and Table S3), whereas the pUL16 C-terminal domain is similar in fold to a variety of bacterial and eukaryotic proteins that have diverse annotated functions ranging from phosphatase activity to lipid binding (Fig S5C,D and Table S3). Residues 1–34 of pUL16 were predicted with low-confidence and are likely to be intrinsically disordered. Residue L490 of pUL21C is predicted to bind a hydrophobic pocket formed by L91, R362 and residues 73–78 of pUL16 (Fig 4H). Loss of pUL16 binding by pUL21C L490E is consistent with the introduction of a charged glutamic acid side chain into this pocket being energetically unfavourable. pUL21 R497 is in close proximity to pUL16 residues T359 and R362. Inverting the charge of pUL21 residue 497 by substituting arginine for glutamate did not prevent pUL16 binding, presumably because the negative glutamic acid side chain could still form polar interactions with these residues. pUL21 E409
is not predicted to lie at the pUL16 binding interface, consistent with mutation of this residue not affecting the pUL21C:pUL16 interaction.

To probe the ability of the pUL21 mutant with reduced CERT binding (pUL21\textsuperscript{V382E}) to stimulate CERT dephosphorylation during infection, a mutant strain of HSV-1 encoding pUL21\textsuperscript{V382E} was generated using two-step Red recombination (54). Dephosphorylated CERT (CERT\textsuperscript{D}) is significantly less abundant in HaCaT cells infected with HSV-1 expressing pUL21\textsuperscript{V382E} or lacking pUL21 expression (ΔpUL21) when compared to wild-type HSV-1 infected cells (Fig 5A,B). In addition to CERT, pUL21 expression reduces the phosphorylation of multiple substrates of the viral kinase pUS3 in HSV-1 infected cells (18), the phosphorylated forms of these substrates being detectable using an antibody that recognises phosphorylated substrates of the cellular kinase Akt (55). While infection with ΔpUL21 HSV-1 causes a dramatic increase in the abundance of multiple phosphorylated pUS3 substrates, the abundance of these phosphoforms is indistinguishable between wild-type and pUL21\textsuperscript{V382E} HSV-1 (Fig 5A). This confirms that the V382E substitution specifically disrupts CERT dephosphorylation, rather than generally inhibiting the ability of pUL21\textsuperscript{V382E} to recruit PP1 to substrates. Similar changes in CERT dephosphorylation, but not in the dephosphorylation of other pUS3 substrates, are observed when Vero cells are infected with pUL21\textsuperscript{V382E} HSV-1 (Fig S6A,B). Immunocytochemistry confirms that both wild-type and V382E pUL21 have the similar subcellular localisation, being observed predominantly at the nuclear rim of infected Vero cells (Fig 5C).

Metabolic labelling was used to monitor the impact of pUL21-mediated CERT dephosphorylation on sphingolipid biogenesis during infection. A pulse-chase experiment was performed where HaCaT cells infected with wild-type or pUL21\textsuperscript{V382E} HSV-1, or mock infected, were incubated for 5 min with alkyne-Sph at 14 hours post-infection (hpi) and its metabolites were monitored for two hours. Cer accumulates and the rate of SM synthesis is significantly decreased in cells infected with wild-type and pUL21\textsuperscript{V382E} HSV-1 when compared to uninfected cells (Fig 5D). Although a substantial decrease in the rate of SM synthesis is seen for both wild-type and mutant HSV-1 infection, the defect is significantly larger in cells infected with HSV-1 pUL21\textsuperscript{V382E} (Fig 5E). The overall abundance of SM plus Cer is similar between infected and uninfected cells, suggesting that Cer to SM conversion is specifically impaired rather than influx of alkyne-Sph into the SM biogenesis pathway being defective (Fig 5F). Taken together, this suggests that pUL21-mediated activation of CERT accelerates Cer to SM conversion during infection, albeit from a much lower base than in uninfected cells.

Having confirmed that pUL21\textsuperscript{V382E} HSV-1 specifically lacks the ability to stimulate CERT dephosphorylation, and that HSV-1 encoding pUL21\textsuperscript{V382E} has a reduced rate of Cer to SM conversion, the impact of this deficit upon virus replication and spread in cultured cells was assessed. Wild-type and pUL21\textsuperscript{V382E} HSV-1 form similar sized plaques on HaCaT and Vero cells (Fig 6A), suggesting that CERT dephosphorylation is dispensable for efficient viral cell-to-cell spread. A single-step growth curve, where Vero and HaCaT cells are infected at a high multiplicity of infection (MOI) and the production of infectious progeny is monitored over time, was used to compare the replication of wild type and pUL21\textsuperscript{V382E} HSV-1 (Fig 6B). Both viruses produce similar abundance of infectious progeny by 24 hours
post infection. In two biologically independent experiments performed for each cell type the kinetics of virus replication appeared to be accelerated, with higher titres of pUL21<sup>V382E</sup> HSV-1 being observed between 6–12 hours post-infection, but the difference in growth rate was not statistically significant for either cell line (two-way ANOVA with Sidak’s multiple comparisons test).

While HSV-1 preferentially remains cell-attached, spreading via direct cell:cell contacts, the cell-free secretion of virions from infected cells is altered when CERT is depleted or overexpressed (56). Cell-free release of pUL21<sup>V382E</sup> HSV-1 was determined by quantitating the amount of infectivity secreted into the medium as a percentage of overall infectivity (secreted plus cell-associated), normalised for each independent replicate to the secretion of a wild-type virus in control experiments performed at the same time. HaCaT cells infected for 12 h with various MOI of pUL21<sup>V382E</sup> HSV-1 did not release significantly more or less infectivity when compared to WT HSV-1 infection (Fig 6C). Similarly, the cell-free release of infectivity at 12 hpi did not differ between Vero cells infected with pUL21<sup>V382E</sup> or WT HSV-1 (Fig S6C).
Discussion

HSV-1 extensively remodels the proteome of infected cells, altering both the abundance (3) and post-translational modification status (2, 18, 57) of multiple cellular proteins. We know much less about HSV-1 mediated changes to the cellular lipidome, although previous studies have identified that HSV-1 infection changes phosphoinositide levels (58) and increases the rate of de novo phospholipid synthesis (59). By combining biochemical and structural studies with cell-based models of infection, we show here that pUL21 accelerates the conversion of Cer to SM in infected cells by promoting dephosphorylation and activation of the Cer transport protein CERT (Fig 5). This is the first observation of a viral protein directly binding CERT and altering its activity. While pUL21-mediated CERT activation causes an apparent modest acceleration in the rate of virus replication, this effect is not statistically significant and CERT activation does not contribute to replication or cell-to-cell spread of HSV-1 in cultured keratinocytes or epithelial cells (Fig 6).

Our previous work showed that pUL21 is a phosphatase adaptor with multiple different targets (18). Mutation of the pUL21 TROPPO motif, required for PP1 binding and thus stimulation of dephosphorylation, dramatically reduced the replication and spread of HSV-1. While the TROPPO motif was identified via its conservation across alphaherpesvirus sequences, the molecular basis of CERT recruitment remained unknown. Our previous immunoprecipitation experiments demonstrated that the N-terminal domain of pUL21 was dispensable for CERT binding (18). Biochemical mapping and SAXS structural characterisation presented here have now identified a specific mutation, V382E, that decreases the affinity of pUL21 for miniCERT by approximately 8-fold (Fig 4E). While this reduction in affinity is moderate when assayed in dilute biochemical solution, it is sufficient to disrupt co-precipitation of CERT from transfected cells (Fig 4A). The V382E substitution abolishes pUL21-mediated dephosphorylation of CERT during infection (Fig 5A,B) and significantly decreases the rate of Cer to SM conversion (Fig 5E). These results are consistent with other binding partners competing to bind CERT and/or pUL21 within the context of infected cells, these competing interactions amplifying the effect of reduced pUL21:CERT binding affinities. The V382E mutation does not prevent pUL21 binding to pUL16 (Fig 4A) nor its ability to stimulate dephosphorylation of other targets (Fig 5A), confirming that pUL21 binds CERT via a molecular surface that is distinct from the binding site(s) of other targets. The wild-type levels of virus replication and cell-to-cell spread observed for pUL21V382E HSV-1 are consistent with our previous in vitro evolution studies of HSV-1 mutants where the pUL21 TROPPO motif was mutated (18). Adaptation of the virus to the loss of pUL21 PP1 binding, via suppressor mutations that reduce the activity of the kinase pUS3, restored virus replication and spread without restoring enhanced CERT dephosphorylation (18). We therefore conclude CERT is not a critical substrate of pUL21 for virus replication and spread in cultured fibroblasts and keratinocytes. The C-terminal domain of pUL21 is highly conserved across HSV-1 and HSV-2 (>93% and 80–84% identity, respectively). V382 is absolutely conserved across these species, and a small hydrophobic residue (valine, leucine or alanine) at this position is very highly prevalent across the genus Simplexvirus (Fig S4A). The C-terminal domain of pUL21 is much less well conserved when considering the entire subfamily Alphaherpesvirinae (>20% sequence identity across the subfamily), with the amino acid residue at position 382 being
correspondingly variable (Fig S4B). This is consistent with our previous observation that the pUL21 homologue ORF38 from varicella-zoster virus (genus: Varicellaviridae) does not bind CERT (18). It is therefore likely that CERT binding and activation is a conserved feature of HSV-1, HSV-2 and other simplexviruses, but does not extend to other genera of alphaherpesvirus. Given the lack of phenotype observed for viruses encoding the CERT-binding pUL21V382E mutant in cultured cells, it is possible that this interaction plays a role in immune regulation or transmission fitness (60). Alternatively, the interaction may enhance virus replication in other cell types. Specifically, we note that HSV-1 is a neurotropic virus and that sphingolipids like SM are highly enriched in neurons, where they are critical for correct neuronal development and function (31). Studying whether pUL21-mediated CERT dephosphorylation affects virus replication in neurons is an important question for future investigation.

Studies of cellular lipid metabolism are complicated by robust cellular feedback mechanisms (61) and challenges in accurately detecting changes in lipid abundance (62). Here, we successfully employed lipid labelling, click chemistry and HPTLC to monitor the kinetics of sphingosine metabolism, revealing that HSV-1 pUL21 significantly increases the rate of CERT-mediated Cer to SM conversion both outside (Fig 1) and within (Fig 5) the context of infection. Furthermore, we observe a highly significant increase in the abundance of labelled Cer when cells are infected with HSV-1, this increase being greater when pUL21-mediated CERT activation has been abolished. The dramatic change in the rate of Cer to SM conversion is most likely explained by the known propensity of HSV-1 to promote dispersal of the Golgi and TGN (4, 63). Such dispersal would alter ER:TGN contact sites, where CERT-mediated transport enables Cer to SM conversion by TGN-resident sphingomyelin synthase (64). Side-by-side kinetic comparison of lipid metabolic enzymes including ceramide synthase confirms that the presence of alkyne groups within acyl tails does not significantly alter lipid catalysis when compared to radiolabelled or natural substrates (41). While the CERT START domain has been demonstrated to bind Cer containing an alkyne group plus a photoactivatable diazirine group (65, 66), we are unaware of any comparisons between CERT lipid transfer activity with natural or radiolabelled Cer versus alkyne-Cer. It is possible that the presence of an alkyne group may alter the affinity of CERT for Cer, and hence that pUL21-mediated CERT activation may cause a greater or lesser change to the rate of unlabelled Cer to SM conversion. Furthermore, our experimental strategy to probe CERT activity exploited the uptake of exogenous alkyne-Sph and its conversion to alkyne-Cer via the salvage pathway mediated by ceramide synthase. This contrasts to the de novo synthesis pathway, where Cer is generated from serine and palmitoyl-coA via intermediates including dihydroceramide (67). While both the de novo and salvage Cer synthesis pathways are known to occur within cells, and the efficient conversion to alkyne-Cer and alkyne-SM clearly indicated efficient salvage of alkyne-Sph in our experiments (Fig 2B,C and 5D), it is possible that the effect of pUL21-mediated CERT activation could differ in magnitude for Cer synthesised via the de novo pathway. Future experiments could use radiolabelled serine to study how pUL21-mediated CERT activity affects the metabolism of Cer produced via the de novo pathway (68).

HSV-1 utilizes PKD-mediated trafficking from the TGN to the plasma membrane (69). Disruption of PKD-mediated trafficking has been shown to alter virus secretion, with siRNA-mediated depletion of CERT increasing the secretion of extracellular (cell-free) HSV-1 particles and CERT overexpression
reducing virus secretion (56). We do not observe changes in the secretion of pUL21V382E HSV-1, which lacks the ability to dephosphorylate CERT and thus activate CERT-mediated Cer transport. While these results may appear at first glance to be contradictory, it should be noted that in our experiments the cells contained wild-type levels of CERT and cells infected with either ΔpUL21 or pUL21V382E HSV-1 have similar levels of active (CERT\(^\text{p}\)) and inactive (CERT\(^\text{o}\)) protein compared to uninfected controls (Fig 5A). In contrast, siRNA depletion would lead to a complete absence of CERT and of CERT-mediated lipid transfer. Furthermore, the previous study demonstrated that treatment of infected cells with HPA-12 did not stimulate virus particle secretion (56). This insensitivity of HSV-1 secretion to CERT pharmacological inhibition, combined with our observation that pUL21-mediated CERT activation does not alter HSV-1 secretion, strongly suggests that CERT catalytic activity is not directly linked to the regulation of virus secretion.

Our observation of labelled Cer accumulation during HSV-1 infection (Fig 5) is consistent with an earlier report that HSV-1 infection causes an approximately 2-fold increase in Cer abundance within BHK-21 cells (34). Cer has many distinctive physical properties that set it apart from other membrane lipids: it has negative intrinsic curvature, it increases the order of phospholipids in membranes, and it makes biological membranes more permeable to even large solutes such as proteins (70). Nascent HSV-1 particles must traverse multiple biological membranes during virus assembly, most notably during capsid egress from the nucleus. Being too large to exit via nuclear pores, capsids leave by budding into and then out of the perinuclear space, the former step being catalysed by the herpesvirus nuclear egress complex (NEC) (71). A recent study identified that the NEC induces lipid ordering to generate the negative curvature required for capsid budding (72). It is tempting to speculate that pUL21 regulates nuclear capsid egress by modulating Cer abundance, an effect that would be distinct from the previously demonstrated role of pUL21 in promoting nuclear egress via regulating the phosphorylation of NEC components (18, 20). In addition to potentially promoting virus nuclear egress, accumulation of Cer can lead to caspase 3 activation and apoptosis via increased mitochondrial outer membrane permeabilization (73). HSV-1 is known to encode proteins that defend against ceramide-induced apoptosis (74) and it is possible that pUL21-stimulated acceleration of Cer to SM conversion serves to limit the pro-apoptotic activity of Cer, although confirmation of this requires further study.

In the process of probing the molecular basis of pUL21 binding to CERT, we serendipitously discovered a single amino acid substitution (L490E) that prevents pUL21 binding to HSV-1 pUL16 (Fig 4B). Both pUL16 and pUL21 are known to facilitate efficient release of virus capsids from the nuclei of infected cells (75), and a recent study suggests that the pUL16:pUL21 interaction may help regulate capsid maturation and stability (76). Our mutagenesis is consistent with previous biochemical experiments that had mapped the binding of pUL16 to the C-terminal region of pUL21 (residues 268–535) and shown that the N-terminal 40 amino acids of pUL16 were dispensable for this interaction (10). Recent advances in deep learning structure prediction enable the accurate prediction of protein structures and complexes, even in challenging cases such as multi-domain viral proteins with no known sequence homologues (77). We therefore generated a model of the pUL16:pUL21C interaction using AlphaFold-Multimer (52). pUL21 residue L490 lies at the centre of the predicted interaction interface, consistent with its
substitution to glutamate inhibiting pUL16 binding (Fig 4B,H). The structure of pUL16 has not been solved experimentally and inspection of the pUL16 structural model provides two interesting observations. Firstly, the closest structural homologue of the predicted pUL16 C-terminal domain is the N-terminal domain of pUL21 (Fig S5B and Table S3). This structural correspondence (DALI Z-score 5.0; 3.6 Å rmsd across 121 Cα atoms) is striking given the low sequence identity (7%) shared by the domains, although it is known that viral proteins can share similar folds despite having unidentifiable sequence similarly (78). While identifiable pUL21 homologues are restricted to alphaherpesviruses (9), homologues of pUL16 can be found across Herpesviridae (79). The similarity of the predicted pUL16 C-terminal domain structure to that experimentally observed for the pUL21 N-terminal domain suggests that the two proteins are distant homologues, having arisen via a gene duplication event in an ancestral (alpha)herpesvirus. The second observation is that several proteins with structural similarity to the predicted pUL16 N-terminal domain have been associated with the transport of lipids or other bulky hydrophobic molecules (Fig S5D and Table S3). For example, the VtrC protein from *Vibrio parahaemolyticus* was crystallised in complex with the large hydrophobic molecule taurodeoxycholate (80). Given the association between pUL21 and the lipid transport protein CERT it is tempting to speculate that pUL16 may also have a role in modifying host or viral lipid membranes, but further biochemical and experimental structural analysis are required assess whether the pUL16 N-terminal domain has any capacity to bind and/or transport lipids.

In summary, we demonstrated that pUL21 dephosphorylates and activates the cellular lipid transport protein CERT, stimulating conversion of Cer to SM. Characterising the solution structure in complex with the PH and START domains of CERT allowed us to identify a single amino acid mutation of pUL21 that disrupts CERT dephosphorylation in infected cells. HSV-1 encoding this pUL21 mutant had similar replication kinetics, virus yields and plaque sizes, confirming that dephosphorylation of other cellular and/or viral targets underpins the important role of pUL21 in HSV-1 replication and spread. The functional rationale for pUL21-mediated modulation of CERT activity by HSV-1 remains elusive, but we have defined the molecular tools that will allow its dissection in other cell types and/or animal models of infection.
Experimental procedures

Plasmids

The StrepII-CERT transient mammalian expression construct was described previously (18). For generation of stable cells, a synthetic gene encoding human CERT\textsubscript{L} (UniProt Q9Y5P4-1) was cloned into a modified version of plasmid PB-T (81) that encodes an N-terminal StrepII tag and a woodchuck hepatitis virus posttranscriptional regulatory element (82) in the 3’ untranslated region. The S132A substitution was introduced into this vector using QuikChange mutagenesis (Agilent) according to the manufacturer’s instructions. CERT\textsubscript{L} destined for expression using \textit{in vitro} transcription/translation system from wheat germ extract was cloned into the plasmid pF3A WG BYDV (Promega) with an N-terminal myc epitope tag.

For purification following bacterial expression, miniCERT\textsubscript{L} (residues 20–131 plus 351–624) was cloned into pOPTH (78), encoding a N-terminal MAH\textsubscript{6} tag. The truncations of CERT\textsubscript{L} were generated by inverse PCR (MR, residues 123–364; START\textsubscript{L}, residues 365–624) or by introduction of a stop codon (PH, residues 1–122) using QuikChange mutagenesis.

Mammalian cell culture

Mycoplasma-free spontaneously immortalised human keratinocyte (HaCaT) cells (84), HaCaT cells stably expressing pUL21 (HaCaT21) (18), African green monkey kidney (Vero) cells (ATCC #CRL-1586) and human embryonic kidney (HEK)293T cells (ATCC #CRL-3216) were maintained in Dulbecco’s Modified Eagle Medium with high glucose (Merck), supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and 2 mM L-glutamine (complete DMEM) in a humidified 5% CO\textsubscript{2} atmosphere at 37°C. For protein purification, Freestyle 293F suspension cells (ThermoFisher) were grown in Freestyle 293F medium (Gibco) on a shaking platform (125 rpm) in a humidified 8% CO\textsubscript{2} atmosphere at 37°C.

Doxycycline-inducible stably transfected Freestyle 293F cells expressing StrepII-CERT\textsubscript{L} and StrepII-CERT\textsubscript{L}\textsubscript{S132A} were generated using a piggyBac transposon-based system (81). A 30 mL suspension culture of Freestyle 293F cells at 1×10\textsuperscript{6} cells/mL were transfected with a 5:1:1 mass ratio of PB-T-CERT\textsubscript{L}\textsubscript{S132A}:PB-RN:PBase (35 μg total DNA) using Freestyle MAX transfection reagent (Invitrogen) as per the manufacturer’s instructions. After two days the cells were transferred to fresh media supplemented with 500 μg/mL Geneticin (Gibco) and the drug selection was continued for two weeks with media replenishment every three days.
**GFP affinity capture**

Monolayers of HEK293T cells grown in 9 cm dishes were transfected with TransIT-LT1 (Mirus) using 7.7 μg of pEGFP-N1 (for GFP alone), pUL21-GFP, or point mutants thereof, following the manufacturer’s instructions. At 24 hours post-transfection, the cells were harvested by scraping into the medium, pelleted (220 g, 5 min, 4°C), washed three times with cold phosphate buffered saline (PBS) and lysed at 4°C in 1 mL lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1:100 diluted EDTA-free protease inhibitor cocktail [Merck]) for 45 min before clarification (21,000×g, 10 min, 4°C). After immunoprecipitation with GFP-Trap beads (ChromoTek) performed in accordance with the manufacturer’s protocol, the samples were eluted by incubation at 95°C for 5 min in 45 μL 2× SDS-PAGE loading buffer. Input and bound samples were separated by SDS-PAGE and analysed by immunoblot.

**GST pulldown**

GST pulldown experiments were carried out in 96-well flat-bottomed plates (Greiner) using magnetic glutathione beads (Thermo Scientific) to capture the GST-tagged pUL21 (bait protein). First, 0.5 nmol of purified bait protein was incubated with the beads at 4°C for 30 min. The beads were washed three times with wash buffer (20 mM Tris pH 8.5, 200 mM NaCl, 0.1% NP-40, 1 mM DTT, and 1 mM EDTA), then incubated at 4°C for 60 min with various truncations of myc-CERTL (prey proteins), expressed using the TNT SP6 High Yield Wheat Germ in vitro transcription/translation system (Promega) in accordance with the manufacturer’s protocol, followed by four washes in wash buffer. Protein was eluted using wash buffer supplemented with 50 mM reduced glutathione before being analysed by SDS-PAGE and immunoblotting.

**Antibodies**

The following antibodies with listed dilutions were used for immunoblotting: rabbit anti-CERT 1:10,000 (Abcam, ab72536), mouse anti-pUL21 1:50 [1F10-D12] (18) for Fig 1F [note: this monoclonal antibody does not recognize pUL21 V382E], rabbit anti-pUL21 1:5000 (10) for Fig 5A and 5A, mouse anti-GAPDH 1:10,000 (GeneTex, GTX28245), mouse anti-Myc 1:4000 (Millipore, 05-724), mouse anti-PP1α 1:1000 (Santa Cruz, sc-271762), rabbit anti-pUL16 1:2000 (85), mouse anti-VP5 1:50 [DM165] (86), rabbit anti-phospho-Akt substrates 1:1000 (Cell Signalling, 9611). Fluorescently labelled secondary antibodies were used at 1:10,000 dilution: LI-COR IRDye 680T donkey anti-rabbit (926–68023) and goat anti-mouse (926–68020), LI-COR IRDye 800CW conjugated donkey anti-rabbit (926–32213) and goat anti-mouse (926–32210). For immunocytochemistry, mouse anti-pUL21 1:1 [L1E4-C10], which was generated in the same immunisation experiments as anti-pUL21 [1F10-D12] (18), and Alexa Fluor 488 conjugated goat anti-mouse 1:1000 (Invitrogen, A21236) were used. For visualising HSV-1 plaques mouse anti-gD (LP2) 1:50 (87) and horseradish peroxidase conjugated rabbit anti-mouse 1:5000 (DaKo, P0161) were used.
Recombinant protein purification following bacterial expression

All recombinant proteins were expressed in *Escherichia coli* T7 Express lysY/I cells (New England Biolabs). Except for GST-PP1γ, cells were grown in 2×TY medium at 37°C to an OD<sub>600</sub> of 0.8–1.2 before cooling to 22°C and inducing protein expression by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). At 16–20 h post-induction, cells were harvested by centrifugation and pellets were stored at -70°C until required. For GST-PP1γ, cells were grown in 2×TY medium at 37°C to an OD<sub>600</sub> of 0.8, followed by induction using 1 mM IPTG. For all recombinant proteins, cells were resuspended in lysis buffer (see below) at 4°C before lysis using a TS series cell disruptor (Constant Systems) at 24 kpsi. Lysates were cleared by centrifugation (40,000 x g, 30 min, 4°C) and incubated with the relevant affinity resins for 1 h at 4°C before extensive washing (≥ 20 column volumes) and elution using the relevant elution buffer (see below). Samples were concentrated and subjected to size exclusion chromatography (SEC, see below). Fractions containing the desired protein as assessed by SDS-PAGE were pooled, concentrated, snap-frozen in liquid nitrogen and stored at -70°C.

The lysis buffer for pUL21-GST (20 mM Tris pH 8.5, 300 mM NaCl, 0.5 mM MgCl<sub>2</sub>, 1.4 mM β-mercaptoethanol, 0.05% TWEEN-20) and GST-PP1γ (50 mM Tris pH 7.5, 500 mM NaCl, 1 mM MnCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 1.4 mM β-mercaptoethanol, 0.05% TWEEN-20) was supplemented with 200–400 U bovine DNase I (Merck) and 200 μL EDTA-free protease inhibitor cocktail (Merck). Cleared lysates were incubated with glutathione Sepharose 4B (Cytiva), washed with wash buffer (20 mM Tris pH 8.5 [pUL21-GST] or 7.5 [GST-PP1γ], 500 mM NaCl, 1 mM DTT, plus 1 mM MnCl<sub>2</sub> [GST-PP1γ only]) and the proteins eluted using wash buffer supplemented with 25 mM reduced glutathione. SEC was performed using a HiLoad Superdex 200 16/600 column (Cytiva) equilibrated in 20 mM Tris pH 8.5, 500 mM NaCl, 1 mM DTT (pUL21-GST) or 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT (GST-PP1γ).

pUL21-H<sub>6</sub>, pUL21<sup>V382E</sup>-H<sub>6</sub> and H<sub>6</sub>-pUL21C were purified in Tris buffer at pH 8.5 in with 500 mM NaCl and H<sub>6</sub>-miniCERT<sub>L</sub> was purified in Tris buffer at pH 7.5 with 150 mM NaCl. Lysis buffer (20 mM Tris, 20 mM imidazole, NaCl, 0.5 mM MgCl<sub>2</sub>, 1.4 mM β-mercaptoethanol, 0.05% TWEEN-20) was supplemented with 200–400 U bovine DNase I (Merck) and 200 μL EDTA-free protease inhibitor cocktail (Merck). Cleared lysates were incubated with Ni-NTA agarose (Qiagen), washed with wash buffer (20 mM Tris, 20 mM imidazole, NaCl) and eluted using elution buffer (20 mM Tris, 250 mM imidazole, NaCl) SEC was performed using a HiLoad Superdex 200 [pUL21-H<sub>6</sub> and pUL21<sup>V382E</sup>-H<sub>6</sub>] or 75 [H<sub>6</sub>-pUL21C and H<sub>6</sub>-miniCERT<sub>L</sub>] 16/600 column (Cytiva) equilibrated in 20 mM Tris, NaCl, 1 mM DTT.

Recombinant protein purification following mammalian cell expression

StreplI-CERT<sup>P</sup> used for phosphatase assays was purified following transient transfection of Freestyle 293F cells, as described before (18). StreplI-CERT<sub>L</sub> and StreplI-CERT<sub>L</sub>S132A were purified from stably transfected Freestyle 293F cells following induction with 2 μg/mL doxycycline (Fisher) for 72 hours.
Next, cells were harvested by centrifugation (220×g, 5 min, 4°C) and washed once with ice-cold PBS before being resuspended in ice-cold lysis buffer [with phosphatase inhibitors, StrepII-CERT\textsubscript{p} only] (100 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, [10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate]). Cells were lysed by passage through a 23G needle six times and lysates were clarified by centrifugation (40,000×g, 30 min, 4°C). The supernatants were sonicated at 50% amplitude for 60 s using a sonicating probe (MSE) and the supernatants were passed through a 0.45 μm syringe filter (Sartorius). StrepII-tagged proteins were captured using 1 mL StrepTrap HP column (Cytiva) that had been pre-equilibrated in wash buffer (100 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT). After extensive washing (20 column volumes) the protein was eluted using wash buffer supplemented with 2.5 mM desthiobiotin. Pooled eluate was applied to a Superose 6 10/300 GL column (Cytiva) column equilibrated in ITC buffer (20 mM Tris pH 8.5, 500 mM NaCl, 0.5 mM Tris(2-carboxyethyl)phosphine [TCEP]) and fractions containing StrepII-CERT\textsubscript{p} as assessed by SDS-PAGE were pooled, concentrated and used for downstream applications.

**Mutagenesis of viral genomes and generation of recombinant HSV-1**

All HSV-1 strain KOS viruses used in this study were reconstituted from a bacterial artificial chromosome (BAC) (88) and the mutated strain was generated using the two-step Red recombination method (54) with the following primers:

**Forward:** 5′-CGGCTCGTAGGCGCTACACAGCGCCACGCCCTGACGAACCTCGGGCCGACGACCCAGTAGGATGACGACGATAAGGG

**Reverse:** 5′-CGTTGATGGCATTGGCCAGACTGGTCTGGGGCCGAGGGTTCGTACAGGGCCGTGGCGCTGTCAACCAAATTCAATTCTGATTAG

The generation of pUL21 deletion mutant (ΔpUL21) was described previously (18). To generate the P0 stocks, Vero cells were transfected with the recombinant BAC DNA together with pGS403 encoding Cre recombinase (to excise the BAC cassette) using TransIt-LT1 (Mirus) following the manufacturer’s instructions. After 3 days the cells were scraped into the media, sonicated at 50% power for 30 s in a cup-horn sonicator (Branson), and titrated on Vero cell monolayers. The subsequent stocks were generated by infecting either Vero (HSV-1 WT) or HaCaT pUL21 cells (HSV-1 mutants) at MOI of 0.01 for 3 days. The cells were then scraped and isolated by centrifugation at 1000×g for 5 min. Pellets were resuspended in 1 mL of complete DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and freeze/thawed thrice at -70°C before being aliquotted, titrated on Vero cell monolayers, and stored at -70°C until required. The presence of the desired mutation in the reconstituted virus genomes was confirmed by sequencing the pUL21 gene.

**Metabolic labelling and lipid extraction for thin layer chromatography**

Metabolic labelling was performed using sub-confluent (60–80% confluence) HaCaT or HaCaT21 cells grown in a 6-well plate. For analysis of stable cell lines, the cells were pre-treated for 30 min with
complete DMEM containing 1 μM N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecanamide (HPA-12, Tokyo Chemical Industry) dissolved in 0.1% dimethyl sulfoxide (DMSO, Merck), or 0.1% DMSO alone, and HPA-12 or DMSO were retained at the same concentrations throughout the subsequent incubation steps. For infection, cells were infected (below) 14 hours before metabolic labelling.

For metabolic labelling, cells were washed twice with warm PBS before incubation in 500 μL pre-warmed DMEM with 1% (v/v) Nutridoma (Merck) supplemented with 5 μM clickable sphingosine (Cayman Chemical) for 5 min (pulse). Next, cells were washed twice with warm PBS and 1 mL of pre-warmed DMEM with 1% (v/v) Nutridoma was added to each well, followed by incubation at 37°C for the indicated times of chase. Alkyne-Sph was stored as a 3.3 mM ethanolic stock solution at -20°C.

At the indicated times of chase, the plate with cells was transferred onto the ice, washed twice with 1 mL ice-cold PBS, scraped into 300 μL of ice-cold PBS and transferred into appropriate 1.5 mL microcentrifuge tubes containing 600 μL of methanol. To each tube 150 μL of chloroform was added, followed by vigorous vortexing. The precipitated protein was pelleted (20,000×g, 1 min) at room temperature (RT). The organic supernatant was transferred to separate 2 mL tubes containing 300 μL chloroform. 600 μL of 0.1% acetic acid in H2O was subsequently added to each tube to induce formation of two phases. Following extensive vortexing, the phases were separated by centrifugation (20,000×g, 1 min, RT) and the lower phase was transferred to a new 1.5 mL microcentrifuge tube. This lipid-containing solvent phase was dried in a UniVapo centrifugal vacuum concentrator (UniEquip) at 30°C for 20 min.

If required for SDS-PAGE analysis, the protein pellet was solubilized with 5 μL 10% (w/v) SDS, diluted in 75 μL lysis buffer (20 mM Tris 7.5, 150 mM NaCl, 1% (v/v) Triton X-100), boiled for 10 min and sonicated at 75% power for 60 s in a cup-horn sonicator (Branson). The protein samples were mixed with 5× loading buffer before being subjected to SDS-PAGE analysis.

Click reaction and thin layer chromatography of labelled lipids

Lipid standards (16:0(Alkyne)-18:1 PC [Avanti], pacFA GalCer [Avanti], d18:1(Alkyne)-6:0 Cer [Cayman Chemicals], 18:0(Alkyne) Sph [Cayman Chemicals]) or extracted lipids (above) were resuspended in 20 μL of 1:1 chloroform:ethanol. To each tube 1 μL of Tris((1-benzyl-4-triazolyl)methyl)amine (TBTA) solution (2.5 mM in DMSO; Merck), 10 μL of Tetrakis(acetonitrile)copper(I) tetrafluoroborate (10 mM in acetonitrile; Merck) and 1 μL of 3-azido-7-hydroxycoumarin solution (1 mM in ethanol; Jena Bioscience) was added. After brief mixing, the reaction was dried in a UniVapo centrifugal vacuum concentrator (UniEquip) for 10 min at 45°C. Lipids were dissolved in 20 μL of 65:25:4:1 chloroform:methanol:water:acetic acid, of which 8 μL was spotted on a 20×10 cm HPTLC Silica gel 60 plates (Merck). When dried, the plate was developed for 5 cm with 65:25:4:1 chloroform:methanol:water:acetic acid, dried again and developed for 9 cm with 1:1 hexane:ethylacetate. Labelled lipids were visualized via UV illumination using a G:BOX Chemi XX9
(Syngene), bands were quantified using the Image Studio Lite software (LI-COR) with local background subtraction, and statistical tests were performed using Prism 7 (GraphPad Software).

Virus infections

Monolayers of indicated cells were infected by overlaying with the appropriate viruses, diluted in complete DMEM to the specified MOI. The time of addition was designated 0 hours post-infection (hpi). Cells were incubated with the inoculum at 37°C in a humidified 5% CO₂ atmosphere for 1 hour, rocking the tissue culture plate housing the monolayers every 15 min, before complete DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin was added to dilute the original infection medium five-fold. Infected cells were incubated at 37°C in a humidified 5% CO₂ atmosphere and harvested at the specified times.

Immunoblotting

For analysis of protein expression and phosphorylation, cells were washed two times with ice-cold 50 mM Tris pH 8.5, 150 mM NaCl and scraped into 100 μL of ice-cold lysis buffer (50 mM Tris pH 8.5, 150 mM NaCl, 1% (v/v) Triton-X100, 1% (v/v) EDTA-free protease inhibitor cocktail [Merck], 10 mM tetratosodium pyrophosphate, 100 mM NaF and 17.5 mM β-glycerophosphate). After 30 min incubation on ice, the lysates were sonicated (two 15 s pulses at 50% power) in a cup-horn sonicator (Branson) followed by centrifugation at 20,000×g for 10 min. For all samples, protein concentrations were determined using a BCA assay (Peirce) and normalized protein lysates were analysed by SDS-PAGE. For enhanced separation of phosphorylated proteins, 7% (w/v) acrylamide gels contained 50 μM MnCl₂ and 25 μM PhosTag reagent (Wako) where indicated. Separated proteins were then transferred to nitrocellulose membranes using the Mini-PROTEAN system (Bio-Rad) and analysed by immunoblotting, signals being detected using Odyssey CLx Imaging System (LI-COR). For quantitation of CERT phosphoform relative abundance, the signal for CERT₀ band alone and for all CERT bands (CERT₀ + CERTₚ) were quantitated using Image Studio Lite software (LI-COR) with local background subtraction. Statistical tests were performed using Prism 7 (GraphPad Software).

Analytical SEC of miniCERT₁:pUL21C complex

Analytical SEC experiments were performed using Superdex 200 10/300 GL (Cytiva) column equilibrated in 20 mM Tris pH 8.5, 500 mM NaCl, 1 mM DTT. To allow complex formation, 200 μL of 140 μM H₆-miniCERT₁ and 680 μL of 55.4 μM H₆-pUL21C were mixed and incubated for 15 min at room temperature prior to injection.

Isothermal titration calorimetry (ITC)

ITC experiments were performed at 25°C using an MicroCal PEAQ-ITC automated calorimeter (Malvern Panalytical). Proteins were transferred into ITC buffer (20 mM Tris pH 8.5, 500 mM NaCl, 0.5 mM TCEP) either by SEC or extensive dialysis prior to experiments. Titrants (StrepII-CERT₁ₚ, StrepII-CERT₁ₚS132A
or H₆-miniCERTₖ) were titrated into titrates (pUL21-H₆, pUL21V382E-H₆ or H₆-pUL21C) using either 19×2 μL or 12×3 μL injections (Table S1). Data were analysed using the MicroCal PEAQ-ITC analysis software (Malvern Panalytical) and fitted using a one-site binding model.

**Multi-angle light scattering (MALS)**

MALS data were collected immediately following SEC (SEC-MALS) by inline measurement of static light scattering (DAWN 8+; Wyatt Technology), differential refractive index (Optilab T-rEX; Wyatt Technology), and UV absorbance (1260 UV; Agilent Technologies). Samples (100 µL) were injected onto an Superose 6 increase (all except H₆-pUL21C) or Superdex 75 increase (H₆-pUL21C) 10/300 GL columns (Cytiva) equilibrated in 20 mM Tris pH 8.5, 500 mM NaCl, 0.5 mM TCEP at 0.4 mL/min. The protein samples were injected at the concentration of 2 mg/mL. Molecular masses were calculated using ASTRA 6 (Wyatt Technology) using a protein dn/dc of 0.186 mL/g.

**Small-angle X-ray scattering (SAXS)**

SAXS experiments were performed in batch mode (H₆-pUL21C and H₆-miniCERTₖ) or inline following SEC (H₆-pUL21C:H₆-miniCERTₖ) at EMBL-P12 bioSAXS beam line (PETRAIII, DESY, Hamburg, Germany) (89, 90).

For SAXS in batch mode, scattering data (I(s) versus s, where s = 4πsinθ/λ nm⁻¹, 2θ is the scattering angle, and λ is the X-ray wavelength, 0.124 nm) were collected from the indicated batch samples and a corresponding solvent blank (20 mM Tris pH 8.5, 500 mM NaCl, 3% (v/v) glycerol, 1 mM DTT). The scattering profiles of H₆-miniCERTₖ (1.73, 2.59, 3.46 and 6.91 mg/mL) and H₆-pUL21C (0.76 mg/mL) were measured in continuous-flow mode using an automated sample changer (30 µL sample at 20°C; 1 mm pathlength). The sample and buffer measurements were measured as 100 ms data frames on a Pilatus 6M area detector for total exposure times of 2.1 and 2.6 s, respectively. For H₆-miniCERTₖ, the resulting SAXS curves were extrapolated to infinite dilution.

For SEC-SAXS experiments, the scattering data were recorded using a Pilatus 6M detector (Dectris) with 1 s sample exposure times for a total of 3600 data frames spanning the entire course of the SEC separation. Proteins were mixed at 1:1 molar ratio, incubated on ice for 15 min, and then concentrated using a centrifugal concentrator to the desired concentration as estimated from absorbance using extinction coefficients (91) calculated assuming a 1:1 molar ratio. 40 µL of purified H₆-pUL21C:H₆-miniCERTₖ (4.3 mg/mL) was injected at 0.35 mL/min onto an Superdex 200 Increase 5/150 GL column (Cytiva) equilibrated in 20 mM Tris pH 8.5, 500 mM NaCl, 3% (v/v) glycerol, 1 mM DTT. SAXS data were recorded from a single peak (frames 1245–1296 s) and solvent blank was collected from pre-elution frames (frames 247–411). Primary data reduction was carried out using CHROMIXS (92) and 2D-to-1D radial averaging was performed using the SASFLOW pipeline (93). Processing and analysis of the SAXS output was performed using the ATSAS 3.0.2 software package (94). The extrapolated forward scattering intensity at zero angle, I(0), and the radius of gyration, Rₛ, were calculated from the
Guinier approximation \((\ln(l) vs s^2, \text{for } sR_p < 1.3)\). The maximum particle dimension, \(D_{\text{max}}\), was estimated based on the probable distribution of real-space distances, \(p(r)\), which was calculated using GNOM (95). A concentration-independent estimate of molecular weight was determined using a Bayesian consensus method (96). All structural parameters are reported in Table S2.

\textit{Ab initio} modelling was performed using GASBOR (49) and figures show the models that best fit their corresponding SAXS profiles (lowest \(\chi^2\)). Pseudo-atomic modelling was performed using CORAL (97). For \(\text{H}_6\)-miniCERT\(_\text{L}\), crystal structures of the CERT PH (PDB ID 4HHV) (24) and START (PDB ID 2E3M) (25) domains were used, the latter including predicted secondary structure as modelled by I-TASSER (98) for intron 11 (residues 371-396), present only in CERT\(_\text{L}\). The miniCERT\(_\text{L}\) 23 amino acid linker and the purification tag (MAH\(_\text{QD}\)) were modelled using CORAL. The model with the best fit was superimposed onto the PH:START crystal structure (PDB ID 5JJD) (46) by aligning the START domains using PyMOL version 2.5.2 (Schrödinger). For \(\text{H}_6\)-pUL21C, the N-terminal (MAH\(_\text{QD}\)) and C-terminal (HGQSV) elements missing from the crystal structure (PDB ID 5ED7) (50) were modelled using CORAL. For \(\text{H}_6\)-pUL21C:\(\text{H}_6\)-miniCERT\(_\text{L}\), the heterodimer was modelled using the SAXS curve of the complex using a fixed conformation of \(\text{H}_6\)-pUL21C and mobile CERT domains, with unstructured elements modelled as above. All the models were assessed for fit to the corresponding SAXS profiles (\(\chi^2\) and CorMap) using CRYSOL (99). Molecular images were generated using PyMOL (Schrödinger).

\textit{Sequence analysis, deep learning structure prediction and structural analysis}

Sequences of the C-terminal domain from pUL21 homologues were identified by querying the NCBI non-redundant protein sequence database (100) with HSV-1 pUL21 (UniProt ID F8RG07) residues 275–535 using BLASTP (101) for simplexviruses (taxid: 10294) and PSI-BLAST with two iterations for alphaherpesviruses (taxid: 10294). Truncated sequences (accession length <80% of HSV-1 pUL21) or those containing ambiguous amino acids (‘X’) were manually removed. Sequences were aligned using COBALT (102) and are provided as supporting information (Data S1).

The structure of HSV-1 pUL16 (UniProt ID P10200) in complex with pUL21C (residues 275–535, UniProt ID F8RG07) was predicted using a locally installed version of AlphaFold-Multimer version 2.2.2 (52). Structural similarity searches and superpositions were performed using the DALI web server (53). Molecular images were generated using PyMOL (Schrödinger).

\textit{Differential scanning fluorimetry}

Differential scanning fluorimetry experiments were carried out using Viia7 real-time PCR system (Applied Biosystems) and 1× Protein Thermal Shift dye (Applied Biosystems). The assay buffer (20 mM Tris pH 8.5, 500 mM NaCl, 1 mM DTT) was mixed with dye stock solution and protein solution (\(\text{H}_6\)-pUL21 or \(\text{H}_6\)-pUL21\(_{V382E}\)) in an 8:1:1 ratio, giving 1 ng protein in a final volume of 20 µL, and measurements were performed in triplicate. Samples were heated from 25 to 95°C at 1 degree per 20 s and fluorescence was monitored at each increment. The melting temperature (\(T_m\)) is the inflection point
of the sigmoidal melting curve, determined by non-linear curve fitting to the Boltzmann equation using Prism 7 (GraphPad Software).

In vitro dephosphorylation assays

Dephosphorylation assays were performed upon 0.5 μM of purified CERT\(^\text{P}\) using a fixed concentration of GST-PP1γ (25 nM) in the presence of pUL21-H\(_6\) or pUL21\(^{V382E}\)-H\(_6\) at the indicated concentrations. Reactions (50 μL) proceeded in assay buffer (150 mM NaCl, 20 mM Tris pH 8.5, 0.1% Tween-20, 1 mM MnCl\(_2\)) for 30 min at 30°C before being stopped by the addition of 50 μL of 2× SDS-PAGE loading buffer and boiling at 95°C for 5 min. Samples were analysed by SDS-PAGE using 7% (w/v) acrylamide gels supplemented with 25 μM PhosTag reagent and 50 μM MnCl\(_2\) and the protein was visualized using InstantBlue Coomassie stain (Expedeon). To measure the ratio of CERT\(^\text{O}\) to total CERT, Coomassie-stained gels were scanned using an Odyssey CLx Imaging System (LI-COR). The signals detected in the 700 nm channel for the CERT\(^\text{O}\) band alone and for all CERT bands (CERT\(^\text{O}\) + CERT\(^\text{P}\)) were quantitated using Image Studio Lite software (LI-COR) with local background subtraction.

Plaque size analysis

Confluent monolayers of Vero or HaCaT cells in 6-well tissue culture plates were infected with 100 plaque forming units of the indicated virus diluted in complete DMEM to a final volume of 500 μL. Following the adsorption for 1 h at 37°C in a humidified 5% CO\(_2\) atmosphere, rocking the plate every 15 min, the cells were overlaid with plaque assay media (DMEM supplemented with 0.3% high viscosity carboxymethyl cellulose, 0.3% low viscosity carboxymethyl cellulose, 2% (v/v) FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin) and incubated for further 48 hours. Next, cells were fixed with 3.7% (v/v) formal saline for 20 min, washed three times with PBS and incubated for 1 h with mouse anti-gD (LP2) (87), diluted 1:50 in blocking buffer (PBS supplemented with 1% (w/v) BSA and 0.1% TWEEN-20). Cells were washed thrice in PBS and incubated for 30 min with horseradish peroxidase conjugated rabbit anti-mouse antibody (DaKo, P0161) diluted 1:5000 in blocking buffer, followed by two subsequent PBS washes and one wash with ultrapure water. Plaques were visualized using the TrueBlue peroxidase substrate in accordance with the manufacturer’s instructions (Seracare). Plaques were scanned at 1200 dpi and plaque areas were measured using Fiji (103, 104).

Single-step (high MOI) virus growth assays

Virus growth assays were performed in technical duplicate for each independent experiment. Monolayers of HaCaT or Vero cells were infected with the indicated viruses diluted in complete DMEM to an MOI of 10. The time of virus addition was designated 0 hpi. After adsorption for 1 h at 37°C in a humidified 5% CO\(_2\) atmosphere, rocking the plates every 15 min, extracellular virus particles were neutralized with an acid wash (40 mM citric acid, 135 mM NaCl, 10 mM KCl, pH 3.0) for 1 min and cells were then washed three times with PBS before being subsequently overlaid with complete DMEM. At designated times post-infection, cells were harvested by freezing the plate at -70°C. When all plates
were frozen, samples were freeze/thawed one more time before scraping and transferring to 1.5 mL tubes and storing at -70°C until titration. Titrations were performed on monolayers of Vero cells. Serial dilutions of the samples were used to inoculate the cells for 1 h, followed by overlaying with DMEM containing 0.3% high viscosity carboxymethyl cellulose, 0.3% low viscosity carboxymethyl cellulose, 2% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Three days later, cells were fixed in 3.7% (v/v) formal saline for 20 min, washed with water and stained with 0.1% toluidine blue. Statistical tests were performed using Prism 7 (GraphPad Software).

**Virus release assays**

Monolayers of HaCaT or Vero cells were infected as described above for single-step virus growth assays, infections being performed in technical duplicate or triplicate for each independent experiment. At 12 hpi, the media (500 μL) were harvested to 1.5 mL Eppendorf tubes, centrifuged for 5 min at 1000×g to remove any detached cells, 300 μL of the supernatant was carefully transferred to fresh tubes and stored at -70°C until titration. The cells were overlaid with 500 μL of fresh DMEM and the plates were immediately frozen at -70°C. Titrations were performed on monolayers of Vero cells as described above. Statistical tests were performed using Prism 7 (GraphPad Software). Figure 6C was generated using SuperPlotsOfData (105).

**Immunocytochemistry**

Cells grown on #1.5 coverslips were infected at an MOI of 1 as described above. At 14 hpi, cells were washed with PBS and incubated with freezing cold (-20°C) methanol for 5 min at -20°C. Coverslips were washed with PBS, followed by incubation with blocking buffer (1% (w/v) BSA in PBS) for 30 min at RT. Primary antibodies (above) were diluted in blocking buffer and incubated with coverslips for 1 h. Coverslips were washed ten times with PBS before incubation for 45 min with the secondary antibodies (above) diluted in blocking buffer. Coverslips were washed ten times in PBS and ten times in ultrapure water before mounting on slides using Mowiol 4–88 (Merck) containing 200 nM 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired using an inverted Olympus IX81 widefield microscope with a 60× Plan Apochromat N oil objective (numerical aperture 1.42) (Olympus) and Retiga EXi Fast1394 interline CCD camera (QImaging).

**Data availability**

The SAXS data measured for each concentration, with an accompanying report, are available in the Small Angle Scattering Biological Data Bank (SASBDB) (106), entries SASDNB7, SASDNC7 and SASDND7. The AlphaFold-Multimer model of the HSV-1 pUL16:pUL21C complex, with associated raw output and quality statistics, has been deposited in the University of Cambridge Data Repository (https://doi.org/10.17863/CAM.88729).
Supporting information

This article contains supporting information (18, 50, 51, 53, 80, 93, 107–112).

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations

A.U., arbitrary units; Cer, ceramide; DAPI, 4′,6-diamidino-2-phenylindole; DP, differential power; dRI, differential refractive index; ER, endoplasmic reticulum; HaCaT21, HaCaT cells stably expressing HSV-1 pUL21; HPA-12, N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecanamide; HexCer, hexosylceramide; hpi, hours post infection; HSV-1, herpes simplex virus 1; MALS, multi-angle light scattering; MOI, multiplicity of infection; PC, phosphatidylcholine; PD, pull-down; pLDDT, predicted Local Distance Difference Test; SAXS, small-angle X-ray scattering; SEC, size-exclusion chromatography; Sph, sphingosine; SM, sphingomyelin; TCEP, Tris(2-carboxyethyl)phosphine; TGN, trans-Golgi network
Figure 1. HSV-1 pUL21 alters the rate of ceramide to sphingomyelin conversion in cultured cells. (A) Simplified schematic diagram of sphingolipid biosynthetic pathways that lead from sphingosine (Sph) to sphingomyelin (SM) and glycosphingolipids (hexosylceramide; HexCer), or to phosphatidylcholine (PC) via a ‘salvage’ pathway. In this salvage pathway Sph-1-phosphate is converted to trans-2-hexadecenal, then trans-2-hexadecenoic acid and trans-2-hexadecenoyl-coA before being converted to palmitoyl-coA (113), which is in turn conjugated to glycerol-3-phosphate to form lysophosphatidic acid before being converted to phosphatidic acid, diacylglycerol and then PC (114). Ceramide (Cer) to SM conversion is accelerated by the transport protein CERT, which is selectively inhibited by HPA-12. (B) Rate of Sph conversion to Cer, SM or PC was measured in HaCaT cells, untreated or treated with 1 μM HPA-12, and in HaCaT cells stably expressing pUL21 (HaCaT21). Cells were incubated with 5 μM ‘clickable’ alkyne-Sph (pulse) for 5 min and harvested for lipid extraction either immediately (0 min) or at the indicated times (chase). Extracted lipids were bioconjugated to 3-azido-7-hydroxycoumarin, separated by HPTLC and detected using UV light. Separated lipids were identified using clickable standards (Stds) and previous literature (42). Data is from one representative experiment of two independent repeats. (C) Quantitation of the lipid intensities from (B) as determined by densitometry and represented as percentage fraction of total signal. (D) Rate of SM synthesis expressed as its fraction in the cumulative signal for SM and Cer. (E) The ratio of alkyne-Sph incorporated into either Cer or SM as a proportion of total alkyne-lipid signal, representing the influx of Sph into the SM biosynthetic pathway. For (D) and (E) the data represents two independent experiments (mean ± SD). Data points are labelled if significantly different to parental HaCaT cells: **, p < 0.01; $$$, p < 0.001; $$$$ p < 0.0001 (two-way ANOVA with Dunnett’s multiple comparisons test). (F) The re-solubilised proteins precipitated during lipid extraction were analysed by SDS-PAGE and immunoblotting using the antibodies listed. Where indicated, the gel was supplemented with PhosTag reagent to retard the migration of phosphorylated proteins, thus enhancing the separation of CERT that is hypo- (CERT⁰) or hyper-phosphorylated (CERT⁰). GAPDH serves as a loading control.

Figure 2. pUL21 binds to the PH and STARTₜ domains of CERTₜ, and to a monomeric form of CERTₜ comprising just the PH and STARTₜ domains (miniCERTₜ). (A) Minimal binding elements of CERTₜ were determined via pull down (PD) experiment using immobilised purified pUL21-GST or GST alone to capture myc-tagged full-length CERTₜ or truncations thereof expressed via in vitro transcription/translation. Captured proteins were subjected to SDS-PAGE and immunoblotting using an anti-myc antibody. Ponceau S (PonS) staining of the immunoblot membrane before blocking shows equal and efficient capture of the bait proteins across the tested conditions. (B) Schematic representation of miniCERTₜ. Dotted lines indicate the regions of full-length CERTₜ that were omitted. (C) SEC-MALS elution profiles (normalized differential refractive index, dRI) of Strepl-CERTₜ (black dotted), Strepl-CERTₜS¹³²A (grey solid) and H₆-miniCERTₜ (green solid). Weight-averaged molecular masses (coloured solid lines) are shown across the elution peaks. The expected molecular masses for trimeric Strepl-CERTₜ and Strepl-CERTₜS¹³²A, and for monomeric H₆-miniCERTₜ, are shown as dotted horizontal lines. Inset shows Coomassie-stained SDS-PAGE of the samples used for SEC-MALS (left), plus SDS-PAGE supplemented with PhosTag reagent (right) of the Strepl-CERTₜ proteins. (D–F)
Representative ITC titration curve of pUL21-H₆ binding to (D) StreplII-CERTₖ, (E) StreplII-CERTₖ5132A, and (F) H₆-miniCERTL. DP, differential power. Insets show normalized binding curves with integrated changes in enthalpy (ΔH) as a function of molar ratio. The affinity (Kₒ), ΔH and stoichiometry (N) for the presented titrations are displayed below. (G) SAXS profile measured for H₆-miniCERTL. The reciprocal-space fit of the p(r) profile to the SAXS data is shown as a yellow line. χ², fit quality; p, Correlation Map (CorMap) probability of systematic deviations between the model fit and the scattering data (93). Inset shows the Guinier plot (sRᵡ < 1.3), which is linear as expected for an aggregate- and repulsion-free system. (H) The real-space distance distribution function, p(r), calculated from the SAXS profile. (I) Dimensionless Kratky plot of the SAXS data. Grey dotted lines indicate the expected maximum of the plot for a compact protein (sRₑ = √3, (sRₑ)²<s(l)/l(0) > = 3eᵢ) (115). (J) Fit of an ab initio dummy-residue model calculated using GASBOR to the SAXS profile, and (K) representative GASBOR dummy-residue model. White spheres indicate modelled water beads of the hydration shell. (L) Fit to the SAXS profile of the pseudo-atomic model of H₆-miniCERTL obtained using CORAL. (M) CORAL pseudo-atomic model of H₆-miniCERTL (green ribbons) superimposed onto the crystal structure of the complex between the CERT PH and START domains (cyan ribbons, PDB ID 5JJD) (46) by aligning the START/STARTL domains. Superposition is shown in orthogonal orientations where the linker regions or termini that were modelled by CORAL are depicted as yellow spheres.

Figure 3. Solution structure of the H₆-pUL21C:H₆-miniCERTL heterodimer. (A) Schematic of pUL21, with N- and C-terminal domains joined by a highly dynamic linker (18). Crystal structures of domains (N, PDB ID 4U4H (51); C, PDB ID 5ED7 (50)) are shown. (B) Superdex 75 10/300 SEC elution profile of the H₆-pUL21C:H₆-miniCERTL complex, pre-formed in the presence of 1.32-fold molar excess of H₆-pUL21C. Dotted lines indicate fractions that were collected and subjected to SDS-PAGE analysis with Coomassie staining, revealing co-elution of the two proteins. (C) Representative ITC titration curve of H₆-pUL21C binding to H₆-miniCERTL. DP, differential power. Inset shows normalized binding curve with integrated changes in enthalpy (ΔH) as a function of molar ratio. The ranges of the vertical axes are identical to Fig 2F. The affinity (Kₒ), ΔH and stoichiometry (N) for the presented titration are displayed below. (D) SEC elution profile (partial integrated scattered X-ray intensity vs. data frame number) obtained during SEC-SAXS analysis of H₆-pUL21C:H₆-miniCERTL complex. Dashed line indicates the calculated radius of gyration (Rₑ) across the frames averaged for structural analyses. (E) Averaged SAXS profile of the H₆-pUL21C:H₆-miniCERTL complex. The reciprocal-space fit of the p(r) profile to the SAXS data is shown as a yellow line. χ², fit quality; p, Correlation Map (CorMap) probability of systematic deviations between the model fit and the scattering data (93). Inset displays the Guinier plot (sRᵡ < 1.3), which is linear as expected for an aggregate- and repulsion-free system. (F) Real-space distance distribution function, p(r), calculated from the SAXS profile. (G) Dimensionless Kratky plot of the SAXS data. Grey dotted lines indicate the expected maximum of the plot for a compact protein (sRₑ = √3, (sRₑ)²<s(l)/l(0) > = 3eᵢ). (H) Ab initio modelling of H₆-miniCERTL:H₆-pUL21C using GASBOR. Fit of the calculated scattering (yellow) to the SAXS profile is shown, as is a representative dummy-residue model (blue spheres) with modelled water beads of the hydration shell (white spheres). (I) and (J) Pseudo-atomic models of the H₆-miniCERTL:H₆-pUL21C complex generated using CORAL.
The fits of the computed scattering (yellow) to the H$_6$-pUL21C:H$_6$-miniCERT$_L$ SAXS profile (blue) are shown. High-quality fits are obtained with models where miniCERT$_L$ binds either the left or right ‘wings’ of the dragonfly-like pUL21C domain (50). pUL21C is shown as a violet ribbon with ‘wings’ and termini labelled, and miniCERT$_L$ as a green molecular surface (PH, light green; START$_L$, dark green). For clarity, regions absent from the crystal structures that were modelled by CORAL are not displayed. Residues from the ‘left’ and ‘right’ wings of pUL21C that were selected for further investigation are shown as sticks with red (I) or pink (J) carbon atoms, respectively. An additional pseudo-atomic model plus its fit to the H$_6$-pUL21C:H$_6$-miniCERT$_L$ SAXS profile is shown in Fig S3, where the fits presented in (I) and (J) are also reproduced for ease of comparison.

Figure 4. Identification of pUL21 point mutants with disrupted binding to CERT or pUL16. (A) and (B) Immunoblot following immunoprecipitation from HEK293T cells transfected with plasmids encoding GFP-tagged pUL21, either wild-type (WT) or with amino acid substitutions at the putative CERT$_L$ binding interface on the left (A) or right (B) wings of pUL21C, or encoding GFP alone. At 24 hours post-transfection cells were infected with ΔpUL21 HSV-1 (MOI = 5) and at 16 hours post-infection cells were lysed, tagged proteins were captured using GFP affinity resin, and the bound proteins were subjected to SDS-PAGE and immunoblotting using the antibodies listed. Ponceau S (PonS) staining of the nitrocellulose membrane before blocking is shown to confirm efficient capture of GFP-tagged proteins. GAPDH (A) and tubulin (B) are used as loading controls. (C) Superdex 75 16/600 SEC elution profile of pUL21$_{V382E}$-H$_6$ following bacterial expression and affinity capture. Indicated peak was pooled and used for subsequent experiments. (D) Differential scanning fluorimetry of WT (purple) and V382E (red) pUL21-H$_6$. Representative curves are shown and melting temperature ($T_m$) is mean ± SD of three technical replicates. Inset shows Coomassie-stained SDS-PAGE of the purified proteins. (E) Representative ITC titration curve of pUL21$_{V382E}$-H$_6$ binding to H$_6$-miniCERT$_L$. DP, differential power. Inset shows normalized binding curve with integrated changes in enthalpy ($\Delta H$) as a function of molar ratio. The ranges of the vertical axes are identical to Fig 2F. The affinity ($K_D$), $\Delta H$ and stoichiometry (N) for the presented titration are displayed below. (F) In vitro dephosphorylation assay using all-purified proteins. 0.5 μM CERT$_P$ was incubated with varying concentrations of pUL21-H$_6$ WT or V382E (two-fold dilution from 4 μM to 62.5 nM) in the presence of 25 nM GST-PP1 for 30 min at 30°C. Proteins were resolved using SDS-PAGE supplemented with PhosTag reagent to enhance separation of CERT that is hyper- (CERT$_P$) or hypo-phosphorylated (CERT$_O$), and protein bands were visualised using Coomassie. Images are representative of two independent experiments. (G) Quantitation of concentration-dependent pUL21-mediated stimulation of CERT dephosphorylation as determined by densitometry. Ratio of CERT$_O$ to total CERT (CERT$_O$/CERT$_P$) for two independent experiments is shown (mean ± SD). Data points are labelled if significantly different: *, p < 0.05; **, p < 0.01; ***, p < 0.001 (two-way ANOVA with Sidak’s multiple comparisons test). (H) Predicted structure of pUL21C (violet ribbon) in complex with pUL16 (cyan ribbon and surface). Residues 1–34 of pUL16, which are predicted to be disordered, are not shown. Residues of the pUL21 right wing that were substituted in (B) are shown as sticks. Inset shows selected pUL16 amino acids residues in close proximity to L490
and R497 of pUL21. (I) Per-residue predicted Local Distance Difference Test (pLDDT) scores (top) and predicted aligned error (PAE) matrix (bottom) for predicted pUL16:pUL21C complex.

Figure 5. Mutating the CERT-binding interface of pUL21 inhibits CERT dephosphorylation and reduces the rate of sphingomyelin synthesis in infected cells. (A) HaCaT cells were infected at MOI = 5 with wild-type (WT) HSV-1, HSV-1 lacking pUL21 expression (ΔpUL21), or a pUL21 point mutant virus (pUL21V382E). Lysates were harvested at 16 hpi in the presence of phosphatase inhibitors and subjected to SDS-PAGE plus immunoblotting using the antibodies listed. Where indicated, the gel was supplemented with PhosTag reagent to enhance separation of CERT phosphoforms. The antibody recognising phosphorylated Akt substrates (Akt sub) illustrates activity of the HSV-1 kinase pUS3, several substrates of which are dephosphorylated in a pUL21-dependent manner (arrowheads) (18). VP5, infection control. (B) Quantitation of the CERT dephosphorylation level (ratio of CERTO to total CERT) in cells infected with WT or mutant HSV-1, as determined by densitometry. Results are presented as mean ± SD from three independent experiments. One-way ANOVA with Tukey’s multiple comparisons test was used for the statistical analysis (ns, non-significant; **, p < 0.01; ***, p < 0.001). (C) Vero cells were infected at MOI = 1, fixed 14 hpi and stained with DAPI (blue) plus an antibody recognising pUL21 (green). (D) Pulse-chase experiment to measure the rate of Sph conversion to Cer, SM and PC. HaCaT cells were infected with WT or pUL21V382E HSV-1 at MOI = 5, or mock infected. Cells were incubated with 5 μM alkyne-Sph (pulse) at 14 hpi for 5 min and harvested for lipid extraction either immediately (0 min) or at the indicated times (chase). Extracted lipids were bioconjugated to 3-azido-7-hydroxycoumarin using click chemistry, separated by HPTLC, visualised using UV light and relative lipid abundances were quantitated by densitometry. Data is from one representative experiment of two independent repeats. (E) Rate of SM synthesis expressed as its fraction in the cumulative signal for SM and Cer. (F) The proportion of alkyne-Sph incorporated into either Cer or SM, representing the temporal influx of Sph into the SM biosynthesis pathway. For (E) and (F) the data represents two independent experiments (mean ± SD). Data points are labelled if significantly different to WT HSV-1: **, p < 0.01; ****, p < 0.0001 (two-way ANOVA with Dunnett’s multiple comparisons test). (G) The resolubilised proteins precipitated during lipid extraction were analysed by SDS-PAGE and immunoblotting using the antibodies listed.

Figure 6. pUL21-mediated dephosphorylation of CERT does not enhance virus replication, secretion or spread in cultured cells. (A) Monolayers of HaCaT or Vero cells were infected with 100 plaque forming units of WT or pUL21V382E HSV-1. Following infection, cells were overlaid with medium containing 0.6% carboxymethyl cellulose and incubated for 48 hours, then fixed and immunostained with chromogenic detection. Relative plaque areas (pixels) were measured using Fiji (103, 104). Bars represent mean plaque sizes, which were compared using unpaired t-test (n=100; ns, non-significant). (B) Single-step (high MOI) growth curves of WT and pUL21V382E HSV-1. Monolayers of HaCaT (continuous line) or Vero (dotted line) cells were infected (MOI = 5) with the viruses shown. Samples were harvested at the indicated times and titres determined by plaque assay using Vero cells. Data are presented as mean values ± SD of technical duplicates from one representative experiment. Difference in replication kinetics across two biological replicates is not statistically significant (two-way ANOVA.
with Sidak’s multiple comparisons test). (C) Virus release into the culture supernatant from HaCaT cells infected with WT or pUL21\textsuperscript{V382E} HSV-1 at various MOIs. Samples were harvested at 12 hpi and virus infectivity in the cells versus the culture medium was measured by titration on Vero cells. The fold change in secretion of infectivity into the culture medium for pUL21\textsuperscript{V382E} versus WT HSV-1 is shown as mean values ± SD of two (MOI = 1 or 3–5) or three (MOI = 10) independent experiments. Technical replicates for each independent experiment are grouped by shape. For MOI 3–5, the data represent one independent experiment performed at MOI = 3 (squares) and one at MOI = 5 (circles). For each MOI, the extent of virus secretion was compared using an unpaired two-tailed t-test with Welch’s correction (ns, non-significant).

**Table 1. Thermodynamic properties of the pUL21:CERT\textsubscript{L} interaction.** As quantitated by isothermal titration calorimetry (ITC). Experiments were performed $n$ (replicates) times and mean ± SD values are shown. Data for individual titrations are presented as supporting information (Table S1).
References


89. Blanchet, C. E., Spilotros, A., Schwemmer, F., Graewert, M. A., Kikhney, A., Jeffries, C. M.,
    Versatile sample environments and automation for biological solution X-ray scattering

90. Graewert, M. A., Franke, D., Jeffries, C. M., Blanchet, C. E., Ruskule, D., Kuhlke, K., Flieger, A.,

91. Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D., and
    Mol. Biol. Clifton NJ.* **112**, 531–552


94. Manalastas-Cantos, K., Konarev, P. V., Hajizadeh, N. R., Kikhney, A. G., Petoukhov, M. V.,
    Molodenskiy, D. S., Panjkovich, A., Mertens, H. D. T., Gruzinov, A., Borges, C., Jeffries, C. M.,
    Svergun, D. I., and Franke, D. (2021) ATSAS 3.0: expanded functionality and new tools for small-


97. Petoukhov, M. V., Franke, D., Shkumatov, A. V., Tria, G., Kikhney, A. G., Gajda, M., Gorba, C.,

    structure and function prediction. *Nat. Methods.* **12**, 7–8

    **28**, 768–773

100. Sayers, E. W., Bolton, E. E., Brister, J. R., Canese, K., Chan, J., Comeau, D. C., Connor, R.,
    Funk, K., Kelly, C., Kim, S., Madej, T., Marchler-Bauer, A., Lanczycki, C., Lathrop, S., Lu, Z.,
    Thibaud-Nissen, F., Murphy, T., Phan, L., Skripchenko, Y., Tse, T., Wang, J., Williams, R.,
    for biotechnology information. *Nucleic Acids Res.* **50**, D20–D26

101. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment


103. Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., and Eliceiri,
    Bioinformatics.* **18**, 529

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Table 1. Thermodynamic properties of the pUL21:CERT\text{L} interaction. As quantitated by isothermal titration calorimetry (ITC). Experiments were performed \( n \) (replicates) times and mean ± SD values are shown. Data for individual titrations are presented as supporting information (Table S1).

<table>
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<tr>
<th>Syringe</th>
<th>Cell</th>
<th>( K_D ) [nM]</th>
<th>( \Delta H ) [kcal/mol]</th>
<th>( \Delta G ) [kcal/mol]</th>
<th>-T( \Delta S ) [kcal/mol]</th>
<th>( N ) (stoichiometry)</th>
<th>( n ) (replicates)</th>
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<tr>
<td>CERT\text{L} \text{p}</td>
<td>pUL21</td>
<td>772.7 ± 274.0</td>
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<tr>
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</table>
A. pUL21 domains

B. Densogram (mL)

C. Time (min)

D. Average intensity

E. Radius of gyration (nm)

F. p(r)

G. $sR_g$(nm)

H. $[N]/[s]p_0$

I. CORAL 1

J. CORAL 3

K. STARTL

L. PH

M. PH

N. PH

O. PH

P. PH

Q. PH

R. PH

S. PH

T. PH

U. PH

V. PH

W. PH

X. PH

Y. PH

Z. PH
Tomasz H. Benedyk: Data Curation, Investigation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Viv Connor: Investigation

Eve R. Caroe: Investigation

Maria Shamin: Investigation

Dmitri I. Svergun: Resources

Janet E. Deane: Funding Acquisition, Resources, Writing – Review & Editing

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